HPr Proteins of Different Microorganisms Studied by Hydrogen-1 High-Resolution Nuclear Magnetic Resonance: Similarities of Structures and Mechanisms†

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ABSTRACT: The HPr proteins of *Streptococcus lactis*, *Streptococcus faecalis*, *Bacillus subtilis*, and *Escherichia coli* were studied by ^{1}H NMR at 360 MHz. The "active-center" histidines of all HPr proteins are characterized by a low pK value between 5.6 and 6.1 and similar spectral parameters. Phosphorylation of the histidyl residues leads to an increase of the pK value of 2–3 units and spectral changes characteristic for N-1 phosphorylation of the histidyl ring. The spectra of the HPr proteins of *S. lactis*, *S. faecalis*, *B. subtilis*, and *Staphylococcus aureus* reveal many similarities, whereas the spectrum of the *E. coli* protein is different with exception of the active-center histidine. The HPr protein of *S. lactis* is formylated at its terminal amino group.

In most anaerobic and facultatively anaerobic microorganisms carbohydrates are transported across the cell membrane by the phosphoenolpyruvate-dependent phosphotransferase system (PTS)[1] (Hengstenberg, 1977; Dills et al., 1980). A constitutive protein of this transport system is the phosphocarrier protein HPr which transfers a phosphoryl group from enzyme I to the carbohydrate-specific components of the PTS, factor III and enzyme II.

Although HPr proteins of different microorganisms could be isolated and purified to homogeneity, no X-ray structure has been published until now; the primary structure is known only for the HPr protein of *S. aureus*. Another approach which can give some insight into structural properties of proteins and their relations to the enzymatic function, namely, high-resolution NMR, was used for investigations on the HPr proteins of *E. coli* and *S. aureus* (Dooijewaard et al., 1979; Maurer et al., 1977; Schmidt-Aderjan et al., 1979; Rösch et al., 1981; Kalbitzer & Hengstenberg, 1981).

Recently we achieved to purify the homologous HPr proteins of *S. faecalis*, *S. lactis*, *B. subtilis*, and *E. coli* in quantities sufficient for ^{1}H NMR experiments. Herewith the basis has been procured to recognize general structural properties of the HPr proteins by comparative ^{1}H NMR studies.

Materials and Methods

(*A*) *Preparation of HPr Proteins.* (*1*) *Bacterial Strains.* *S. faecalis* 26487 and *S. lactis* 48847 were obtained from the Institut für Milchforschung, Streptococcenzentrale, Kiel, West Germany. *B. subtilis* W 1360 was a gift of Professor Winkler, Bochum. *E. coli* j 623 was kindly provided by Professor Gershanowitch, Moscow.

(*2*) *Cell Cultivation.* A 100-L sample of sterilized medium (0.1% Tryptone, 1% yeast extract, 1% glucose, 0.02% $MgSO_4 \times 2H_2O$, 0.1% KCl) was seeded with a 3-L overnight culture of bacteria. The fermenter was run at 37 °C, the pH was adjusted to 7.5 with NaOH, and aeration was kept moderate to avoid foaming. After 6–7 h of growth, the cells reached the stationary phase OD_{580} 10–11 and were harvested with a Westfalia continuous flow centrifuge. The yield was about 1 kg of wet cell paste.

(*3*) *Cell Disruption.* A 250-g sample of cells was suspended in 800 mL of standard buffer (Beyreuther et al., 1977) and then passed through the continuous flow device of a Dynomill KDL (Bachofen, Basel, Switzerland).

(*4*) *Purification of the Proteins.* Crude extracts were centrifuged at 11000*g* for at least 2 h. The supernatant (about 1200 mL) was applied to a DE 23 (Whatman) column (12 × 12 cm). After elution with 3 L of standard buffer a gradient of 6 L of 0–0.5 M NaCl in standard buffer was applied. Fractions of 120 mL were collected, and the HPr activity was localized by a mutant complementation assay of Hengstenberg et al. (1969). The fractions containing HPr activity were pooled, adjusted to pH 5 with acetic acid, and heated to 65 °C for 5 min. The precipitate was removed by centrifugation at 11000*g* for 1 h. The supernatant was adjusted to pH 7 with Tris base and concentrated to 20 mL with an Amicon pressure dialysis (UM 2) membrane. The dialyzate was applied to a Sephadex G-75 column (5 × 90 cm) and eluted with standard buffer. Fractions containing HPr activity were determined, and the purity of the protein was monitored by acrylamide gel electrophoresis at pH 9.5 in the native state and in $NaDodSO_4$ gels. Fractions containing pure HPr protein were pooled, concentrated, and desalted by gel filtration over a G-25 column run with 0.05 M NH_4HCO_3 buffer. The HPr-containing eluate was freeze-dried. For pure *S. lactis* HPr, an additional DE 52 column was necessary as described by Beyreuther et al. (1977) for the preparation of *S. aureus* HPr.

(*5*) *Yield of Freeze-Dried Protein from Various Bacterial Species.* *B. subtilis* (80–100 mg), *S. faecalis* (80–100 mg), and *S. lactis* (about 30 mg) were obtained from 250 g of cells (wet weight). All column steps were run at 4 °C.

