^{1}H Nuclear Magnetic Resonance Studies on the Structure and Mechanism of the HPr Protein of *Staphylococcus aureus*†

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ABSTRACT: ^{1}H NMR studies of the phosphocarrier protein HPr and its three nitrotyrosyl derivatives revealed some structural features which may finally lead to an explanation of the mechanism of the phospho-transfer reaction. Titration studies on mononitrated, dinitrated, and trinitrated derivatives—i.e., derivatives with Tyr-56, Tyr-56 and Tyr-37, and Tyr-56, Tyr-37, and Tyr-6 modified—have been performed. The three tyrosyl residues seem to be in positions completely different from each other with respect to their solvent accessibility; Tyr-56 seems to be located near the surface of the protein, Tyr-6 seems to be completely buried, and Tyr-37 takes an intermediate position. Tyr-6 contributes to the core structure of the protein. A resonance at −0.18 ppm could be shown to correspond to a CH_3 group of a valine. Nuclear Overhauser experiments revealed its being close to Tyr-6. One of the resonances tentatively assigned to methionine SCH_3 groups titrates in the dinitrated derivative with the same pK as nitrotyrosyl residue 37. The titration behavior of the active-center histidyl residue suggests a hydrogen bond to the imidazole ring, possibly from Tyr-56 or Arg-17.

An indispensible component of the phosphoenolpyruvate (PEP)[1] dependent phosphotransferase system (PTS) of *Staphylococcus aureus* as well as other bacteria is the soluble phosphocarrier protein HPr (Postma & Roseman, 1976; Hengstenberg, 1977; Saier, 1979). The staphylococcal HPr is well characterized by biochemical as well as NMR methods. It consists of 70 amino acids, resulting in a molecular weight of 7650; five of the amino acids contain aromatic side chains: a phenylalanyl residue at position 29, a histidyl residue at position 15, and three tyrosyl residues at positions 6, 37, and 56 (Beyreuther et al., 1977). NMR studies could show that His-15 is the amino acid to which the phosphoryl group is covalently bound during the phosphotransfer, presumably at the N-1 position (Gaßner et al., 1977). The titration behavior of the aromatic residues (Maurer et al., 1977; Rösch, 1978) as well as the complete assignment of their ^{1}H NMR resonances (Schmidt-Aderjan et al., 1979) have been reported.

Very little is known about the tertiary structure and the mechanism of the phosphoryl transfer from enzyme I to HPr protein and from HPr protein to factor III as described by

$$\text{PEP} + \text{enzyme I} \overset{Mg^{2+}}{\rightleftharpoons} \text{P–enzyme I} + \text{pyruvate}$$

$$\text{P–enzyme I} + \text{HPr} \rightleftharpoons \text{P–HPr} + \text{enzyme I}$$

$$\text{P–HPr} + {}^1/_3\text{factor III} \rightleftharpoons {}^1/_3\text{P–factor III} + \text{HPr}$$

$$\text{P–factor III} + \text{sugar} \underset{\text{enzyme II}}{\overset{Mg^{2+}}{\rightleftharpoons}} \text{P–sugar} + {}^1/_3\text{factor III}$$

In this paper we describe an attempt to get information on structural features of HPr protein by ^{1}H NMR experiments. Thus we studied the titration behavior of the aromatic residues in HPr protein and its nitrotyrosyl derivatives. In addition we performed decoupling and nuclear Overhauser effect experiments to assign a resonance shifted to high field and learn which residue causes this ring current shift.

Materials and Methods

HPr Protein. HPr protein was purified as described earlier (Beyreuther et al., 1977). After the second DEAE-cellulose column the protein solution was concentrated to about 15 mL, desalted on a Sephadex G-25 column (3 × 25 cm), and eluted with NH_4HCO_3, pH 8.5. The protein was lyophilized and prepared for the NMR experiments as described below.

Nitro Derivatives of HPr Protein. The preparation and analysis of monitrated, dinitrated, and trinitrated HPr protein have been described (Schmidt-Aderjan et al., 1979).

^{1}H NMR Spectroscopy. All NMR spectra were recorded on a commercially available Bruker HX-360 spectrometer equipped with a 72K Nicolet data system and a Diablo disk unit working in the Fourier mode (part of these experiments were performed with a 40K Aspect 2000 system, which replaced te Nicolet system). HPr protein and its nitro derivatives were dissolved in 99.75 % D_2O after the lyophilization step. Buffers used were either sodium borate, 50 mM, or potassium phosphate (purified with Chelex 100), 20 mM, adjusted to the desired pH value with NaOD and DCl. All pH values reported are direct meter readings, uncorrected for the deuterium isotope effect. pH was determined before and after each NMR measurement. The spectra were discarded if the difference was larger than 0.05 pH unit.

Titration parameters and curves were obtained with a fit program using a nonlinear least-squares method according to

$$\delta = \delta_{A^-} + \sum_i \Delta_i \frac{10^{pK_i - pH}}{1 + 10^{pK_i - pH}}$$

where i is the number of titrating groups, δ is the chemical shift observed for a specific proton, δ_{A^-} is the chemical shift in the deprotonated state, and $\delta_{A^-} + \sum_i \Delta_i$ is the chemical shift in the protonated state of the amino acid. δ_{A^-}, Δ_i, and pK_i are the fitted parameters.

The residual HDO peak in the spectra was suppressed with a presaturation pulse applied to the sample for 0.5 s directly before the sampling pulse. The sample temperature was controlled to ±1 °C by a commercial temperature control unit

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[1] Abbreviations used: HPr, histidine-containing protein; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; PTS, phosphotransferase system; PEP, phosphoenolpyruvate; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect.

