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Phosphoenolpyruvate-Dependent Phosphotransferase System. ^{1}H NMR Studies on Chemically Modified HPr Proteins†

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ABSTRACT: The low-p*K* tyrosyl residue present in the heat-stable proteins (HPr) of all Gram-positive bacteria studied until now has been labeled by tetranitromethane in the HPr of *Bacillus subtilis* and *Streptococcus faecalis*. The nitrotyrosyl derivatives obtained are fully active in the complementation assay. The labeled tyrosyl residues could be identified as Tyr-37 in both proteins. Reinvestigation of the low-p*K* tyrosyl residue in HPr of *Staphylococcus aureus* resulted in the same assignment. In all three proteins an interaction between nitrotyrosine-37 and the active center His-15 could be observed, leading to an increase in the p*K* of His-15 and a change of its chemical shift parameters. The ^{1}H NMR lines of the complete aromatic spin system of HPr of *B. subtilis* could be assigned by the nitration studies. Labeling of Arg-17 in HPr of *S. aureus* and *S. faecalis* by 1,2-cyclohexanedione in the presence of borate ions causes an almost complete inhibition of its enzymatic activity. In the NMR spectrum the labeling of the arginyl residue influences the resonance lines of His-15: two new resonance lines for the C-2 protons of equal intensity are observed, a fact that could be explained by two different conformations in slow exchange. The p*K* value of His-15 was not changed by the labeling, excluding Arg-17 as responsible for the low p*K* of His-15.

HPr[1] is a constitutive protein of the phosphoenolpyruvate-dependent phosphotransferase system (PTS), a carbohydrate transport system found in virtually all anaerobic and facultatively anaerobic bacteria [for a review see, e.g., Hengstenberg (1977), Dills et al. (1980), or Robillard (1982)]. HPr transfers the phosphoryl group from enzyme I to factor III or enzyme II, which finally becomes bound to the transported carbohydrate during the vectorial phosphorylation. Enzyme I, HPr, and factor III carry the phosphoryl group covalently bound to a histidyl residue. NMR studies showed that in the HPr proteins from all microorganisms studied until now (*Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus faecalis*, *Streptococcus lactis*, *Escherichia coli*) the phosphoryl group is bound at the N-1 of histidyl ring. Because of its low p*K* the histidyl ring is deprotonated at physiological pH before the phosphoryl transfer, and the phosphorylation of the histidyl ring leads to an unusually large increase in p*K* resulting in a complete protonation of the phosphohistidyl residue (Gassner et al., 1977; Dooijewaard et al., 1979; Kalbitzer et al., 1982). The phosphoryl group is bound to His-15 in HPr of *S. aureus*; the similarity of p*K* values and chemical shifts of the active center histidines suggests a similarity of the overall structure around these histidines (Kalbitzer et al., 1982). The heat-stable proteins of all studied microorganisms except that of *E. coli* contain a tyrosyl residue with a low p*K* value whose chemical shift values are again very similar (Maurer et al., 1977; Kalbitzer et al., 1982). The tyrosyl residue is accessible by tetranitromethane in HPr of *S. aureus*, its nitration re-

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[1] Abbreviations: HPr, heat-stable protein(s); PTS, phosphoenolpyruvate-dependent phosphotransferase system; NMR, nuclear magnetic resonance; *E. coli*, *Escherichia coli*; *S. aureus*, *Staphylococcus aureus*; *S. faecalis*, *Streptococcus faecalis*; *S. lactis*, *Streptococcus lactis*; *B. subtilis*, *Bacillus subtilis*; TFA, trifluoroacetic acid; HPTLC, high-performance thin-layer chromatography; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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sulting in an interaction influencing the pK values and chemical shifts of His-15 and the tyrosyl residue (Rösch et al., 1981). The tyrosyl residue was assigned to Tyr-56 by Schmidt-Aderjan et al. (1979). An interaction between the tyrosyl residue and the active center histidine could be observed in HPr of *S. lactis* even in the native state (Kalbitzer et al., 1982). As a possible interpretation of the NMR results a hydrogen bond between both residues has been suggested (Rösch et al., 1981).

In these studies we have attempted to find out whether the interaction between these residues is a singular phenomenon or is of more general importance. Moreover, modification experiments should elucidate the role of Arg-17, whose positive charge could be responsible for the low pK of His-15.

Materials and Methods

Preparation of HPr Proteins

The HPr were prepared as described by Kalbitzer et al. (1982).

Nitration and Assignments of Nitrotyrosine Residues of HPr Proteins

Mononitrotyrosyl HPr of S. faecalis. A total of 10 mg (1.25 μmol) of lyophilized HPr was dissolved in 8 mL of 0.1 M NaCl. Nitration was performed in the dark with automated titration equipment from Radiometer (Copenhagen). The pH was kept at 10 by addition of 0.1 M NaOH. Tetranitromethane (Serva, Heidelberg) was used in 40-fold molar excess and applied as a 10% alcoholic solution. After the mixture was reacted for 90 min at 25 °C under vigorous stirring, it was centrifuged at 40000g and chromatographed on a G-75 column (5 × 85 cm) eluted with 50 mM NH_4HCO_3, pH 8.5. Fractions containing HPr and derivatives were localized by the *S. aureus* mutant complementation assay (Hengstenberg et al., 1969) and adsorbed on a DEAE column (1.5 × 20 cm; DE-52, Whatman) that was equilibrated with 50 mM NH_4HCO_3, pH 8.5. Nitrotyrosyl derivatives were eluted with a linear gradient of 500 mL of 0.05–0.4 M NaCl in 80 mM NH_4HCO_3. Fractions of 6 mL were collected. HPr and nitrotyrosyl HPr (slightly yellow) were detected by their biological activity. After desalting, mononitrotyrosyl HPr was recovered in 40% yield as freeze-dried material.