(*6*) *Purification of E. coli HPr.* (*a*) *Cell-Free Extract.* A 300-g sample of wet cell paste, strain j 623, suspended in 600

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[1] Abbreviations: 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*; *S. typhimurium*, *Salmonella typhimurium*; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl.

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mL of standard buffer was passed through the Dynomill KDL as described by Beyreuther et al. (1977). Crude extract was centrifuged at 14000*g* for 1 h at 8 °C.

(*b*) *DEAE-cellulose Chromatography.* The cell-free extract was applied to a DE 25 (Whatman) column (12 × 20 cm), which was first washed with 1.5 L of standard buffer. HPr was eluted with a salt gradient of 7 L of 0–0.8 M NaCl standard buffer. HPr activity was usually detected in the first third of the gradient.

(*c*) *Acid Precipitation.* The HPr pool of the DE 23 column was adjusted to pH 4.2 with HCl, and the resulting precipitate was removed by centrifugation at 14000*g* for 1 h. The supernatant was readjusted to pH 7.2.

(*d*) *Heat Precipitation.* The above pool was heated to 72 °C for 3 min and centrifuged as above. The clear supernatant was concentrated to 20 mL by pressure dialysis by using an Amicon UM2 membrane.

(*e*) *Sephadex G-75 Chromatography.* The dialyzate from the previous step was run on a G-75 column (5 × 90 cm) and eluted with 0.05 M NH_4HCO_3, pH 8.7.

(*f*) *DEAE 52 Chromatography.* The fractions from the G-75 step were applied to a DE 52 (Whatman) column (1.5 × 20 cm) and eluted with a gradient of 500 mL of 0–0.3 M NH_4HCO_3, pH 8.7.

(*g*) *Hydroxylapatite Chromatography.* The eluate from above was adjusted to pH 7.5 and loaded on a HA Ultragel (LKB) column. HPr protein eluted at 0.05 M NH_4HCO_3, pH 8.7. The protein was pure on native acrylamide gels run at pH 9.3 in Tris–glycine buffer and by isoelectrofocusing. The yield of pure freeze-dried protein was 13 mg corresponding to 13% recovery. The total protein in the crude extract was 16 g.

(*7*) *Assay for HPr Activity.* A semimicrocuvette was loaded with the following components: 400 μg of enzyme I, 5 μg of enzyme II (wet membrane fragments), 2.4 μmol of PEP monocyclohexylammonium salt, 0.5 μmol of glucose, 0.2 mol of NADP (Boehringer Mannheim), 5 μmol of $MgCl_2$, 2.5 μg of glucose-6-P dehydrogenase (Boehringer) from yeast, and 100 μL of sample containing HPr. The total volume was 380 μL, and the incubation time was 30 min. The reaction was stopped with 500 μL of 10% Na_2CO_3, and the increase in OD at 366 nm was recorded.

(*B*) *Preparation of Enzyme I.* (*1*) *Enzyme I from either S. aureus or S. faecalis*, eluted from the DE 23 column at 0.3–0.4 M NaCl activity, was assayed with the *S. aureus* mutant complementation assay. The protein was precipitated with 70% ammonium sulfate and dialyzed against 0.05 M sodium acetate buffer, pH 5. The supernatant was concentrated by pressure dialysis and run on a G-100 column (pH 5) (5 × 100 cm). Active material from that column was dialyzed against water and freeze-dried (Alpert, 1980).

(*2*) *Enzyme I of E. coli.* A 40-mL sample of crude extract of the HPr preparation was fractionated on a (5 × 90 cm) Sephadex G-75 column calibrated with marker proteins. Enyzme I was detected close to the void volume of the column with the colorimetric assay just described. The elution buffer was 0.05 M NH_4HCO_3, pH 8.7. The pooled fractions were adjusted to pH 5 with acetic acid. After centrifugation the supernatant was freeze-dried. Enzyme I prepared by this method was suitable for the HPr assay and for *E. coli* HPr phosphorylation.

(*C*) *Phosphorylation of HPr Protein.* Phosphorylation of HPr was done with enzyme I and PEP in the NMR tube. HPr (5 mg) in 0.5 mL of D_2O adjusted to pH 8.5 with NaOD, 0.5 μmol of $MgCl_2$ in D_2O, and 0.4 μmol of PEP in D_2O were mixed in the NMR tube. The reaction was started by addition of 30 μg of enzyme I in D_2O. Spectra were recorded after the time indicated below.