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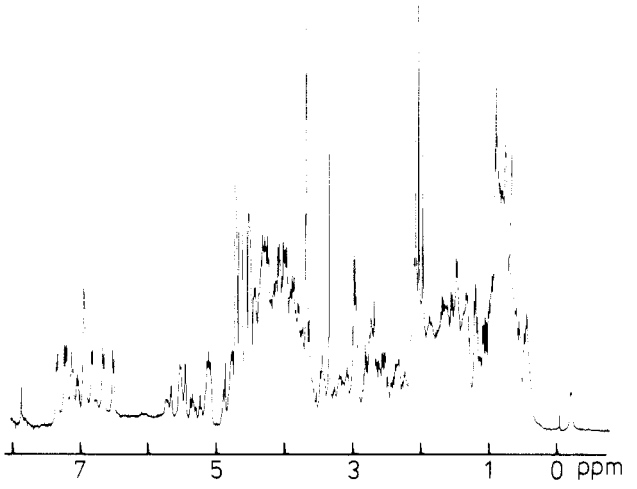

FIGURE 1: ^{1}H NMR spectrum of native HPr protein, 20 mg/mL, pH 7.0, T = 37 °C, spectral width 3300 Hz, 16K computer memory, 60° pulse angle, 3.3-s repetition time. Sample in 0.1 M KCl and 0.2 mM EDTA.

(Bruker SV 200) with a continuous flow of dry air.

All spectra were referenced to internal DSS (4,4-dimethyl-4-silapentane-1-sulfonate).

Coupling constants were determined by use of the noniterative part of the LAOCOON program as adopted to the Bruker Aspect 2000 computer.

NOE experiments were performed in the gated mode, with the FID taken immediately after the irradiation pulse. The NOE experiment made use of a difference method in order to minimize long-term instabilities of the spectrometer: Four FID's with the irradiation frequency on resonance were accumulated and stored on disk; then four FID's with the second frequency 50 Hz downfield from the resonance were accumulated and stored on disk. A new cycle started with transferring the on-resonance spectra from the disk to the computer memory and adding the next four on-resonance spectra and so forth.

Results

Native HPr Protein. Figure 1 shows the ^{1}H NMR spectrum of native HPr protein at pH 7.0. The results of the present titration study—shown in Table I—are in good agreement with the ones published earlier (Maurer et al., 1977).

The chemical shifts of the proton resonances of Tyr-56 [as assigned by Schmidt-Aderjan et al. (1979)] in the protonated as well as the deprotonated form of the amino acid are very close to the corresponding values determined in a tetrapeptide (Bundi & Wüthrich, 1979) and of free tyrosine (Roberts & Jardetzky, 1970). For $C_{2,6}H$ the difference in δ_{AH} and δ_{A^-} compared to the tetrapeptide is 0.11 and 0.14, respectively; for $C_{3,5}H$ these differences are both 0.13; the titration step is 0.03 ppm smaller for the $C_{2,6}H$ of Tyr-56 in HPr protein and is the same for $C_{3,5}H$ of Tyr-56 in HPr protein as for the tyrosyl residue in the tetrapeptide. pH values differ by 0.3 pH unit, that of Tyr-56 in HPr protein being the higher one. The dissimilarities between Tyr-37 of HPr protein and the tyrosyl residue in the tetrapeptide are larger: the titration step is 0.08 ppm larger for the $C_{2,6}H$ of Tyr-37 and 0.26 ppm larger for the $C_{3,5}H$ of Tyr-37. The pK value of the phenol group of Tyr-37 is 1.2 ppm units higher than that for the tyrosyl residue in the model peptide. Tyr-6 of HPr protein is completely different from the tyrosyl residue in the model peptide—it does not titrate in the pH range 5.5–10.5.

δ_{AH} of the resonance of C_2H of His-15 in HPr protein is 0.11 ppm lower than for histidine in the model peptide; δ_{A^-} is about the same in both systems, resulting in an about 15% smaller titration step for the C_2H of His-15. The values of δ_{AH} and δ_{A^-} for the resonance of C_4H in the histidine of HPr protein are 0.06 and 0.29 higher, respectively, than in the model peptide, resulting in a titration step which is reduced to one-third of its value in the peptide. The pK value of His-15 is 1.2 pH units lower than in the model compound.

Table I: Titration Parameters of the Aromatic Protons of Native HPr Protein and Model Compounds

group	proton	pK	δ_{AH}	δ_{A^-}	$\delta_{AH} - \delta_{A^-}$
Tyr-56	$C_{2,6}H$	10.65	7.26	7.12	0.14
	$C_{3,5}H$		6.99	6.70	0.29
Tyr-37	$C_{2,6}H$	11.52	6.99	6.74	0.25
	$C_{3,5}H$		6.70	6.13	0.57
Tyr-6	$C_{2,6}H$		6.87	6.87	0
	$C_{3,5}H$		6.56	6.56	0
His-15	C_2H	5.8	8.50	7.76	0.74
	C_4H		7.41	7.26	0.15
Amino Acids in Tetrapeptides[a]					
Tyr	$C_{2,6}H$	10.3	7.15	6.98	0.17
	$C_{3,5}H$		6.86	6.57	0.29
His	C_2H	7.0	8.61	7.75	0.86
	C_4H		7.35	6.97	0.38
Free Amino Acids[b,c]					
Tyr	$C_{2,6}H$	10.0	7.18	7.04	0.14
	$C_{3,5}H$		6.90	6.67	0.23
His	C_2H	6.0	8.70	7.68	1.02
	C_4H		7.49	6.93	0.56

[a] Bundi & Wüthrich (1979). [b] Roberts & Jardetzky (1970). [c] *Handbook of Biochemistry* (1970).

All three tyrosyl residues in HPr protein show an AA'BB' type spectrum for the protons of the phenol ring, indicating the possibility of 180° flips for those rings around their C_β–C_γ bonds. The frequency of reorientation is high in comparison to the differences in chemical shift between C_2H and C_6H on one hand and C_3H and C_5H on the other hand, as is the case for tyrosyl residues in other proteins, e.g., BPTI (Wüthrich, 1976).

