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Phosphoenolpyruvate-Dependent Phosphotransferase System of *Staphylococcus aureus*: ^{1}H Nuclear Magnetic Resonance Studies on Phosphorylated and Unphosphorylated Factor III^{lac} and Its Interaction with the Phosphocarrier Protein HPr†

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ABSTRACT: The trimeric phosphocarrier protein factor III specific for galactosides was investigated by ^{1}H NMR spectroscopy. The protomer contains four histidyl residues with acidic pK values in the range 5.6–6.2. One of the histidyl residues, His-B, carries the phosphoryl group. The pK value of His-B increases from 6.0 to 8.6 upon phosphorylation. To determine the position of the phosphoryl group with respect to the nitrogens required the isolation of a peptide T-2 containing the phosphorylated active-center histidine and one of the other histidines. The pK value and the chemical shift of the phosphopeptide clearly indicated the phosphorus to be bound to the N-3 atom of the imidazole ring. The temperature dependence of the factor III spectrum demonstrates multiple conformations which exchange rapidly on the NMR time scale. Titration of factor III with HPr protein showed an upfield shift of the active-center histidine, indicating complex formation between both proteins. Phosphorylation of both proteins abolished the interaction, which is plausible from mechanistic considerations.

In 1964, Kundig and co-workers discovered that in *Escherichia coli* carbohydrates are transported across the cell membrane by the phosphoenolpyruvate-dependent phosphotransferase system (PTS)[1] (Kundig et al., 1964). In subsequent years, this transport system has been found in many different microorganisms (Hengstenberg, 1977), e.g., in *Staphylococcus aureus*. The lactose-specific PTS of *S. aureus* may be described by eq 1–4. Both proteins involved in reaction 3 can be obtained in pure form and have a relatively low molecular weight of 7685 (HPr protein) (Beyreuther et al., 1977) and 33 000 (factor III) (Hays et al., 1973), respectively. It has been shown by NMR methods that HPr protein has the phosphoryl group covalently bound to nitrogen 1 of the single histidyl residue during the phosphoryl transfer (Gassner et al., 1977). Selective nitration of the three tyrosyl residues of HPr combined with ^{1}H NMR studies gave some insight into the structure of this protein (Schmidt-Aderjan et al., 1979; Rösch et al., 1981). Factor III^{lac} is composed of three identical protomers. Each subunit can be loaded with one phosphoryl group, supposedly bound to the N-3 atom of one of the histidyl residues (Hays et al., 1973). It has been shown that phosphorylation leads to a destabilization of the trimeric structure of FIII and that phosphorylated FIII in contact with the cell membrane may dissociate in its subunits (Hengstenberg, 1977).

$$\text{PEP} + \text{enzyme I} \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} \text{P–enzyme I} + \text{pyruvate} \quad (1)$$

$$\text{P–enzyme I} + \text{HPr} \rightleftharpoons \text{P–HPr} + \text{enzyme I} \quad (2)$$

$$\text{P–HPr} + \text{factor III} \rightleftharpoons \text{P–factor III} + \text{HPr} \quad (3)$$

$$\text{P–factor III} + \text{galactoside} \underset{Mg^{2+}}{\overset{\text{enzyme II}}{\rightleftharpoons}} \text{galactoside-6-P} + \text{factor III} \quad (4)$$

In this paper, we report about the effect of phosphorylation on the structure of FIII and the interaction with the phosphocarrier protein HPr as studied by NMR methods.

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[1] Abbreviations used: PTS, phosphoenolpyruvate-dependent phosphotransferase system; NMR, nuclear magnetic resonance; HPr, histidine-containing phosphocarrier protein; P-HPr, phosphorylated HPr protein; FIII, factor III^{lac}; P-FIII, phosphorylated FIII; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; PEP, phosphoenolpyruvate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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Materials and Methods

HPr Protein. HPr protein was purified according to Beyreuther et al. (1977).

FIII Protein. FIII protein was purified according to Deutscher (1979).

Enzyme I. Enzyme I was partially purified. The following purification procedures were used. A crude extract prepared from cells washed with EDTA (300 g of the strain S 305 A) was chromatographed on DEAE-cellulose (Whatman DE 23). Enzyme I was eluted by a salt gradient, 0–0.6 M NaCl, in standard buffer (0.05 M Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM DTT, and 0.1 mM PMSF). Fractions containing enzyme I were pooled, and an ammonium sulfate precipitation (60% saturation) was followed. The pellet was resuspended in standard buffer and applied to a Sephadex G-100 column. The enzyme I pool was again concentrated by precipitation with ammonium sulfate (60% saturation). Before use, enzyme I was desalted with a Sephadex G-25 column eluted with 0.05 M NH_4HCO_3, pH 7.5.

Phospho-Factor III. Phospho-factor III was prepared according to Deutscher (1979). The final preparation used for the NMR experiments contained about 10% unphosphorylated FIII.