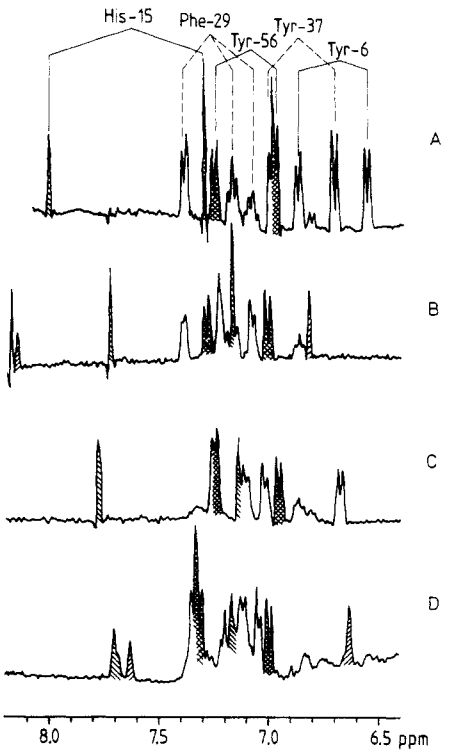

FIGURE 1: Downfield region of the 360-MHz spectra of different HPr proteins. HPr protein of *S. aureus* (A), *S. lactis* (B), *B. subtilis* (C), and *S. faecalis* (D). The resonance lines are assigned according to Schmidt-Aderjan et al. (1979). Temperature 308 K; pH 6.2 (A), 7.7 (B), 8.4 (C), and 8.8 (D), respectively. Resolution enhanced by Gaussian multiplication (Ferrige & Lindon, 1978). His-A (\\\), His-B (///), Tyr-A (XXX).

(*D*) *Preparation of the ^1H NMR Samples.* Freeze-dried protein (5–10 mg) was dissolved in 500 μL of 0.01 M phosphate buffer, pH 8, in 99.75% D_2O. The phosphate buffer was purified from possible contaminations by divalent ions by passage through a Chelex 100 column. After passage through the column, the buffer was dried, dissolved in D_2O, and dried again. An appropriate amount of the obtained powder was used for preparing the buffer for the NMR measurements.

(*E*) *NMR Measurements.* 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 digital resolution was 0.2 Hz. 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, and the pH values were measured with a combination glass electrode of Ingold, Frankfurt, FRG, and are not corrected for the isotope effect. The pH dependence of the chemical shifts was fitted to a modified Henderson–Hasselbalch equation as described by Kalbitzer & Rösch (1981).

Results

Histidyl Residues. Figure 1 shows the downfield part of the ^1H NMR spectra of HPr proteins of *S. aureus* (A), *S. lactis* (B), *B. subtilis* (C), and *S. faecalis* (D) and Figure 3A,B that of the *E. coli* protein. The chemical shift values of the histidine C-2 and C-4 proton resonances cannot be compared directly in these figures, because the pH values differ slightly

Table I: Titration Parameters of the Histidyl Residues of Different HPr Proteins[a]

HPr of	proton	His-A			His-B		
		pK	δ_{AH} (ppm)	δ_{A^-} (ppm)	pK	δ_{AH} (ppm)	δ_{A^-} (ppm)
S. aureus[b]	C-2	5.8	8.50	7.76			
	C-4		7.41	7.26			
S. lactis	C-2	6.0	8.38	7.73	8.1	8.53	7.58
	C-4		7.48	7.17		6.98	6.56
B. subtilis	C-2	5.8	8.53	7.78			
	C-4		7.40	7.14			
S. faecalis	C-2	6.1	8.37	7.69	8.0	8.52	7.54
	C-4		7.47	7.15		7.01	6.57
E. coli[c]	C-2	5.6 (5.6)	8.56 (8.62)	7.84 (7.88)	6.0 (6.0)	8.71 (8.78)	7.79 (7.88)
	C-4		7.39 (7.43)	7.25 (7.33)		7.01 (7.10)	6.63 (6.72)

[a] δ_{AH}, δ_{A^-}, and pK are the results of a nonlinear least-squares fit to a modified Henderson–Hasselbalch equation. [b] Values as given for His-15 by Rösch et al. (1981). [c] Values in parentheses from Dooijewaard et al. (1979).

Table II: Titration Parameters of the Histidyl Residues in Phosphorylated HPr Proteins[a]

HPr of	proton	His-A			His-B		
		pK	δ_{AH} (ppm)	δ_{A^-} (ppm)	pK	δ_{AH} (ppm)	δ_{A^-} (ppm)
S. aureus[b]	C-2	8.3	8.58	7.9			
	C-4		7.35	6.98			
S. faecalis	C-2	7.8	8.52	7.82	8.0	8.52	7.54
	C-4		7.16	6.82		7.01	6.57
E. coli[c]	C-2	8.0 (7.8)	8.57 (8.69)	7.92 (8.00)	6.0 (6.0)	8.71 (8.78)	7.79 (7.88)
	C-4		7.29 (7.38)	6.98 (7.03)		7.01 (7.10)	6.63 (6.72)

[a] δ_{AH}, δ_{A^-}, and pK are the results of a nonlinear least-squares fit to a modified Henderson–Hasselbalch equation. [b] Values as given for His-15 by Gassner et al. (1977). [c] Values in parentheses from Dooijewaard et al. (1979).