Decoupling experiments and comparison of relative intensities led to the assignment of three resonances of the phenyl ring of Phe-29 (Rösch, 1978): the resonance at 7.4 ppm corresponds to $C_{2,6}H$, the one at 7.2 ppm to $C_{3,5}H$, and the one at 7.1 ppm to C_4H. This spectral type indicates rotational mobility of the phenol ring around the C_β–C_γ bond with a frequency of reorientation high in comparison to the differences in chemical shifts of the C_2H and C_6H resonances and the C_3H and C_5H resonances.

Mononitrated HPr Protein. The aromatic part of the spectrum of mononitrated HPr protein at pH 8.0, i.e., HPr protein with an NO_2 group attached to C_3 of Tyr-56 (Schmidt-Aderjan et al., 1979), is shown in Figure 2a; Figure 2b shows the same spectral part of unmodified HPr protein. The C_2H resonance of His-15 and the C_2H resonance of (NO_2)Tyr-56 as well as the resonances of $C_{2,6}H$ of Phe-29 and C_6H of (NO_2)Tyr-56 overlap completely at this particular pH; the doublet originating from C_5H of (NO_2)Tyr-56 is located at 6.89 ppm.

Table II shows the titration parameters as derived from a one-group titration fit. A comparison between (NO_2)Tyr-56 in HPr protein and free nitrotyrosine shows close similarities between the two systems. In particular, the heights of the titration steps in free (NO_2)Tyr as well as (NO_2)Tyr-56 are governed by the relation $C_5H > C_6H > C_2H$. [Of course, a strict comparison should involve a nitrotyrosyl residue in a model peptide, but those data are not available in the literature. The comparison with free nitrotyrosine may be justified by

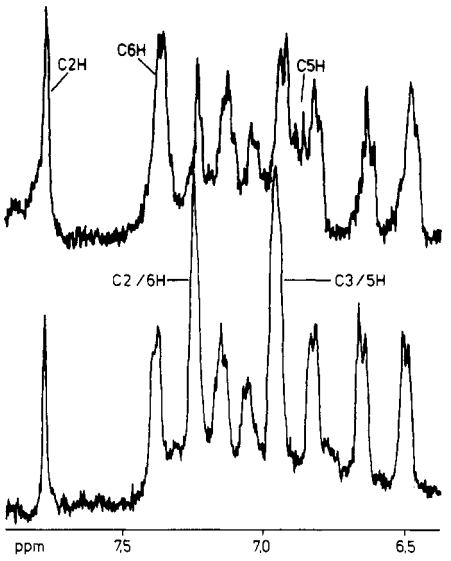

FIGURE 2: (a, upper) ^{1}H NMR spectrum of the aromatic part of mononitrated HPr protein, pH 8.0, 10 mg/mL, 2000 scans; other parameters as in Figure 1. (b, lower) The same part of the ^{1}H NMR spectrum of native HPr protein; parameters as in Figure 1.

Table II: Titration Parameters As Determined with Mononitrated HPr Protein

group	proton	pK	δ_{AH}	δ_{A^-}	$\delta_{AH} - \delta_{A^-}$
(NO_2)Tyr-56	C_2H		7.92	7.75	0.17
	C_5H	7.12	7.32	6.86	0.46
	C_6H		7.73	7.34	0.39
His-15	C_2H	6.71	8.38	7.72	0.66
	C_4H		7.36	7.27	0.09
	Free Amino Acid[a]				
(NO_2)Tyr	C_2H		7.99	7.71	0.28
	C_5H	7.1	7.14	6.73	0.41
	C_6H		7.54	7.20	0.34

[a] Snyder et al. (1975).

Table III: Titration Parameters As Determined with Dinitrated HPr Protein[a]

group	proton	pK	δ_{AH}	δ_{A^-}	$\delta_{AH} - \delta_{A^-}$
(NO_2)Tyr-56	C_2H		7.95	7.75	0.03/0.17
	C_5H	6.4/5.8	7.22	6.89	0.21/0.12
	C_6H		7.69	7.36	0.20/0.13
(NO_2)Tyr-37	C_2H		8.06	7.81	0.25
	C_5H	7.69	7.01	6.55	0.46
	C_6H		7.18	6.98	0.20
His-15	C_2H	6.6/5.8	8.76	7.77	0.05/0.94
	C_4H		7.53	7.27	0.08/0.34
Met	CH_3	7.76	2.04	1.90	0.14

[a] Two values are given for pK and $\delta_{AH} - \delta_{A^-}$ according to the two-group titration fit.

the fact that the differences between the titration parameters of free tyrosine and the tyrosyl residue in the model peptide are minor (see Table II).]

It should be emphasized at this point that the titration parameters in Table II resulted in a rather poor fit to the experimental data, but these data were not sufficient to attempt a curve-fit with more than one titrating group.

A comparison of other resonance positions in the aromatic as well as in the aliphatic part of the spectrum of (NO_2)-HPr protein with the corresponding parts of the unmodified protein revealed no visible changes.

Dinitrated HPr Protein. The aromatic part of the ^{1}H NMR spectrum of the dinitrated HPr protein, i.e., HPr protein with an NO_2 group attached to the C_3 of Tyr-56 and an NO_2 group attached to the C_3 of Tyr-37 (Schmidt-Aderjan et al., 1979), is shown in Figure 3 at various pH values.

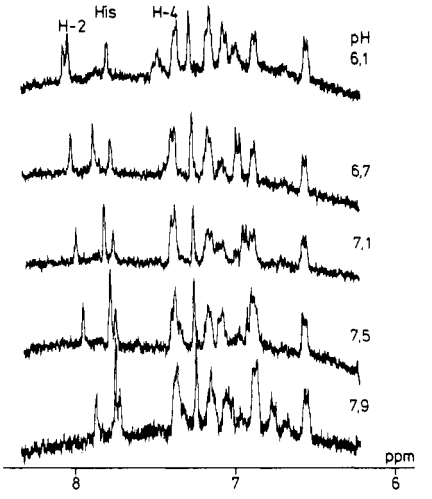

FIGURE 3: Aromatic part of the ^{1}H NMR spectrum of dinitrated HPr protein at various pH values; the lowest spectrum was taken first, the upper last; spectral parameters as in Figure 1; spectral width 3700 Hz, 16K computer memory, repetition time 2.2 s, pulse angle 80°, T = 37 °C, 2000 scans. Buffer: 50 mM sodium borate.