Tryptic Peptides of HPr of S. faecalis. A total of 4 mg of lyophilized mononitrotyrosyl HPr was dissolved in 500 μL of 0.05 M NH_4HCO_3, pH 8.5, and digested with 100 μg of trypsin for 2 h (Jany et al., 1976). The reaction was followed by the decrease of the biological activity after heating the sample to 100 °C to destroy trypsin. Tryptic peptides were separated on a Sephadex G-25 column (1.5 × 80 cm) eluted with 50 mM NH_4HCO_3. Fractions containing the nitrotyrosine peptide were detected at 280 nm. Aliquots (15 μL) were spotted on Nanosiligur HPTLC plates (Macherey and Nagel, Düren) and developed with the solvent system 1-propanol/H_2O (70:30 v/v). Peptides can be visualized with fluorescamine (Hoffmann-La Roche, Basel) as described by Beyreuther et al. (1977). The nitrotyrosine-containing peptide can be specifically recognized as a yellow spot by exposing the plate to an ammonia atmosphere. Further purification of the nitrotyrosyl peptide was achieved on a DE-52 column (1.5 × 15 cm) eluted with a linear gradient of 500 mL of 0.05–0.5 M NH_4HCO_3. The yield from 4 mg of protein was approximately 0.2 mg of pure peptide. The peptides from unmodified HPr were isolated analogously.

Mononitrotyrosyl HPr of B. subtilis. B. subtilis HPr (1 μmol) was nitrated with a 25-fold molar excess of tetranitromethane at pH 8.5 for 90 min. The reagent (10% ethanol solution) was added in two batches; the second batch was added after 30 min. The purification and detection of the modified HPr was performed as described for HPr of *S. faecalis*. The recovery was around 25% of the starting material.

Tryptic Peptides from Mononitrotyrosyl HPr from B. subtilis. The isolation was performed as described above. A typical yield was 0.4 mg from 8 mg of mononitrotyrosyl HPr.

Mononitrotyrosyl HPr from S. aureus. A total of 10 mg of HPr in 8 mL of 0.1 M Tris-HCl, pH 8.5, was treated with 120 μL of 10% tetranitromethane in ethanol under vigorous stirring for 60 min. The molar excess was 20-fold per mole of tyrosine in the protein. Under these conditions a mixture of 37-mononitrotyrosyl HPr and 56-mononitrotyrosyl HPr was obtained in a ratio that varied from experiment to experiment. The yield of mixed derivatives was approximately 20%. The two mononitrated derivatives could be separated by DEAE high-performance liquid chromatography (HPLC) on a 5-μm Serva Polyol column (4.6 × 250 mm) (Serva, Heidelberg) with a linear gradient of potassium phosphate, pH 6.8, of 0.02–0.16 M in 40 min at a flow rate of 1 mL/min. 56-Mononitrotyrosyl HPr eluted at a lower salt concentration than 37-mononitrotyrosyl HPr. The peaks were detected by their UV absorption. Nitrotyrosine-containing fractions were yellow colored upon addition of ammonia. Desalting was performed by adsorption of the aqueous solution of nitrotyrosine-containing protein on a small precolumn that was filled with 30-μm RP-8 (Serva, Heidelberg). Desorption was achieved with a linear gradient of 0–60% acetonitrile in 0.1% TFA in 30 min at a flow rate of 1 mL/min.

Discrimination between Mononitrotyrosyl and Dinitrotyrosyl Derivatives of HPr. Acrylamide gel electrophoresis at pH 9.3 as described by Schmidt-Aderjan et al. (1979) was a suitable tool to distinguish between HPr and its nitrotyrosyl derivatives. At pH 9.3 nitrotyrosyl HPr derivatives have an increased charge, which results in higher electrophoretic mobility.

HPLC Fingerprinting of HPr from S. aureus and Its Nitrotyrosyl Derivatives. A total of 20 nmol of HPr was dissolved in 300 μL of 50 mM NH_4HCO_3; 17 μmol of $CaCl_2$ and 10 μg of trypsin were added for the overnight digestion of the protein at 37 °C. A total of 100 μL of the peptide sample was analyzed on an RP-8 column (4.6 × 250 mm) filled with Nucleosil RP-8 (10 μm) (Machery and Nagel, Düren) by gradient elution: solvent A was 0.1% TFA, solvent B was 85% acetonitrile, and the gradient used was 0–60% solvent B in 60 min. Peptides were detected at 230 nm at 0.16 absorbance units full scale (AUFS) and collected manually according to the absorption response of the detector.

Assignment of Tyrosine-Containing Peptides to the Primary Structure of HPr from S. aureus. The peaks with high UV absorbance (peaks I, II, and III) were collected, dried in a vacuum centrifuge, and subjected to an amino acid analysis. Peak I contained the peptide whose amino acid composition corresponded to peptide 29–43 containing Tyr-37, peak II corresponded to peptide 1–17 containing Tyr-6, and peak III corresponded to peptide 50–60 containing Tyr-56.

Assignment of Nitrotyrosine-Containing Peptides of HPr from S. aureus. The two mononitrotyrosyl derivatives of *S. aureus* HPr isolated by DEAE HPLC were digested with trypsin as described above. Peaks with high UV absorbance were analyzed for nitrotyrosine after acid hydrolysis. Nitrotyrosine-containing peptides showed a characteristic shift to higher hydrophobicity compared to peptide 1–17, whose tyrosine was not modified during the nitration procedure at pH 8.5 (Schmidt-Aderjan et al., 1979).

Table I: Part of the Primary Structure of Heat-Stable Proteins from Some Gram-Positive Bacteria

	30	39
B. subtilis	-Tyr-Asp-Ala-Asp-Val-Asn-Leu-Glu-Tyr-Met-Lys-	
S. faecalis	-Phe-Asn-Ser-Asp-Ile-Asn-Leu-Glu-Tyr-Lys-	
S. aureus[a]	-Phe-Asp-Ser-Ile-Asp-Gln-Gly-Gly-Tyr-Asp-	

[a] From Beyreuther et al. (1977).

HPLC Equipment. A low-pressure gradient former M250-B from Gynkothek, München, a Jasco Twincle HPLC pump, a Rheodyne 7125 sample injector, a Jasco Uvidec 100-III UV monitor (all from Biotronik, München), and a column oven from Knauer, Berlin, were used.

Modification of the Arginyl Residue

The arginyl residues were modified according to Patthy & Smith (1975). A total of 1 μmol of HPr in 1 mL of 0.1 M $Na_2B_4O_7$, pH 8.5, was incubated with 80 μL of a freshly prepared 1,2-cyclohexanedione solution (1 M). After 2 h at 37 °C the reaction was stopped by freezing in dry ice. To monitor the biological HPr activity during the course of the reaction, aliquots were taken, diluted 1:10, frozen, and subjected to the mutant complementation assay.