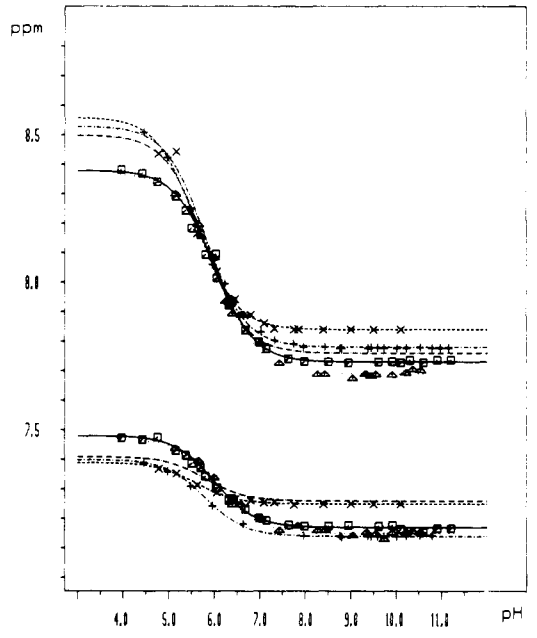

FIGURE 2: pH dependence of chemical shift of the C-2 H and C-4 H resonances of His-A and His-15, respectively. The theoretical curves were computed with the parameters of Table I. Experimental conditions as described under Materials and Methods. HPr protein of *S. lactis* (□), *B. subtilis* (+), *S. faecalis* (△), and *E. coli* (×). The titration curve for the HPr protein of *S. aureus* (no data points) was computed from the parameters given by Rösch et al. (1981) (–·–).

from spectrum to spectrum. The spectra of the HPr proteins of *S. lactis*, *S. faecalis*, and *E. coli* contain four singlet resonances and that of *B. subtilis* two resonances, which can easily be identified as the C-2 and C-4 proton resonances of histidyl residues by the pH dependence of their chemical shift, their multiplicity, and their intensity.

The obtained titration parameters are summarized in Table I where the histidyl residues are arbitrarily named His-A and His-B. All four HPr proteins contain a histidyl residue

Table III: Titration Parameters of Tyrosyl Residues of Different HPr Proteins[a]

HPr of	protons	Tyr-A		
		pK	δ_{AH} (ppm)	δ_{A^-} (ppm)
S. aureus[b]	C-2/6	10.7	7.26	7.12
	C-3/5		6.99	6.70
B. subtilis	C-2/6	10.9	7.25	6.96
	C-3/5		6.96	6.68
S. lactis[c]	C-2/6	6.0/10.2	7.28/7.29	7.09
	C-3/5		7.03/7.01	6.70
S. faecalis	C-2/6	10.6	7.30	6.97
	C-3/5		7.01	6.70

[a] δ_{AH}, δ_{A^-}, and pK are results of a nonlinear least-squares fit to a modified Henderson–Hasselbalch equation. [b] Values as given for Tyr-56 by Rösch et al. (1981). [c] Results of a computer fit to an equation allowing two different pK values (Kalbitzer & Rösch, 1981).

(His-A) with a low pK value of about 6 and the HPr proteins of *S. lactis* and *S. faecalis* an additional histidyl residue (His-B) with a pK value of about 8. The pK value of His-B of the HPr protein of *E. coli* (6.0) differs clearly from that of the other HPr proteins. The experimental data for the pH dependence of chemical shifts of the ring protons of His-A are presented in Figure 2 together with curves computed from the parameters of Table I.

The phosphorylation of the HPr protein of *S. faecalis* and *E. coli* with PEP and enzyme I in the NMR sample tube leads to changes in the chemical shifts of the C-2 and C-4 proton resonances of His-A and the pK value of this histidyl residue, whereas His-B remains uninfluenced. This affirms that His-A becomes phosphorylated in HPr protein of *S. faecalis* and *E. coli* by enzyme I (Table II). Moreover, the changes of the spectral parameters of His-A agree well with those observed on the model peptide Gly-Gly-His-Ala upon phosphorylation (Kalbitzer & Rösch, 1981).

Other Aromatic Residues. The spectra of the four HPr

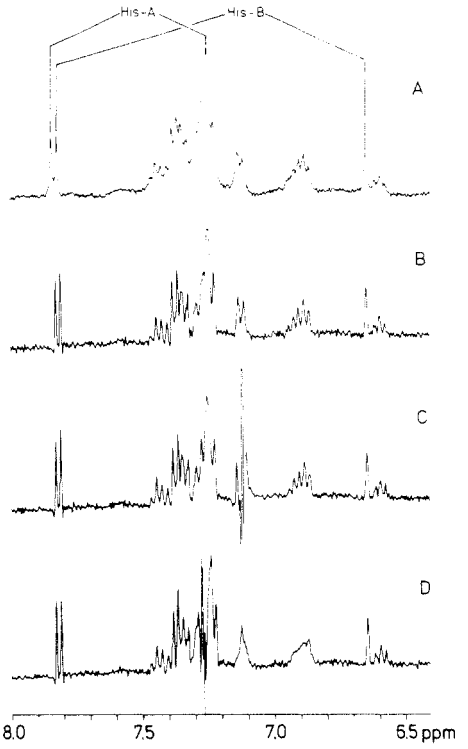

FIGURE 3: HPr protein of *E. coli*. Signal to noise improved by multiplication of the free induction decay with an exponential function (A); resolution enhanced by Gaussian multiplication (Ferrige & Lindon, 1978) (B–D). Decoupling at 7.12 (C) and 7.27 ppm (D), respectively.

proteins depicted in Figure 1 contain at least one pair of doublet resonances with an integral corresponding to approximately two protons each. From the pH dependence of their chemical shifts and their mutual J coupling (as proved by decoupling experiments), they can be assigned to C-2/6 and C-3/5 protons of a tyrosyl residue. The parameters obtained from the titration data are compiled in Table III and are very similar for the tyrosyl residues (Tyr-A) in each of the HPr proteins.