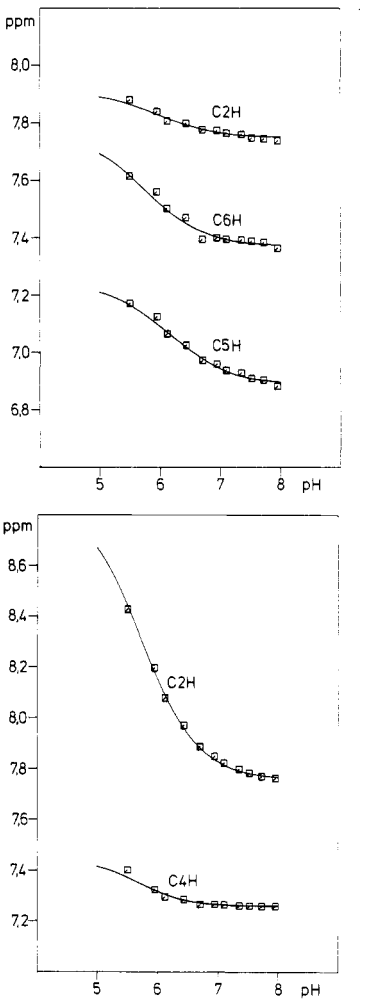

FIGURE 4: Titration curves of the protons of the aromatic rings of (NO_2)Tyr-56 and (NO_2)Tyr-37 (upper) and of His-15 (lower) in dinitrated HPr protein.

We examined the spectra in the pH range 5.5–7.9. Chemical shift values are plotted vs. pH values in Figure 4 for all resonances originating from protons of the phenol rings of the

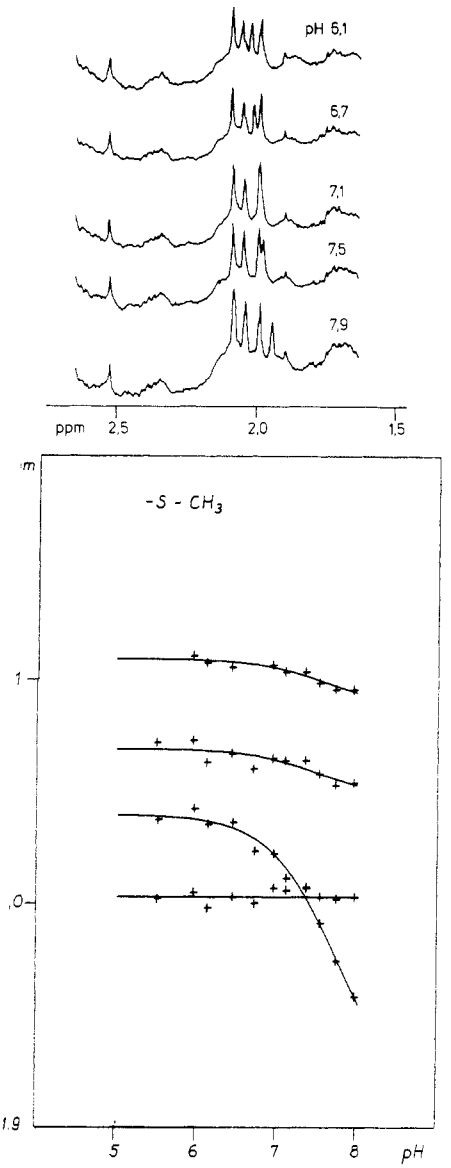

FIGURE 5: (a, upper) Region aroung the SCH_3 resonances of methionines of dinitrated HPr protein. Spectral parameters as in Figure 1. (b, lower) Titration curves of the four resonances shown in (a).

two nitrotyrosyl residues; titration parameters are collected in Table III. (NO_2)Tyr-37 shows a pK value about 0.6 pH unit higher than that of free nitrotyrosine. Also, the heights of the titration steps for the three protons do not obey the order $C_5H > C_6H > C_2H$, as found in free nitrotyrosine, but instead in order $C_5H > C_2H > C_6H$, due to a rather low titration step of C_6H—0.2 ppm as compared to 0.34 ppm in free nitrotyrosine (Snyder et al., 1975).

(NO_2)Tyr-56 exhibits two titration steps: one near the pH value of free nitrotyrosine (7.1 for free nitrotyrosine, about 6.4 for (NO_2)Tyr-56), the other one the same as that of His-15 in native HPr protein, within experimental error. The titration parameter for His-15 and (NO_2)Tyr-56 were evaluated with a two-group titration fit.

Another titrating group can be observed in the region around 2 ppm. Here we see—in native HPr protein as well as in its NO_2 derivatives—three resonances of total intensity 12, i.e., one resonance with intensity six and two with intensity three. (The intensities were calibrated with the C_2H peak of His-15 as standard for one proton.) In addition, the line width of these resonances is very small compared to the other resonances in the spectrum; therefore we assign them tentatively to the four methionine SCH_3 groups found in HPr protein. One of these