Assay for HPr Activity

In all cases the mutant complementation assay was used as described by Hengstenberg et al. (1969), employing extracts of the strain S-797A, which is defective in HPr.

NMR Spectroscopy

The NMR samples were prepared by dissolving the freeze-dried protein in 500 μL of an appropriate buffer in 99.75% D_2O. The ^1H NMR spectra were recorded with a Bruker HX-360 spectrometer operating at 360 MHz. The HDO signal was suppressed by a selective presaturation pulse of 0.8-s duration. The sample temperature was kept constant at 308 K. All chemical shift values are referred to 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) as internal standard.

The pH was varied by adding an appropriate amount of DCl or KOD to the samples. The pH was measured with a combination glass electrode (Ingold, Frankfurt, FRG) and was not corrected for the isotope effect. The pH dependence of the chemical shift was fitted to a modified Henderson–Hasselbalch equation as described, e.g., by Kalbitzer & Rösch (1981). The errors given correspond to a 95% confidence level when the *t* test is applied to the experimental data.

Results

Nitrotyrosyl Derivatives of HPr from B. subtilis

Nitration of HPr from B. subtilis. According to preliminary sequence data (Beyreuther, personal communication) and published NMR data (Kalbitzer et al., 1982) HPr from *B. subtilis* contains two tyrosyl residues at positions 29 and 37 in the sequence (Table I). Nitration at pH 8.5 mainly resulted in the formation of 37-mononitrotyrosyl HPr as could be shown by the isolation of the tryptic peptides and their amino acid analysis. The analysis revealed 1 mol of tyrosine and 1 mol of nitrotyrosine per peptide (Table II). The Edman degradation gave a tyrosine as the N-terminal amino acid of the peptide.

The biological activity in the *S. aureus* mutant complementation assay was identical for mononitrotyrosyl HPr and unmodified HPr. The dinitrotyrosyl derivative of HPr showed a decrease in activity of approximately 25%.

Table II: Amino Acid Composition of Nitrotyrosine-Containing Peptides of Mononitrotyrosyl HPr from *B. subtilis* and *S. faecalis*

	composition of peptides from		calcd from sequence of	
amino acid	*B. subtilis*	*S. faecalis*	*B. subtilis*	*S. faecalis*
Lys	0.79	0.93	1	1
His				
Arg				
Asp	3.20	2.60	2	3
Thr	0.06			
Ser	0.09	0.63		1
Glu	0.92	1.93	1	1
Pro				
Gly	1.02	0.47		
Ala	0.99	0.29	1	
Val	0.74	0.39	1	
Met	0.73		1	
Ile	0.04	0.89		1
Leu	0.79	0.95	1	1
Tyr	0.58		1	
Phe		1.0		1
NO_2-Tyr	0.85	0.64	1	1
Asn			1	

^1H NMR Spectroscopy. The nitration of the tyrosyl residue does not result in an overall change of the NMR spectrum as expected for a global change of the protein conformation. The resonance lines in the low-field part of the spectrum of HPr of *B. subtilis* shown in Figure 1 can be assigned to one histidyl residue, one phenylalanyl residue, and two tyrosyl residues by their integrals, their multiplicity, the pH dependence of their chemical shifts, and their coupling pattern (as revealed by homodecoupling experiments). The assignment to specific amino acids in the sequence is trivial for the histidyl and phenylalanyl residues because there is only one of each in the sequence (Beyreuther, personal communication). After nitration of Tyr-37 the doublet of 6.68 ppm corresponding to the C-3/5 protons of one of the tyrosyl residue remains unchanged, whereas the doublet at 6.96 ppm corresponding to the C-3/5 protons of the other tyrosyl residues disappears (Figure 1A,B). Simultaneously, the signal at 7.25 ppm originating from the C-2/6 protons of both tyrosines decreases in intensity, and three new signals appear at 7.75, 7.35, and 6.87 ppm, positions where the signals of the nitrotyrosine are to be expected (Snyder et al., 1975). Therefore, the assignments of Figure 1A are correct. Figure 1B shows two additional resonances at 7.67 and 6.94 ppm. Most probably they come from denatured HPr because of their typical line position and the pH dependence of their chemical shift. The tryptic peptide (Figure 1C) was isolated from the same sample whose spectrum is shown in Figure 1B. Its spectrum shows that it contains about one nitrotyrosine and one tyrosine, but no phenylalanine or histidine (Figure 1C) and no methionine (this part of the spectrum is not shown). This agrees very well with the sequence data mentioned above.

Table III summarizes the results obtained by observing the change of chemical shifts with the pH. In accordance with general practice, the assignment of the histidyl C-2 and C-4 resonances, the tyrosyl C-2/6 and C-3/5 resonances, and the nitrotyrosyl C-6 and C-5 resonances was made according to their chemical shifts, which does not completely exclude that they may be interchanged pairwise (e.g., the high-field histidyl line could come from the C-2 proton instead of the C-4 proton and vice versa).

Nitrotyrosyl Derivatives of HPr from S. faecalis

Nitration of HPr from S. faecalis. According to its primary structure (K. Beyreuther et al., unpublished results) and to

Table III: Titration Parameters of the Aromatic Residues in Native and Mononitrated HPr from *B. subtilis* and *S. faecalis*