The HPr protein of *E. coli* shows a doublet resonance at 7.12 ppm coupled to a resonance line at 7.27 ppm (Figure 3). Its intensity corresponds to 1.8 ± 0.2 protons as compared to the intensity of the histidyl C-2 H resonances. No pH dependence of the chemical shift can be detected up to pH 11.

The resonances of the ring protons of Tyr-A of the protein of *S. lactis* are especially well observable because they do not overlap with other lines in the spectrum. At acidic pH a deviation of their chemical shift values from the curve predicted by the simple Henderson–Hasselbalch equation occurs; this titration behavior can only be described correctly under the assumption of a second pK value in this pH range. The phosphorylation of the active-center histidine of the *E. coli* protein causes a downfield shift of the resonances at 7.27 and 7.12 ppm of 0.01 and 0.04 ppm, respectively.

Downfield Shifted $C_\alpha H$ Resonances. The NMR spectra of the HPr protein of *S. lactis* and *S. faecalis* contain a doublet resonance with a pH-dependent chemical shift and a coupling constant which is very similar in both proteins (J = 10.0 ± 0.5 Hz). The chemical shifts at low pH are 5.97 and 6.01 ppm and at high pH are 5.88 and 5.87 ppm for the HPr protein of *S. lactis* and *S. faecalis*, respectively. The corresponding pK values can be computed as 8.4 and 8.0, close to that of His-B in both proteins. These resonances can only be found in those proteins which also contain the high pK His-B, i.e., in *S. lactis* and *S. faecalis*.

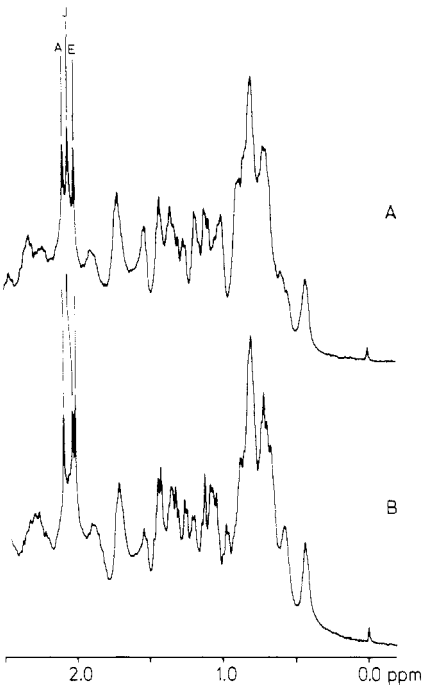

FIGURE 4: High-field region of the 360-MHz spectrum of HPr protein of *S. lactis* at pH 6.1 (A) and pH 8.5 (B).

Table IV: Chemical Shifts of the Methionyl CH_3 Resonances of Different HPr Proteins

	HPr protein of				
S-CH_3 of	*S. aureus*	*B. subtilis*	*S. lactis*	*S. faecalis*	*E. coli*
Met-A	2.101	2.098	2.097	2.097	
Met-B	2.059				
Met-C	2.059				
Met-D				2.040	
Met-E		2.021	2.021		
Met-F		2.005			
Met-G	1.998				
Met-H					1.965
δ_{AH}(Met-J)			2.107	2.102	
δ_{A^-}(Met-J)			2.031	2.040	
pK(Met-J)			5.8	5.9	

Methionyl Residues. The HPr protein of *S. aureus* contains four singlets around 2 ppm which were tentatively assigned to the methyl groups of the four methionyl residues (Rösch et al., 1981). Resonance lines are observable in the same chemical shift range for the HPr protein of *B. subtilis*, *S. lactis*, *S. faecalis*, and *E. coli* with the characteristics of methionyl CH_3 groups, i.e., a singlet structure and an integral corresponding to three protons (see, e.g., Figure 4). In Table IV the chemical shift values of these resonance lines are arranged into groups with about the same chemical shift values and arbitrarily labeled as Met-A, -B, etc.

Formyl Group. Only the HPr protein of *S. lactis* shows an additional ^{1}H NMR resonance at 8.18 ppm which does not shift with the pH. The intensity of this line equals that of the observed histidyl C-2 H or C-4 H resonances and therefore corresponds to one proton. The resonance line of the proton in the N-terminal CHO group is observable at 8.143 ppm (pH 6.9–12) in *N*-formyl-L-methionyl-L-valine. It exhibits only a slight pH dependence of the chemical shift at low pH values (8.126 ppm at pH 2.0). The common amino acids do not show any resonance line with these characteristics. Therefore one may conclude that the resonance at 8.18 ppm is due to a CHO

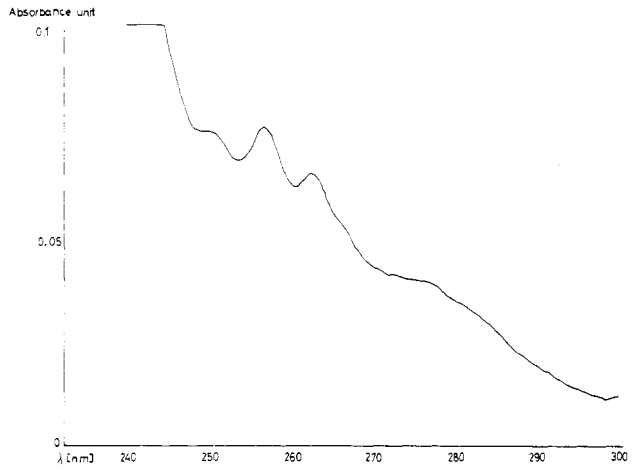

FIGURE 5: UV spectrum of HPr protein of *E. coli*.

group bound to the terminal amino group of this HPr protein.