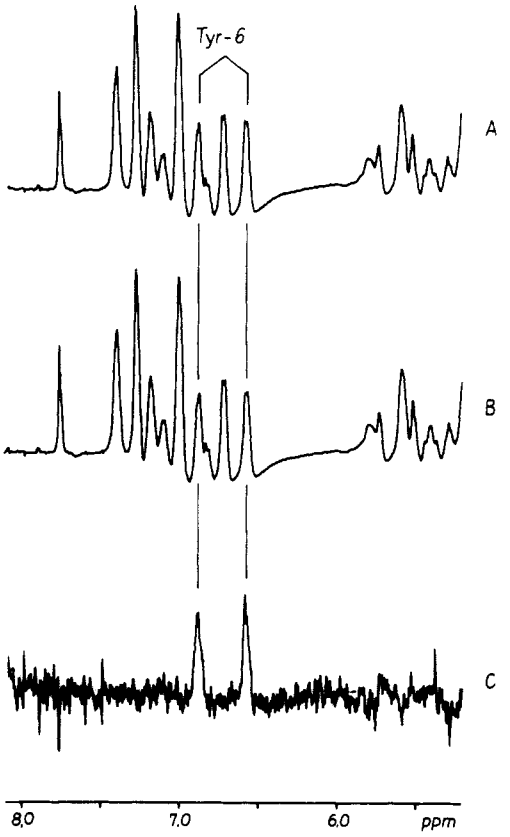

FIGURE 6: (A) Aromatic part of the ^1H NMR spectrum of native HPr protein with a presaturation pulse applied 50 Hz downfield from the resonance at −0.18 ppm; 40 mg/mL HPr protein, pH 7.0, presaturation time 0.8 s, T = 30 °C, spectral width 3200 Hz, 8K computer memory, repetition time 6.1 s, 400 scans, pulse width 90°. Buffer: 50 mM potassium phosphate and 0.2 mM EDTA. (B) Same as (A) but presaturation pulse on resonance at −0.18 ppm. (C) Difference (A) − (B).

resonances titrates with a pK of 7.76, which is the same within experimental error as the one calculated for (NO_2)Tyr-37 (Figure 5 and Table III). Two others titrate with a very small titration step and a similar pK value; other changes have been not observed in the spectrum.

The intensities of C_2H and C_4H of His-15 in dinitrated HPr shown in Figure 3 decreased rapidly in the course of this experiment. In native HPr at neutral pH, the half-life of C_2H for proton–deuterium exchange is several weeks, and a decrease of the C_4H peak has never been observed. Fitting the decrease of these two peaks in dinitrated HPr proteins to a decaying exponential yields half-lives of 5.5 and 11.5 h for C_2H and C_4H, respectively.

Trinitrated HPr Protein. The spectrum of the protein derivative with all three tyrosines modified by addition of a NO_2 group to their C_3 atoms did not allow an assignment of resonances because of the comparatively small amount of modified protein available. The doublet shifted to high field disappeared completely in this derivative.

Double-Resonance Experiments. To determine what kind of an amino acid the doublet shifted to −0.18 ppm originates from as well as which aromatic residue causes the ring current shift we performed driven NOE experiments as described by Wagner & Wüthrich (1979) and under Material and Methods. Figure 6A represents the aromatic part of the spectrum with the saturation field 50 Hz downfield from the resonance at −0.18 ppm; Figure 6B shows the same part of the spectrum with the saturation field on resonance at −0.18 ppm; Figure 6C is the difference (A) − (B). The only resonances showing up in the difference spectrum belong to Tyr-6. The time course

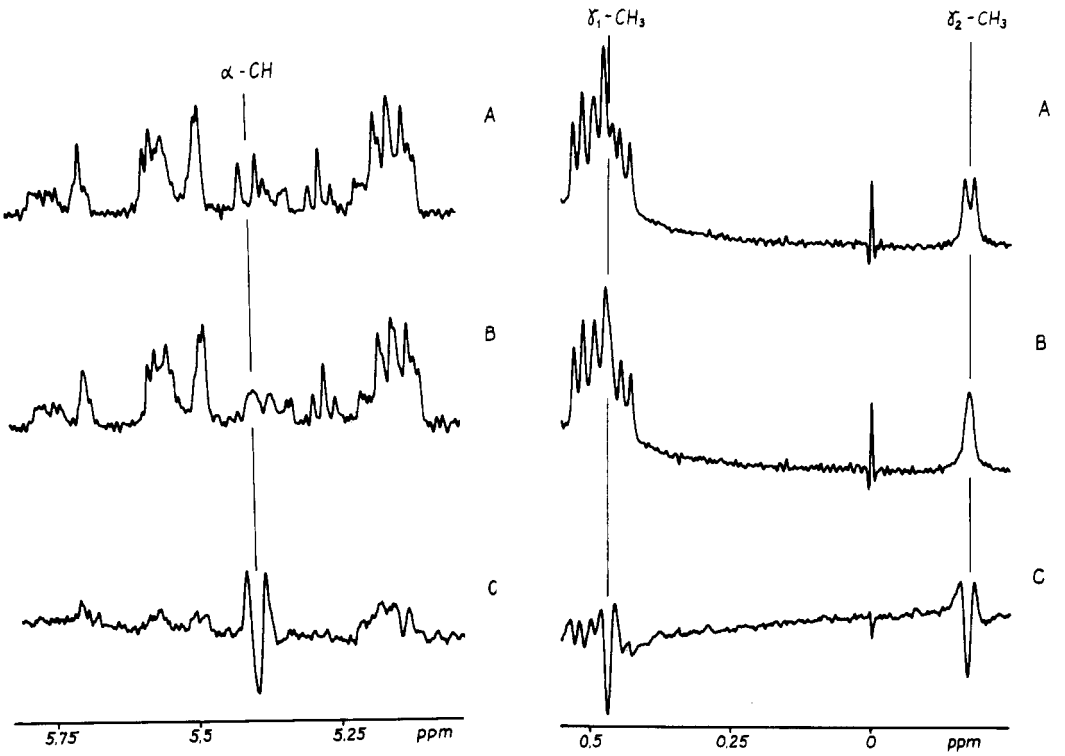

FIGURE 7: (A) ^{1}H NMR spectrum of native HPr protein resolution enhanced by Gaussian multiplication (Ferrige & Lindon, 1978) of the FID with −5 Hz; conditions as in Figure 6 except for pulse repetition time 3.3 s, 16K computer memory, 180 scans. (B) Same as (A) but irradiated at 1.48 ppm. (C) Difference (A) − (B).

of the NOE with various lengths of the presaturation pulse led to the conclusion that spin diffusion did not contribute significantly to the observed effect.