		native HPr[a]						mononitrotyrosyl HPr[d]					
		B. subtilis			*S. faecalis*			*B. subtilis*			*S. faecalis*		
residue	protons	p*K*	δ_{AH}	δ_{A^-}	p*K*	δ_{AH}	δ_{A^-}	p*K*	δ_{AH}	δ_{A^-}	p*K*	δ_{AH}	δ_{A^-}
Phe-6	C-2/6	*b*	7.022 ± 0.003	*b*				*b*	7.03 ± 0.01	*b*			
	C-4		6.865 ± 0.003	*b*				*b*	6.867 ± 0.003	*b*			
	C-3/5		7.120 ± 0.003	*b*					7.15 ± 0.01	*b*			
His-7	C-2				7.9 ± 0.1	8.526 ± 0.003	7.541 ± 0.003				7.6 ± 0.1	8.53 ± 0.01	7.53 ± 0.03
	C-4					7.010 ± 0.003	6.571 ± 0.001					7.017 ± 0.005	6.55 ± 0.02
His-15	C-2	5.8 ± 0.1	8.53 ± 0.01	7.777 ± 0.001	6.2 ± 0.1	8.34 ± 0.04	7.688 ± 0.001	6.2 ± 0.2	8.4 ± 0.1	7.776 ± 0.002	6.5 ± 0.1	8.27 ± 0.05	7.653 ± 0.005
	C-4		7.399 ± 0.005	7.141 ± 0.001		7.45 ± 0.02	7.149 ± 0.002		7.35 ± 0.07	7.141 ± 0.003		7.39 ± 0.02	7.103 ± 0.005
Tyr-29	C-2/6	>11[c]	7.255 ± 0.003	*c*				>10[e]	7.259 ± 0.003	*e*			
	C-3/5		6.676 ± 0.003	*c*					6.681 ± 0.003	*e*			
Tyr-37	C-2	10.9 ± 0.1	7.247 ± 0.002	6.96 ± 0.06	10.4 ± 0.2	7.303 ± 0.001	6.97 ± 0.08	6.8 ± 0.4	7.91 ± 0.07	7.75 ± 0.02	6.7 ± 0.2	7.90 ± 0.01	7.714 ± 0.007
	C-6		7.247 ± 0.002	6.96 ± 0.06	10.4 ± 0.2	7.303 ● 0.001	6.97 ± 0.08		7.69 ± 0.05	7.35 ± 0.01		7.71 ± 0.04	7.393 ± 0.007
	C-3		6.958 ± 0.001	6.68 ± 0.09		7.010 ± 0.002	6.70 ± 0.05						
	C-5		6.958 ± 0.001	6.68 ± 0.09		7.010 ± 0.002	6.70 ± 0.05		*f*	6.87 ● 0.03		7.3 ± 0.1	6.89 ± 0.03

[a] The original data from Kalbitzer et al. (1982) were reanalyzed for obtaining the confidence intervals; the experimental conditions are given in Figures 1 and 2, respectively. [b] No change of chemical shift expected. [c] No change of chemical shift observable up to pH 11. [d] For experimental conditions see Figures 1 and 2, respectively. [e] No change of chemical shift observable up to pH 10. [f] No value obtained because of severe overlapping of lines.

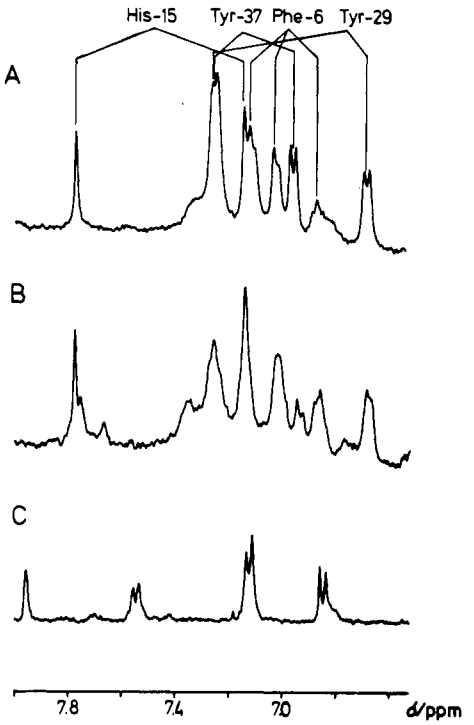

FIGURE 1: Aromatic region of the ^1H NMR spectra of HPr of *B. subtilis* and its nitrotyrosyl derivative. (A) Native HPr of *B. subtilis* (5 mg) in 0.01 M phosphate buffer in D_2O, pH 8.0. The indicated assignments of resonance lines are discussed in the text. (B) Mononitrated HPr (3 mg) in D_2O, pH 9.9. (C) Fragment 28–37 of the mononitrated HPr (0.5 mg) in D_2O, pH 2.0. For all spectra, line broadening by exponential multiplication was 0.5 Hz and the temperature was 308 K.

the NMR data (Kalbitzer et al., 1982) HPr from *S. faecalis* has a single tyrosyl residue in position 37 (Table I). Therefore, nitration can be conducted at high pH, giving a higher yield of the nitrotyrosyl derivative. As described for the nitrotyrosyl derivative of HPr from *B. subtilis*, no significant change in biological activity was detected.

^1H NMR Spectroscopy. As in the case of HPr of *B. subtilis*, the NMR spectra of the streptococcal enzyme show no indication of a larger conformational change after nitration of the tyrosyl residue. The resonance lines of one tyrosyl residue and two histidyl residues were identified previously (Kalbitzer et al., 1982). With the known sequence data (Beyreuther et al., unpublished results) the tyrosyl resonance lines can be assigned to Tyr-37, the only tyrosyl residue in the protein. The resonance lines of the histidyl residue that has a low p*K* value and that can be phosphorylated by enzyme I (Kalbitzer et al., 1982) can now be assigned to His-15. The remaining pair of singlet lines has to come from His-7 (Figure 2A).

As expected, the nitration of the tyrosyl residue leads to the disappearance of the two doublets at 7.30 and 7.01 ppm and to the appearance of three new lines at 7.77, 7.44, and 6.98 ppm (Figure 2B). Again, their multiplet structure (one singlet, two doublets) and their integrals corresponding to one proton each (as compared to the histidyl C-2 H resonances) agree well with the pattern expected for a nitrotyrosyl residue. The variation of the pH allows the calculation of the chemical shifts in the protonated and the deprotonated forms together with the p*K* values (Table III).

Nitrotyrosyl Derivatives of HPr from S. aureus

Nitration of HPr from S. aureus. In an earlier study Schmidt-Aderjan et al. (1979) described the specific mononitration of HPr from *S. aureus* which resulted in the assignment of Tyr-A to Tyr-56 in the amino acid sequence. Evidence for the assignment came from peptide separation by fingerprinting on cellulose thin-layer plates followed by analyzing the nitrotyrosine-containing spots. The assignment, however, did not fit the data from our studies on *S. faecalis*