Discussion

Heterogeneity of HPr Proteins of E. coli. Anderson et al. (1971) reported that HPr proteins of *E. coli* contain no tyrosine. In contrast, Roosien et al. (1979) prepared *E. coli* HPr containing about 0.5 mol of tyrosine per mol of protein as shown by NMR spectroscopy and amino acid analysis. They suggested a principal heterogeneity of the HPr proteins of *E. coli* caused by the existence of two different genes, one of them coding for one HPr protein containing four phenylalanyl residues and one tyrosyl residue and the other containing five phenylalanyl residues and no tyrosyl residue. The HPr protein we isolated contained no tyrosine. During a few preparations we observed a tyrosine-containing contaminant together with HPr which could be separated by chromatography on an LKB TSK 2000 SW column. The UV spectrum of pure HPr protein (Figure 5) is almost identical with the spectrum of Anderson et al. (1971). The amino acid analysis does not show tyrosine. Attempts to isolate a nitrated derivative failed. From data available for HPr from *Salmonella* (S. Roseman, personal communication), no tyrosine was detected in *Salmonella* HPr.

The overall features of the NMR spectra we recorded (Figure 3) appear similar to those reproduced by Dooijewaard et al. (1979) and Roosien et al. (1979). The results we obtained differed slightly in the chemical shift values for the histidyl resonances (Table I). The better resolution of our spectra may suggest that our procedure of protein purification yielded a more homogeneous HPr protein of *E. coli*.

Active-Center Histidine. All proteins listed in Table I contain a histidyl residue with a low pK value (between 5.6 and 6.1) and similar chemical shifts.

For the proteins of *S. aureus* (Gassner et al., 1977), *S. faecalis* (this paper), and *E. coli* (this paper; Dooijewaard et al., 1979), it could be shown that this histidine is phosphorylated during the phosphoryl transfer. For the HPr protein of *B. subtilis* and *S. lactis* the same should hold by analogy.

The abnormally low titration step of the active-center histidyl C-4 H resonances found in the HPr proteins of *S. aureus* (0.15 ppm) (Gassner et al., 1977; Rösch et al., 1981) and of *E. coli* (0.10 and 0.14 ppm, respectively) (Table I) seems to be characteristic for these HPr proteins only. The studies on other HPr proteins reveal a titration step of 0.31, 0.26, and 0.32 ppm for the histidyl residues of the HPr protein of *S. lactis*, *B. subtilis*, and *S. faecalis*, respectively, not very different from the value of 0.38 ppm found in the model peptide Gly-Gly-His-Ala by Bundi & Wüthrich (1979). Therefore the low titration step can no longer be interpreted as a general feature of the active-center histidines in HPr proteins connected closely with the enzymatic mechanism common to all the HPr proteins as it was tentatively done before (Dooijewaard et al., 1979).

Upon phosphorylation the pK value increases dramatically: at neutral pH, the protonation state of the histidyl residues changes almost completely from deprotonated to protonated, the histidyl ring itself carrying now a positive charge. In contrast to the low titration step of the histidyl resonances discussed above, the change of pK seems a feature common to all HPr proteins studied until now. Moreover, the active-center histidine of factor III, to which the phosphoryl group is transferred, exhibits a similar change of pK, i.e., a low pK (6.0) in the unphosphorylated form and a high pK (8.6) upon phosphorylation (Kalbitzer et al., 1981). This supports the suggestion that the change of pK and the change of protonation state at physiological pH, respectively, play an important role for the phosphoryl transfer reaction.

The phosphorylation of His-A of *S. faecalis* leads to a change of the chemical shift values of the histidyl ring protons which are again indicative for a phosphorylation at the N-1 position of the histidyl ring (Kalbitzer & Rösch, 1981); i.e., all HPr proteins studied so far carry the phosphoryl group at the N-1 position in contrast to factor III, which carries the phosphoryl group at the N-3 position (Kalbitzer et al., 1981).

Interaction between the Active-Center Histidine and a Tyrosyl Residue in HPr Protein. The HPr proteins of *S. lactis*, *B. subtilis*, and *S. faecalis* contain a tyrosyl residue with chemical shift parameters similar to those of Tyr-56 of the HPr protein of *S. aureus* (Rösch et al., 1981).

In the HPr protein of *S. lactis* the resonance lines of the ring protons of Tyr-A are particularly well resolved and do not overlap with other resonance lines. The variation of the pH reveals a small titration step at acidic pH values which can be described by pK values identical with that of His-A within experimental error (Tables I and III).

All these observations are in line with the results on the HPr protein of *S. aureus*. It was concluded from the interaction between His-15 and NO_2-Tyr-56 in the mono- and dinitrated protein (Rösch et al., 1981) that His-15 is in the vicinity of Tyr-56.