In the aliphatic part of the difference spectrum, peaks appeared at 1.48 and 0.47 ppm. Decoupling at various frequencies around 1.48 ppm and with different power levels showed that a resonance at this frequency couples with the doublet at 0.18 ppm, another doublet at 0.47 ppm, and a third doublet at 5.4 ppm (Figure 7). Decoupling of the doublet at 0.18 and 0.47 ppm could be obtained with a modest power level, thus defining precisely the coupling resonance at 1.48 ppm. From its position as well as its intensity, the doublet at 0.18 ppm belongs to a CH_3 group; the doublet at 0.47 ppm is within the range of CH_3 groups too. The resonance at 1.48 ppm, which must originate from a single proton because of the doublet splitting of the coupling resonances, is in the β-CH region, whereas the doublet at 5.4 ppm is in the α-CH region. The only amino acid with a β-CH group directly coupling to two CH_3 is valine; therefore the resonances in question originate from one of the four valine residues of HPr protein.

Compared to the amino acid in a model peptide (Bundi & Wüthrich, 1979) the γ-CH_3 resonances are shifted upfield by about 1.1 and 0.5 ppm, respectively. The β-CH resonance is shifted upfield by 0.7 ppm whereas the α-CH is shifted downfield by 1.2 ppm (see Table IV).

The $-CHCH_3$ coupling constant which we were able to determine agrees very well with the corresponding coupling constant in the model peptide (6.5 ± 0.5 Hz vs. 6.9 ± 0.5 Hz). In contrast, $J_{\alpha,\beta}$, the coupling constant between α-CH and β-CH, is about 4 Hz higher in HPr protein than in the model peptide (10.8 ± 0.5 Hz vs. 6.9 ± 0.5 Hz).

We could not observe a collapse of the multiplet structure originating from the β-CH group, but this could be expected in light of the fact that these group couples to two nonequivalent CH_3 groups and one CH group, yielding a multiplet consisting of 32 lines even in first-order spectra with an intensity of one proton. An attempt to observe the collapse of the β-CH multiplet of a valine residue in lysozyme failed also (Chapman et al., 1978).

Table IV: Spectral Data of the Valine Residue in HPr Protein with a Resonance of a γ-CH_3 Group Shifted to High Field As Compared to the Model Compound

proton	chemical shift (ppm)	J (Hz)
	Valine in HPr Protein	
α-CH	5.40	10.8 ± 0.5
β-CH	1.47	
γ_1-CH_3	0.47	6.5 ± 0.5
γ_2-CH_3	−0.18	6.5 ± 0.5
	Valine in Tetrapeptide[a]	
α-CH	4.18	6.9 ± 0.5
β-CH	2.13	
γ-CH_3	0.97	
	0.94	6.9 ± 0.5

[a] Bundi & Wüthrich (1979).

Discussion

Histidyl Residue. The two major features of histidyl residue 15 of native HPr protein distinguishing it from the histidyl residue in the linear tetrapeptide are its lower pK value (diminished by more than one pH unit) and the lower titration steps for both C_2H and C_4H (diminished by 0.1 and 0.2 ppm, respectively). Similar behavior has been observed for histidyl residues in a variety of different proteins; a few examples for comparison with His-15 in HPr protein are given in Table V. All authors claim that a possible reason for the low pK values of those histidyl residues is the presence of positive charges nearby [also see Markley (1975)], e.g., Lys-47 and -50 in the case of metmyoglobin (Hayes et al., 1975) and arginine in neurotoxin II of *Naja naja oxiana* for His-4 (Arseniev et al., 1976). In fact, an arginine residue (Arg-17) is close to the active-center histidyl residue of HPr protein. Other possible explanations are the formation of a hydrogen bond with the imidazole ring as a proton acceptor or a hydrophobic envi-

Table V: Comparison of Various Histidyl Residues As Found in the Literature

compound	group	proton	pK	δ_{AH}	δ_{A^-}	$\delta_{AH} - \delta_{A^-}$
soybean trypsin inhibitor	His^{H_2}	C_2H	5.28	8.32	7.75	0.57[a]
sperm whale metmyoglobin	His-48	C_2H	5.41	8.76	7.93	0.83[b]
horse metmyoglobin	His-48	C_2H	5.68	8.64	7.90	0.74[b]
human metmyoglobin	His-48	C_2H	5.88	8.54	7.78	0.76[b]
hen egg white lysozyme	His-15	C_2H	5.8			[c]
neurotoxin II from *Naja naja oxiana*	His-31	C_2H	5.93	9.01	8.08	0.93[d]
		C_4H	5.95	7.73	7.37	0.36[d]
	His-4	C_2H	5.02	9.26	7.91	1.35[d]
		C_4H	5.13	7.11	6.87	0.24[d]
HPr protein from *E. coli*	active-center His	C_2H	5.6	8.62	7.88	0.74[e]
		C_4H		7.43	7.33	0.10[e]

[a] Markley (1973). [b] Hayes et al. (1975). [c] Meadows et al. (1967). [d] Arseniev et al. (1976). [e] Dooijewaard et al. (1979).

ronment of the histidyl residue (Tanford, 1962). Although the presence of a positive charge close to His-15 of HPr protein could account for the lowering of its pK value, it could not explain the low titration step of both the C_2H and C_4H groups. The active center histidine of HPr protein of *E. coli* has, according to Dooijewaard et al. (1979), exactly the same properties as His-15 of HPr protein from *S. aureus*. The authors claim that a local movement of an aromatic ring during pH titration could be the reason for the behavior of this histidyl residue. Still another explanation would be the formation of a hydrogen bond in which the unprotonated form of the histidyl residue acts as a proton acceptor. According to Arseniev et al. (1976), this should result also in a downfield shift of C_2H and C_4H in the unprotonated form of the residue and in a lowering of their titration steps; these are precisely the two additional effects we observe with His-15 in HPr protein.