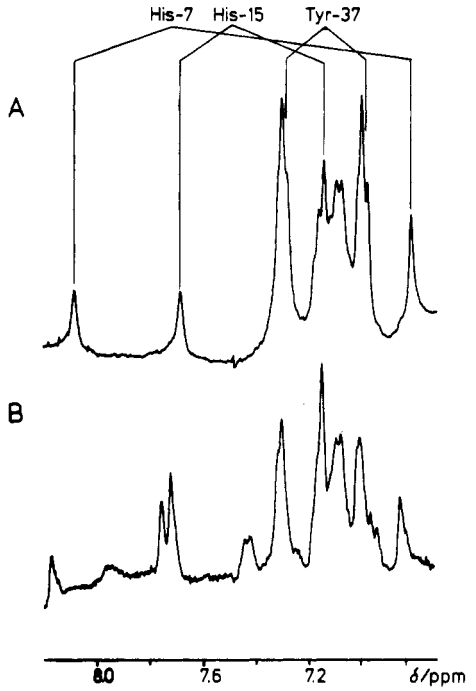

FIGURE 2: Aromatic region of the ^1H NMR spectra of HPr of *S. faecalis* and its nitrotyrosyl derivatives. (A) Native HPr of *S. faecalis* (5 mg) in 0.01 M phosphate buffer in D_2O, pH 7.9. The indicated assignments of resonance lines are discussed in the text. (B) Mononitrated HPr (3.5 mg) in a buffer solution containing 0.02 M phosphate and 0.1 M KCl in D_2O, pH 7.4. For all spectra, line broadening by exponential multiplication was 0.5 Hz and the temperature was 308 K.

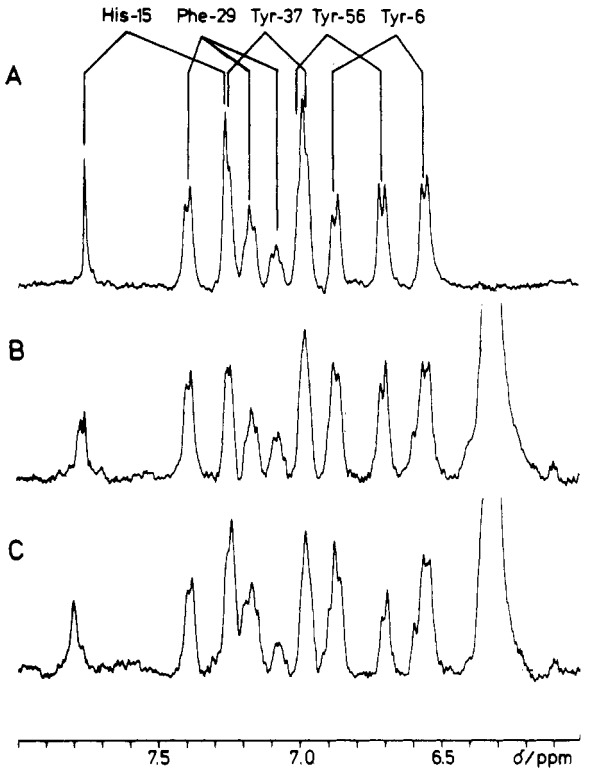

FIGURE 3: Aromatic region of the ^1H NMR spectra of HPr of *S. aureus* labeled at Arg-17 by cyclohexanedione. (A) Native HPr of *S. aureus* dissolved in 0.1 M sodium borate buffer, pH 8.6. (B) Same as (A) but with cyclohexanedione added to an end concentration of 0.15 M, pH 8.3. (C) Same as (B) but with the pH changed to 7.5. For all spectra, line broadening by exponential multiplication was 2 Hz and the temperature was 308 K.

and *B. subtilis* HPr where NMR data and protein chemical studies revealed that Tyr-A (from the low-p*K* tyrosine) must be assigned to Tyr-37. A reinvestigation of the nitration reaction of *S. aureus* HPr showed that mononitrotyrosyl HPr from *S. aureus* could be nitrated either at Tyr-37 or at Tyr-56. Both derivatives were produced simultaneously, their concentration ratio varying from experiment to experiment. HPLC fingerprinting of *S. aureus* and its nitrotyrosyl derivatives clearly showed the tyrosine-containing peptides I, II, and III, which were assigned to the peptides containing Tyr-37, Tyr-6, or Tyr-56 according to the amino acid analysis. Only peptide II contained an arginyl residue, which clearly demonstrated cleavage of the Arg–Pro bond at position 17–18, which has also been observed during sequence studies of *E. coli* HPr (Weigel et al., 1982). Nitration of a tyrosine residue showed a characteristic change in the retention time of the peptide during the gradient elution: the nitrotyrosyl peptides elute later in the gradient. In addition, nitrotyrosine could be detected by amino acid analysis in the correspondent peptide. To exclude any uncertainties in nitration conditions, the same sample used to record the NMR spectrum of the mononitrated HPr from *S. aureus* was digested with trypsin and analyzed with HPLC fingerprinting. The peak pattern was superimposable to that from 37-nitrotyrosyl HPr that had been obtained by separation of 56-mononitrotyrosyl HPr and 37-mononitrotyrosyl HPr on a DEAE column at pH 6.8. Separation of these HPr derivatives on a native gel as done earlier might not resolve the two isomers because of the low p*K* values of nitrotyrosine A and B of 6.4 and 7.7, respectively (Rösch et al., 1981).

^1H NMR Spectroscopy. The nitration of Tyr-37 resulted in the same spectral changes as described previously by Schmidt-Aderjan et al. (1979) for the nitration of Tyr-56 (spectrum not shown). The discrepancy of the assignment can probably be explained from the fact that the location of the modification is strongly dependent on the experimental conditions (as described above) and that probably the NMR spectroscopy and amino acid analysis were done on samples prepared under slightly different conditions (e.g., pH). As mentioned above, in our case the same sample the NMR spectra were taken from was used for the amino acid analysis. This excludes the above errors, and the resonance lines previously assigned to Tyr-56 must to be reassigned to Tyr-37 and vice versa.