Structural Similarities of HPr Proteins. The comparison of the sequences of homologous proteins is a well-established method for recognizing general structural features which may be important for the proper function of the enzyme. The comparison of NMR spectra of homologous proteins can supply similar information. In spite of the fact that similarity of the ^1H NMR spectrum does not necessarily imply similarity of the three-dimensional structure (which is also true for similarities in the sequence), it is not probable to find nearly identical chemical shift values for the proton resonance lines of two residues (e.g., histidyl residues) in homologous proteins if they differ in their chemical environment, i.e., in their neighbors in the sequence and three-dimensional structure.

Apart from the active-center histidine and Tyr-A (Tyr-56), there are many similarities in the spectra of some of the HPr proteins. Three of the HPr proteins studied contain a second histidyl residue, His-B. The spectral parameters and pK values are remarkably similar in two of them, the proteins of *S. lactis* and *S. faecalis*. In contrast, His-B of *E. coli* is characterized by a pK value about 2 units lower (Table I).

The signals tentatively assigned to the methionyl CH_3 groups exhibit similar features (Table IV); e.g., the resonance named Met-A is present in the spectra of all HPr proteins save that of *E. coli*, and the pH-dependent resonance named Met-J is

present in the spectra of the *S. lactis* and *S. faecalis* protein.

Evolutionary Aspects and Mechanistic Considerations of the HPr Proteins. Saier (1977) has proposed an evolutionary pathway of the PTS, which starts from a primordial gene coding for a simple soluble sugar phosphorylation enzyme. He speculated that the more efficient membrane associated systems may have developed by gradual substitution of hydrophilic amino acids by hydrophobic ones, finally resulting in an elaborate topological structure of an enzyme II. This is able to bind sugar from the outside and to release it as a phosphorylated product in the inside of the cell membrane.

Our own evolutionary speculation will be restricted to the HPr component alone. The most accurate method so far to study bacteria relatedness is to compare the structure of ribosomal RNA. This allowed construction of a phylogenetic tree of bacteria (Fox et al., 1980). In this scheme Gram-positive organisms like *S. aureus*, *S. faecalis*, *S. lactis*, and *B. subtilis* are closely related whereas *E. coli* belongs to a different branch.

HPr proteins of *S. faecalis*, *S. lactis*, *B. subtilis*, and also *Clostridia* (W. Hengstenberg, unpublished results) are so similar that they are interchangeable with HPr from *S. aureus* in the mutant complementation assay. HPr from *E. coli* cannot substitute HPr from *S. aureus* in the above assay. In our opinion HPr protein as well as the entire PTS must be a very old system already present in obligate anaerobic bacteria (*Clostridia*); it is well expressed in facultative anaerobic microorganisms such as *S. aureus*, *S. faecalis*, *S. lactis*, and *B. subtilis*. HPr of *E. coli* or *Salmonella* is a variant which is present in an organism with a well developed oxidative phosphorylation system (Cox & Gibson, 1974). The active-center region of the HPr proteins studied so far exhibits great similarity as derived from an analysis of the NMR spectra.

All HPr proteins contain at least one histidyl residue with a low p*K*, which is phosphorylated during the phosphotransfer reaction. Since the low p*K* tyrosine is present in all HPr proteins which can complete the *S. aureus* mutant complementation assay system and since this tyrosine residue is close to the active-center histidine as shown by its titration behavior, it could serve as a species specific recognition site for enzyme I and factor III protein during the phosphotransfer reaction. This means that the conservation of the NMR data of the low p*K* histidine and the low p*K* tyrosine (Tyr-56 and Tyr-A, respectively) throughout the HPr proteins so far studied indicates the significance of the above group during the chemistry of the phosphotransfer reaction. The amino acids which are probably not directly involved in the chemical mechanism, i.e., the high p*K* tyrosine residue and histidine-B in the *S. faecalis* and *S. lactis* HPr protein, have been changed during the process of evolution. Sequence studies on the HPr proteins of *B. subtilis* and *S. faecalis* to compare the active centers in terms of primary structures are in progress. From the sequence data already available from *S. aureus* and *Salmonella* HPr, it is evident that the primary structure of these proteins is almost identical around His-15: *S. aureus*, -Gly-Ile-His_{15}-Ala-Arg-Pro-; *Salmonella*, -Gly-Leu-His_{15}-Thr-Arg-Pro-. In the *S. aureus* protein the low p*K* tyrosyl residue has been assigned to tyrosine-56 by nitration studies (Schmidt-Aderjan et al., 1979). For clarification of whether Tyr-A is located in the same position or not, nitration studies with HPr of *S. faecalis* and *B. subtilis* are in progress.