Only the last two reasons could explain sufficiently the behavior of His-15 in *S. aureus* (as well as the active center histidine in *E. coli*), the formation of a hydrogen bond between the histidyl and some other residue being the more likely in light of structure and mechanism of other proteins (e.g., serine proteases) (Schultz & Schirmer, 1979). The fact that during alkaline denaturation around pH 10 one can observe two distinct resonances for the C_2H of His-15 of *S. aureus* HPr protein (Maurer et al., 1977) could then be due to the breakage of the hydrogen bond. A hydrogen bond involving the active-center histidyl residue would of course have implications on the mechanism of the protein.

Tyrosyl Residues. The observed mutual effect of the deprotonation of the histidyl residue and (NO_2)Tyr-56 in the mononitrated and, more clearly, in the dinitrated protein suggests some kind of interaction between these residues. This could be an influence of the degree of protonation of Tyr-56 as well as His-15 on the structure of the vicinity of the other residue or a more direct interaction, e.g., via a hydrogen bond between those two residues. It should be emphasized in this connection that no other visible changes in the spectrum occur in the pK region of either of those residues, so that an overall conformational change or even a conformational change of a rather large volume in the protein structure can be excluded. Influences of hydrogen bonds on the pH titration curves of histidyl residues have been described for many other proteins (Markley, 1975; Dwek, 1973).

The enhancement of the rate of proton/deuterium exchange observed for C_2H and C_4H of His-15 in dinitrated HPr may be ascribed to the induction of a positive charge near the imidazole ring by either (NO_2)Tyr-56 or (NO_2)Tyr-37 as demonstrated by Vaughan et al. (1970).

In any case, a contribution of Tyr-56 to the active-center structure or even more directly to the mechanism of the phosphoryl transfer should be suspected. The virtual titration of at least one of the singlet resonances tentatively assigned to the CH_3 groups of the four methionine residues in HPr protein with the pK value of (NO_2)Tyr-37 may be due to a changing ring current shift caused by (NO_2)Tyr-37. The change in ring-current shift during deprotonation of tyrosine can be explained by the changing density of π electrons in the ring. The same effect could well be present in the native protein where it might be obscured by the denaturation of HPr protein at a pH value lower than the pK of Tyr-37.

Valine Residue. It could have been suspected from the titration experiments with HPr protein and its nitro derivatives that Tyr-6 causes the ring-current shift of the valine CH_3 group along the following line of argument: in the native protein no change of the chemical shift of the doublet at −0.18 ppm could be observed in the pH range 5.5–10.5 (where the protein denatures and the doublet disappears). This excludes His-15 as a possible candidate, leaving Tyr-56, Tyr-37, Tyr-6, and Phe-29. Nitration of Tyr-56 did not change its position either, nor did titration of the mononitrated HPr protein in the pK region of (NO_2)Tyr-56, excluding this residue as a candidate. No effect was observed caused by nitration of Tyr-56 as well as Tyr-37 in the dinitrated HPr protein and during titration in their pK region, leaving only Tyr-6 and Phe-29 as possible candidates. Nitration of Tyr-6 finally caused the disappearance of the doublet. This result could be evaluated very clearly by the NOE experiments performed.

An estimation of the upper limit of the distance between the valine CH_3 group and Tyr-6 may be performed as follows: The difference in chemical shift of this CH_3 group in HPr protein and in the model peptide (Bundi & Wüthrich, 1979) is about −1.13 ppm. A benzene ring placed 3.5 Å from a proton would cause a shielding effect of this magnitude along its 6-fold symmetry axes C_6, i.e., along the axes perpendicular to the benzene ring where the largest magnetic shielding due to the ring current induced by the applied static field is to be expected (Johnson & Bovey, 1958); this value therefore can be used as an upper limit on the distance between the valine CH_3 group and the phenol ring of Tyr-6 in HPr protein if one neglects all other influences on the chemical shift of the CH_3 group, which is reasonable according to Perkins & Wüthrich (1979).

Structural Interpretation. According to Schmidt-Aderjan et al. (1979), Tyr-56 is most accessible to the nitration procedure, Tyr-37 less, and Tyr-6 least of all. This in itself suggests that Tyr-56 is a residue at the surface of HPr protein, Tyr-6 in the most buried of the three, and Tyr-37 is in a position somewhere intermediate between these extreme cases. This statement gets support from the fact that Tyr-56 resembles the tyrosyl residue in the model peptide most closely, Tyr-37 less, and Tyr-6, which does not titrate with H_3O^+, least.

In addition, a valine residue, which is an amino acid with a hydrophobic side chain, well-known to be in the interior of proteins (Schulz & Schirmer 1979), has been demonstrated to be close to Tyr-6.

The valine residue near Tyr-6 could well be the one next to this residue in the primary structure, i.e., Val-7 (Beyreuther et al., 1977). The only objection to this stems from the predicted secondary structure of this region: Beyreuther et al. (1977) predict, according to the Chou–Fasman rules, a β-pleated sheet from residue 5 to residue 9. Model building clearly shows that this makes it impossible for a CH_3 group of Val-7 to come close enough to the phenol ring of Tyr-6 to explain the observed ring current shift.