Cyclohexanedione-Labeled HPr of S. aureus

The labeling of the arginyl group by cyclohexanedione was performed inside the NMR tube. Figure 3 shows the spectra of native protein and of the same protein after incubation with cyclohexanedione for about 2 h. Although no overall spectral changes are observable, some distinct changes occur after labeling of the arginyl group. The intensity of the His C-2 H resonance line at 7.758 ppm decreases in intensity simultaneously to the growing of two new lines at 7.775 and 7.788 ppm. After a 2-h incubation time the intensity ratio is about 1:1:1; with prolonged incubation time the line at 7.758 ppm almost vanishes. Analogously, the C-4 H line at 7.254 ppm decreases with time, and a new line at 7.240 ppm appears (Figure 3). The time dependence of the intensity and the unchanged chemical shift lead to the conclusion that the lines at 7.758 and 7.254 ppm are assignable to the histidyl C-2 and C-4 protons in the unlabeled protein in the reaction mixture and that the two high-field-shifted and the down-field-shifted resonances come from the C-2 and C-4 protons in labeled HPr, respectively. The results of a pH variation are depicted in Figure 4. The rightmost point in every trace corresponds to the resonance position of the histidyl resonances prior to the

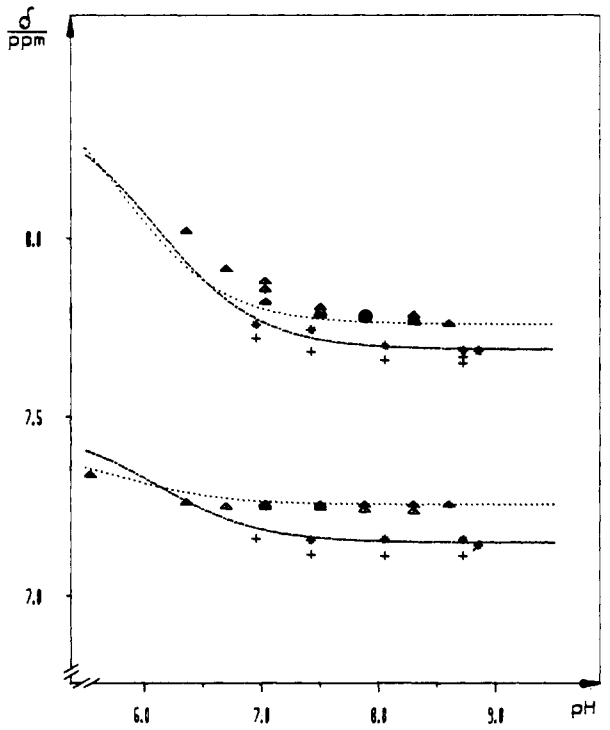

FIGURE 4: pH dependence of chemical shifts of the ring protons of His-15 in native and cyclohexanedione-labeled HPr of *S. aureus* and *S. faecalis*. Experimental conditions are described in Figures 3 and 5. Chemical shift values of the C-2 H and C-4 H resonances of His-15 in unlabeled HPr of *S. aureus* (▲) or in HPr of *S. aureus* labeled at Arg-17 by cyclohexanedione (△) are shown. The values at pH 8.6 were taken prior to the addition of cyclohexanedione. Curves were computed with the p*K* value and chemical shifts given by Rösch et al. (1981). Chemical shift values of the C-2 H and C-4 H resonances of His-15 in unlabeled HPr of S. *faecalis* (*) or in HPr of *S. faecalis* labeled at Arg-17 by cyclohexanedione (+) are shown. As above, the values at pH 8.9 were taken prior to the addition of cyclohexanedione. Curves were computed with the p*K* value and chemical shifts given by Kalbitzer et al. (1982).

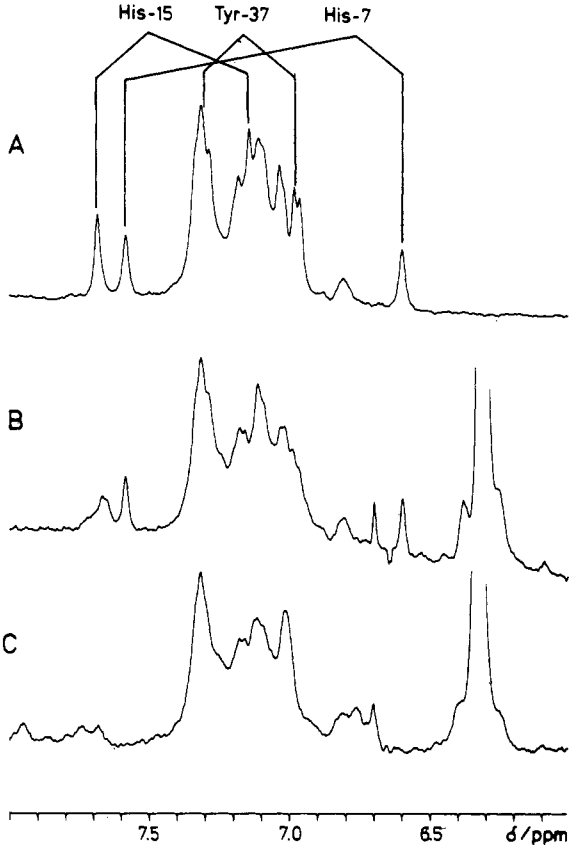

FIGURE 5: Aromatic region of the ^{1}H NMR spectra of HPr of *S. faecalis* labeled at Arg-17 by cyclohexanedione. (A) Native HPr from *S. faecalis* dissolved in 0.1 M sodium borate buffer, pH 8.7. (B) Same as (A) but with cyclohexanedione added to an end concentration of 0.1 M and incubated for 2 h at 308 K and pH 8.7. (C) Same sample as in (B) but at pH 7.4. For all spectra, line broadening by exponential multiplication was 2 Hz and the temperature was 308 K.

addition of cyclohexanedione. One pair of curves shown in Figure 4 was computed with the p*K* value and chemical shifts reported by Rösch et al. (1981) for HPr of *S. aureus*. The experimental points assigned to native HPr agree very well with the theoretical graphs. At low pH the line width increased so much that the lines resolved at high pH could not be observed separately. Besides the effect on the His-15 resonance lines, the addition of cyclohexanedione to the reaction mixture leads to spectral changes at 6.88 and 6.59 ppm that disappear after a longer exposure of the sample at pH 5.5.

Cyclohexanedione-Labeled HPr of S. faecalis

As above, HPr was labeled inside the NMR tube by cyclohexanedione. Comparison of the spectra of native HPr of *S. faecalis* and of HPr reacted with cyclohexanedione (Figure 5) reveals the same principal pattern observed for the staphylococcal enzyme: The C-2 H line of His-15 at 7.687 ppm decreases in intensity concomitantly to the appearance of two new lines at 7.669 and 7.651 ppm with almost equal intensity. In the same way the C-4 H line at 7.15 ppm decreases in intensity, and a new line becomes visible at 7.11 ppm. The time dependence of the intensity and the chemical shift allow the assignment of the resonances at 7.69 and 7.15 ppm to the C-2 H and C-4 H histidyl protons of unlabeled protein even in the reaction mixture. The additional resonances are assignable to His-15 in the labeled protein. The variation of chemical shifts is plotted on Figure 4 as function of the pH for the histidyl protons. The rightmost points are again measured before adding cyclohexanedione. Only at pH 8.7 is the resolution good enough for distinguishing all three histidyl C-2 H resonance lines. At lower pH values the line width is too large compared to the peak separation. The theoretical pH dependence of chemical shift was computed with the p*K* values for His-15 of HPr of *S. faecalis* given by Kalbitzer et al. (1982) and agrees quite well with the experimental points obtained in the reaction mixture.