Concluding Remarks

The most accurate method to study relatedness of active centers in proteins is, of course, X-ray crystallography. But this method requires protein crystals, the production of which is still the bottle neck during the determination of tertiary structures. NMR methods are still nonstandard. However, under certain circumstances, if crystals are not available, if the protein is small enough, and if the active center residues are readily observable in the spectrum, NMR spectroscopy is a powerful tool to investigate the relatedness of active centers in proteins. The study of several analogous proteins may have the advantage that one is able not only to determine the functional amino acid, which forms a covalent intermediate, but also to detect other functional residues which participate in the catalytic mechanism. Preliminary studies on the modification of Arg-17 with cyclohexanedione in borate buffer show that this procedure lowers the biological activity of the HPr proteins to less than 20% of the starting activity without especially disturbing the tertiary structure of the protein as concluded from NMR spectra. This means that Arg-17, which is probably present in all HPr proteins, is a good candidate for lowering the p*K* of His-15 during the phosphotransfer reaction. A possible involvement of the low p*K* tyrosyl residue (tyrosine-56 in *S. aureus*) has been mentioned by Rösch et al. (1981). The cotitration of the low p*K* tyrosine in *S. lactis* HPr with His-A may be another indication for the possible involvement of the tyrosine residue in the phosphoryl transfer mechanism.

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Nitrogen-15 Nuclear Magnetic Resonance of Arsanilazotyrosine-248 Carboxypeptidase A and Its Complex with β-Phenylpropionate. Structure and Dynamics in Solution†

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ABSTRACT: Nitrogen-15 nuclear magnetic resonance has been used to study the structure of arsanilazocarboxypeptidase A and its complex with the inhibitor β-phenylpropionate. Derivatives selectively enriched with ^{15}N were prepared to facilitate observation of the ^{15}N resonances. The results are consistent with the conclusions reached previously from absorption spectroscopic studies and, in addition, provide new information regarding the properties of the azoenzyme and its inhibitor complex. Direct evidence has been obtained for formation of an intramolecular complex between the catalytically essential zinc ion and azoTyr-248, and it has been possible to estimate the degree of complexation. Moreover, the zinc complex involves the distal (N_β) nitrogen of the azo linkage, whereas a model compound, tetrazolyl-*N*-acetyltyrosine, complexes to zinc through the proximal (N_α) nitrogen. The ^{15}N NMR spectra give specific information regarding the intramolecular hydrogen bonding in the azoenzyme. The free azophenol form of the azoenzyme, like that of the model compound arsanilazo-*N*-acetyltyrosine, exists predominantly with the tyrosine phenolic proton intramolecularly hydrogen bonded to N_β of the azo linkage to form a six-membered ring structure. A similar hydrogen bond is also present in the apoazoenzyme and in the azoenzyme–(Gly + L-Tyr) complex, but not in the complex between the azoenzyme and β-phenylpropionate. In the latter complex, there appears to be a new and strong hydrogen bond between the phenolic proton of Tyr-248 and the carboxylate group of enzyme-bound β-phenylpropionate. Thus, azoenzyme-bound β-phenylpropionate, but not azoenzyme-bound Gly + L-Tyr, is apparently able to compete effectively with, and displace, the azo nitrogen as the hydrogen-bond acceptor of the phenolic proton of Tyr-248.

X-ray diffraction studies of carboxypeptidase A have identified only two amino acid residues with side chains close enough to the enzyme-bound pseudosubstrate Gly-L-Tyr to function in catalysis (Lipscomb et al., 1968, 1970). Both of these residues, Glu-270 and Tyr-248, have, in fact, also been demonstrated to be unusually reactive and essential for catalysis through specific chemical modification experiments (Riordan & Hayashida, 1970; Petra & Neurath, 1971; Hass & Neurath, 1971; Nau & Riordan, 1975; Simpson et al., 1963; Riordan & Vallee, 1963; Muszynska & Riordan, 1976). However, in the absence of substrate, X-ray diffraction has localized the Tyr-248 residue at the surface of the molecule, some 12 Å removed from the proposed catalytic site and, moreover, approximately 17 Å from the essential zinc atom (Reeke et al., 1967; Lipscomb et al., 1968). The remarkable movement of Tyr-248 to close to the active site on binding of the pseudosubstrate Gly-L-Tyr has been cited as an example of the induced-fit theory of enzyme–substrate interaction (Koshland, 1958). Whatever the merit of that interpretation, the data clearly underscore the inherent potential for conformational flexibility of globular proteins. Moreover, because understanding of enzyme catalysis is linked to knowledge of structure and because structural change could well be integral to the catalytic act, it further underscores the need for methods that can detect and monitor such conformational variations in solution.

Coupling of carboxypeptidase A with diazotized *p*-arsanilic acid specifically labels Tyr-248 (Figure 1) without significantly affecting its catalytic properties (Johansen & Vallee, 1971, 1973; Johansen et al., 1972). The resulting azoenzyme is intensely chromophoric and has proved to be very useful as a conformational probe.

In aqueous solution, the chromophoric zinc–azoenzyme appears to exist in at least three interconvertible, pH-dependent species involving changes in the azoTyr-248[1] residue. Each of these species exhibits characteristic absorption (Johansen & Vallee, 1973, 1975), circular dichroic (Johansen & Vallee, 1973, 1975), and resonance Raman spectra (Scheule et al., 1977). Furthermore, their separate existence can be inferred

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[1] For simplification of the presentation, "Zn-azo-CPA" and "azoenzyme" are terms used interchangeably with arsanilazotyrosine-248 zinc carboxypeptidase A, "apoazoenzyme" and "apo-azo-CPA" are used interchangeably with apoarsanilazotyrosine-248 carboxypeptidase, and "azoTyr-248" is used interchangeably with arsanilazotyrosine-248.

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