A Possible Mechanism. Gassner et al. (1977) claim that His-15 of HPr protein of *S. aureus* is phosphorylated at the N-1 position. Although some caution has been expressed by Dooijewaard et al. (1979) that the comparison of free phosphohistidine with a phosphohistidyl residue in HPr protein, on which the argument by Gassner et al. (1977) is founded, might be invalid because histidyl residues in peptides may well behave differently, recent titration studies on a phosphorylated model peptide suggested that it is indeed the N-1 atom which is phosphorylated in HPr protein (H. R. Kalbitzer and P. Rösch, unpublished experiments). The results of the present experiments suggest that a hydrogen bond involving the active-center histidyl residue contributes to the mechanism of the phosphoryl transfer. Dooijewaard et al. (1979) postulated two active-center conformations—one suitable for the acceptance of the phosphoryl group from enzyme I, the other for the donation of the phosphoryl group to factor III. This would be in line with the assumption that a hydrogen bond stabilizes one conformation of the protein in the absence of enzyme I and factor III. More specifically: if on the enzyme the N-1 atom of the histidyl residue is preferentially protonated at physiological pH values—as opposed to free histidine—a weak hydrogen bond to N-3 of His-15 could stabilize the active-center structure suitable for accepting the phosphoryl group from enzyme I in N-1, which is a nucleophilic atom with its two free electrons.

There are at least two possible candidates for the donation of a proton: arginine at position 17 and, as suggested by the present results, Tyr-56. (A similar hydrogen bond—His–Tyr—is found, e.g., in GAPDH (Schultz & Schirmer, 1979).

It is very interesting here to note that tyrosyl residues analogous to Tyr-56 of HPr protein of *S. aureus* have now been found by NMR studies of HPr protein of other species (H. R. Kalbitzer et al., unpublished results). In addition, Roossien et al. (1979) claim to have observed a tyrosyl analogous to Tyr-6 of HPr protein of *S. aureus* in the *E. coli* HPr protein. They conclude this from an assignment of the $C_{2,6}H$ and $C_{3,5}H$ resonances by decoupling experiments. It may well be that this assignment needs some reevaluation: A close look at their spectra with and without irradiation at 7.0 ppm shows that during irradiation a peak at about 7.25 ppm appears. If one assigns these peaks to a tyrosyl residue—instead of the peaks at 7.0 ppm and 6.7 ppm as done by those authors—then one would have a tyrosyl residue at exactly the same position as Tyr-56 in the NMR spectrum of the HPr protein of *S. aureus*. Completely in line with this suggestion is the observation by Dooijewaard et al. (1979), that a peak at 7.24 ppm shifts upon phosphorylation of the protein.

These observations would shed new light on a mechanism common to the *S. aureus* protein and the *E. coli* protein, which were thought to be not similar as judged from their amino acid composition and their molecular weight.

References

Arseniev, A. S., Balashova, T. A., Utkin, Y. N., Tsetlin, V. I., Bystrov, V. F., Ivanov, V. T., & Ovchinnikov, Y. A. (1976) *Eur. J. Biochem. 71*, 595–606.

Beyreuther, K., Raufuss, H., Schrecker, O., & Hengstenberg, W. (1977) *Eur. J. Biochem. 75*, 275–286.

Bundi, A., & Wüthrich, K. (1979) *Biopolymers 18*, 285–297.

Chapman, G. E., Abercrombie, B. D., Cary, P. D., & Bradbury, E. M. (1978) *J. Magn. Reson. 31*, 459–469.

Dooijewaard, G., Roossien, F. F., & Robillard, G. T. (1979) *Biochemistry 18*, 2996–3001.

Dwek, R. A. (1973) *NMR in Biochemistry*, Clarendon Press, Oxford, England.

Ferrige, A. G., & Lindon, J. C. (1978) *J. Magn. Reson. 31*, 337–340.

Gassner, M., Stehlik, D., Schrecker, O., Hengstenberg, W., Maurer, W., & Rüterjans, H. (1977) *Eur. J. Biochem. 75*, 287–296.

Handbook of Biochemistry (1970) 2nd ed., The Chemical Rubber Co., Cleveland, Ohio.

Hayes, M. B., Hagenmaier, H., & Cohen, J. S. (1975) *J. Biol. Chem. 250*, 7461–7472.

Hengstenberg, W. (1977) *Curr. Top. Microbiol. Immunol. 77*, 97–126.

Johnson, C. E., & Bovey, F. A. (1958) *J. Chem. Phys. 29*, 1012–1014.

Markley, J. L. (1973) *Biochemistry 12*, 2245–2250.

Markley, J. L. (1975) *Acc. Chem. Res. 8*, 70–80.

Maurer, W., Rüterjans, H., Schrecker, O., Hergsternberg, W., Gassner, M., & Stehlik, D. (1977) *Eur. J. Biochem. 75*, 297–301.

Meadows, D. H., Markley, J. C., Cohen, J. S., & Jardetzky, O. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 1307–1314.

Perkins, S. J., & Wüthrich, K. (1979) *Biochim. Biophys. Acta 576*, 409–423.

Postma, R. W., & Roseman, S. (1976) *Biochim. Biophys. Acta 457*, 213–257.

Roberts, G. C. K., & Jardezky, O. (1970) *Adv. Protein Chem. 24*, 447–545.

Roosien, F. F., Dooijewaard, G., & Robillard, G. T. (1979) *Biochemistry 18*, 5793–5797.

Rösch, P. (1978) Thesis, Heidelberg.

Saier, M. H. (1979) *Bacteriol. Rev. 41*, 856–871.

Schmidt-Aderjan, U., Rösch, P., Frank, R., & Hengstenberg, W. (1979) *Eur. J. Biochem. 96*, 43–48.

Schulz, G. E., & Schirmer, R. H. (1979) *Principles of Protein Structure*, Springer, New York.

Snyder, G. H., Rowan, R., Karplus, S., & Sykes, B. D. (1975) *Biochemistry 14*, 3765–3777.

Tanford, C. (1962) *Adv. Protein Chem. 17*, 70–165.

Vaughan, J. D., Mughrabi, Z., & Wu, E. C. (1970) *J. Org. Chem. 35*, 1141–1145.

Wagner, G., & Wüthrich, K. (1979) *J. Magn. Reson. 33*, 675–680.

Wüthrich, K. (1976) *NMR in Biological Research: Peptides and Proteins*, North-Holland Publishing Co., Amsterdam.