Tryptic Peptide of HPr from S. aureus

The ^{1}H NMR spectrum of the active center peptide (peptide 1–28) differs clearly from a typical random-coil spectrum, indicating that at least some secondary structure has been preserved. The p*K* value of His-15 could be estimated from the pH dependence of chemical shifts of the correspondent C-2 H and C-4 H resonance lines as 7.0 ± 0.3.

DISCUSSION

Assignments. The nitration studies allow the complete assignment of the aromatic spin systems in HPr of *B. subtilis* to specific amino acids in the sequence. The same is true for HPr of *S. aureus*. HPr of *S. faecalis* contains two histidyl residues, one tyrosyl residue, and two phenylalanyl residues. With the now established primary structure (Beyreuther, unpublished results) and the results of the phosphorylation studies published earlier, the assignments of the histidyl resonance lines that have been suggested from similarities in chemical shifts and their pH dependence (Kalbitzer et al., 1982) can be verified. The assignment of the resonance lines of the only tyrosyl residue is straightforward whereas the

phenylalanyl resonances cannot be assigned by the nitration studies. Although the primary structure of HPr of *S. lactis* is not yet known, the similarity of the chemical shifts and of the corresponding pH dependence of His-A in phosphorylated and unphosphorylated HPr of *S. lactis* to that of His-15 in the HPr of all Gram-positive bacteria examined until now (Kalbitzer et al., 1982) suggests that His-A is identical with His-15 in HPr of *S. lactis*. The fact that the HPr of these bacteria can be replaced mutually in the complementation assay supports this assumption, because the interaction with two different proteins, with enzyme I and factor III, suggests a similarity of the structures at least in the regions responsible for the protein–protein interaction. The same line of argument leads to the conclusion that Tyr-A probably corresponds to Tyr-37. HPr of *S. faecalis* contains a histidyl residue (His-7) with very unusual pK and chemical shifts, which are almost identical with the values found for His-B of HPr of *S. lactis*. By analogy His-B can be assigned to His-7 in the sequence. Inversely, the above assignments allow the prediction that HPr of *S. lactis* probably contains two histidyl residues at positions 7 and 15 of the sequence and a tyrosyl residue at position 37.

Tyr-37 and Its Interaction with His-15. In all examined HPr of Gram-positive bacteria Tyr-37 has a relatively low pK value and can be deprotonated without denaturation of the whole proteins, as concluded from the spectral data. Tyr-37 can be nitrated in the HPr of *S. aureus* (Schmidt-Aderjan et al., 1979; this work), of *S. faecalis*, and of *B. subtilis* without signs of a global denaturation in the NMR spectrum and without significant changes of their phosphocarrier activity. Taking both pieces of evidence together, it follows that Tyr-37 is located at the surface of these proteins and that it is accessible by water molecules as well as by the larger molecule tetranitromethane.

In all mononitrated HPr an interaction between Tyr-37 and the active center histidine His-15 is clearly observable. The most obvious influence of the nitration can be seen on the pK of His-15: It increases from 5.8 to 6.7 in the HPr of *S. aureus* (Rösch et al., 1981), from 5.8 to 6.2 in the HPr of *B. subtilis* (Table III), and from 6.2 to 6.5 in the HPr of *S. faecalis* (Table III) when fitted by assuming only one pK value. Some of the chemical shift values calculated for very low and very high pH values are influenced by the nitration of the proteins, but the effects are mostly rather small. From the HPr of *S. lactis* no nitration data exist. But even in the unmodified protein a mutual influence between His-15 and Tyr-37 could be observed (Kalbitzer et al., 1982). Clearly, the interaction between His-15 and Tyr-37 first observed in the protein of *S. aureus* is not a singular phenomenon but appears to be common to all HPr of Gram-positive bacteria. The increase in pK of His-15 could easily be explained as a direct effect of the negative charge of the nitro group: According to the unmodified Tanford–Kirkwood theory the expected change of pK_i, ΔpK_i, of the ith group by a charged group j can be calculated by $\Delta pK_i = -W'_{ij}z_iZ_j/2.303kT$, where W'_{ij} is the free energy of interaction for a pair of point charges i and j corrected for the accessibility of site j, z_i is the unit charge, and Z_j is the fractional charge of site j (Tanford & Kirkwood, 1957; Matthew et al., 1979). Qualitatively, a negative charge near the histidyl ring would stabilize its positively charged low-pH form, but one should not forget that the electrostatic interaction is a long-range interaction depending on different factors quite difficult to determine a priori. In the worst case an effect as observed in our nitration studies could be caused by a charge as much as 0.9 nm apart (Matthew et al., 1979). The large pK change found in the HPr from *S. aureus* (Rösch et al., 1981) would impose an smaller upper limit of about 0.4 nm for the separation of the charged groups.

The nitration of Tyr-37 in HPr of *S. faecalis* seems also to affect the pK value of His-7 but not its chemical shifts (Table III). The change of pK is simply due to the differences in ionic strength, because it could be shown that the pK value of His-7 is very sensitive to the ionic strength compared to His-15 (data not shown).

Interaction between Arg-17 and His-15. The modification of Arg-17 by cyclohexanedione leads to a remarkable decrease of the HPr activity in the complementation assay when borate ions are present (Muss et al., unpublished results). As the phosphorylation assay indicates, this is caused at least partly by the inhibition of the phosphorylation of His-15 by enzyme I and/or by the decreased stability of phosphohistidine against hydrolytic cleavage. There are several reasons for this effect that may be discussed: (1) a general conformational change (protein denaturation) induced by the chemical agent; (2) steric hindrance by the bulky group of the label; (3) a specific effect because the positively charged arginyl residue plays a role in the phosphoryl transfer or in the stabilization of the phosphohistidine complex. A denaturation of a larger part of the total protein can be ruled out because there is no sign for such a process in the NMR spectra and because the inactivation is 70% reversible when the label is removed by hydroxylamine (Muss et al., unpublished results). The remaining 30% could be due to irreversible side reactions (Patthy & Smith, 1975). This corresponds to the observation that after prolonged reaction times the NMR signals of Tyr-6 in the staphylococcal enzyme and of His-7 in the streptococcal enzyme (Figure 3 and 5) are partly disturbed and that the spectral changes are not reversed by reacting the protein with hydroxylamine.

The activities of HPr labeled by cyclohexanedione in the absence of borate ions and of HPr where the label was removed by hydroxylamine are almost identical; only after complexing with borate does the labeling of Arg-17 lead to a drop in the enzymatic activity to about 20% of the initial activity (Muss et al., unpublished results). This means that Arg-17 is not involved in any covalent bond during the catalytic process and that the dihydroxycyclohexylene group of the label does not disturb the enzymatic activity simply by steric hindrance. However, the additional binding of borate affects the activity either because the label is now bulky enough to block the active center or because the compensation of the positive charge, which is even present in the (dihydroxycyclohexylene)arginyl residue, by the negative charge of the borate group cancels an electrostatic interaction necessary for the proper enzymatic process.

One possibility for such a specific electrostatic interaction could be the suggested lowering of the pK value of the active center histidine by Arg-17 (Rösch et al., 1981). Figure 4 demonstrates that the pK value of His-15 does not increase very much after labeling. The maximum increase of the pK can be estimated from Figure 4 as about 0.2 unit for the streptococcal enzyme. For the staphylococcal protein there even appears to be a small decrease of pK. Therefore, this mechanism of inactivation can be ruled out.

The labeling of Arg-17 leads to distinct changes of the chemical shifts for the C-2 H as well as for the C-4 H resonance lines of His-15 in both studied proteins (Figures 3–5). The C-2 H lines are split in two lines of approximately the same integral (hardly visible in the figures because of the large scale), indicating two conformations with about the same population with a half-life of more than 34 and 25 ms for the

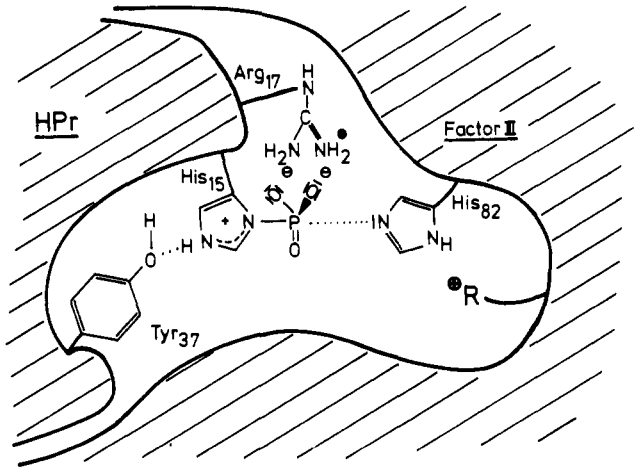

FIGURE 6: Possible mechanism of the phosphoryl transfer from HPr to factor III.

staphylococcal and the streptococcal enzymes, respectively. Although these two states could simply be explained by two different conformations of the labeled arginine or even worse by two chemically distinct species, the most attractive interpretation would be that the bulky label at the active center "freezes" two conformations of the native HPr that are usually in fast exchange and are therefore not distinguishable in the NMR spectra. This argument is supported by the observation that even in native HPr two conformations can be observed at low temperatures that seem again to be limited mainly to the active center (unpublished results). These two conformations could be related to the recently described regulation of the HPr activity by phosphorylation of a serine residue (Deutscher & Saier, 1983) or to a conformational change after phosphorylation of the active center histidine as postulated by Dooijeward et al. (1979) for the *E. coli* protein. A simpler explanation would be that the flipping of the histidyl ring is hindered by the label. In any case, the differences between the two conformations can only be very small or only local because the ^{1}H NMR spectra exclude the existence of two largely different conformations.

The Low pK Value of His-15. The active center histidine (His-15) in all HPr studies (Gassner et al., 1977; Doijewaard et al., 1979; Kalbitzer et al., 1982) has rather low pK, between 5.6 in *E. coli* and 6.2 in *S. faecalis*. In the model peptide Gly-Gly-His-Ala a pK value of 7.0 and 6.9 was found (Bundi & Wüthrich, 1979; Kalbitzer & Rösch, 1981), a value much higher than that found in the proteins. Accordingly, in peptide 1–28 of HPr of *S. aureus* His-15 now has a pK value around 7, indicating either that the amino acids causing the low pK are located in the other part of the sequence or that they are close enough only in the native structure.

There are three main factors influencing the intrinsic pK value of an individual group in a protein, namely, hydrogen bonding, water accessibility, and electrostatic interactions. A natural candidate for explaining the low pK of His-15 seems to be the positively charged arginyl residue Arg-17 (Rösch et al., 1981) that is in the neighborhood of His-15. The effect of the electrostatic interaction on the pK is rather sensitive to the ionic strength if both residues interacting are located at the surface of the protein (Tanford & Kirkwood, 1957; Matthew et al., 1979).

His-15 itself is located at the surface of the protein because it must be accessible during the phosphoryl transfer from enzyme I to factor III and can be labeled by phosphoamidate (Gassner et al., 1977). The same is true for Arg-17 (see above). Nevertheless, the pK value of His-15 in HPr of *S. aureus* and *S. faecalis* is only slightly dependent on the ionic strength I, increasing only by less than 0.1 unit from I = 0.001 M to I = 0.1 M. The introduction of the negatively charged borate group in the cyclohexanedione HPr derivatives has again only a minor influence on the pK of His-15. Both these pieces of evidence speak against the hypothesis that the low pK of His-15 is mainly caused by the electrostatic interaction with Arg-17. There remains the possibility that the low pK is due to positively charged groups located inside the protein or by hydrogen bonding to another residue. A possible candidate would be Tyr-37, which is contained in the HPr proteins of all Gram-positive bacteria examined until now and is replaced in the HPr of *E. coli* (Weigel et al., 1982) by a seryl residue again carrying an –OH group. In Figure 6 a possible structure of the complex of phospho HPr with factor III prior to the phosphoryl transfer is depicted that would be consistent with the data know today.

Registry No. PTS, 56941-29-8; Tyr, 60-18-4; His, 71-00-1; Arg, 74-79-3.

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