Tryptic Fragment T-2. A 10-mg sample of phospho-factor III (labeled with ^{32}P) or 10 mg of FIII were dissolved in 0.5 mL of 0.05 M NH_4HCO_3, pH 8.7. A 500-μg sample of highly purified trypsin (kindly provided by Dr. K. Jany) was added. After 30 min at 37 °C, an additional 200 μg of trypsin was added, and after further 30 min, the reaction mixture was applied to a Sephadex G-50 column (1.6 × 100 cm). A second purification step on a DEAE-cellulose column (Whatman DE 52) eluted with a gradient of 0.1–0.4 M NaCl was necessary to obtain pure T-2 peptide. The last purification step even separated phosphorylated and unphosphorylated tryptic peptide T-2. The peptide solution was concentrated by pressure dialysis and then applied on a Sephadex G-25 column eluted with 0.05 M $NaDCO_3$ in D_2O (pH 8.4).

^{32}P-Labeled Phosphoenolpyruvate (^{32}PEP). ^{32}PEP was synthesized and prepared according to Lauppe et al. (1972).

NMR Spectra. All spectra were recorded with a Bruker HX-360 spectrometer operating at an ^{1}H resonance frequency of 360 MHz. The HDO signal was suppressed by selective presaturation. 4,4-Dimethyl-4-silapentane-1-sulfonic acid (DSS) was used as an internal standard. The pH values were measured with a combination glass electrode of Ingold, Frankfurt, West Germany, and are not corrected for the deuterium isotope effect. The sample temperature was kept at 308 K if not stated otherwise.

Evaluation of the Data. The pH dependence of chemical shifts was fitted as usual according to the modified Henderson–Hasselbalch equation

$$\delta(\mathrm{pH}) = \delta_{AH} + (\delta_{A^-} - \delta_{AH})10^{\mathrm{pH}-\mathrm{p}K}/(1 + 10^{\mathrm{pH}-\mathrm{p}K}) \quad (5)$$

where δ, δ_{AH}, and δ_{A^-} stand for the observed chemical shift and the chemical shift in the fully protonated and in the fully deprotonated form, respectively. The changes in chemical shift during the titration of FIII with HPr may be described by

$$\delta = \delta_{FIII} + \tfrac{1}{2}(\delta_{FIII-HPr} - \delta_{FIII})\{1/(3K[FIII]_T) + [HPr]_T/(3[FIII]_T) + 1 - [[1/(3K[FIII]_T) + [HPr]_T/(3[FIII]_T) + 1]^2 - 4[HPr]_T/(3[FIII]_T)]^{1/2}\} \quad (6)$$

under the assumptions that HPr binds independently to the subunits of FIII and that fast-exchange conditions apply for the exchange between free FIII and the FIII in the FIII–HPr complex. δ_{FIII} and $\delta_{FIII-HPr}$ are the chemical shift values in free FIII and the FIII–HPr complex, K is the association constant for the binding of HPr to one of the subunits of FIII, and $[HPr]_T$ and $[FIII]_T$ are the total concentrations of HPr and FIII, respectively.

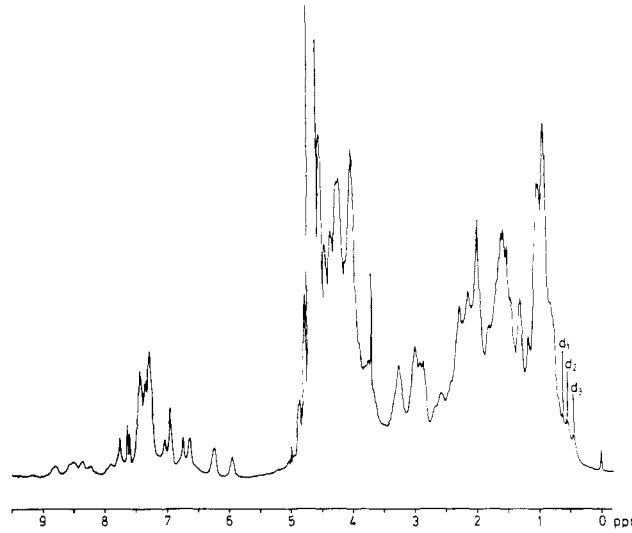

FIGURE 1: 360-MHz spectrum of native FIII. FIII (10 mg/mL) in 6 mM phosphate buffer, 0.1 M KCl, and 0.5 mM EDTA, pH 7.6. About 3000 spectra were accumulated at 308 K. The NH protons were preexchanged in D_2O at room temperature for 12 h.

Results

Unphosphorylated Factor III. Figure 1 shows the ^{1}H NMR spectrum of native (unphosphorylated) factor III. As expected for a protein of a molecular weight of 33 000, the resonance lines are so broad that J couplings are not resolved. The low field part of the spectrum consists of the resonance lines of amide protons and the 41 protons of the aromatic rings of two tyrosyl, five phenylalanyl, and four histidyl residues (Hays et al., 1973).

Spectral Changes in the pH Range of pH 6–9. FIII is soluble in millimolar concentrations around pH 8.5. Its solubility decreases drastically at acidic pH. Below pH 5.8, the obtainable concentrations are too low to get ^{1}H NMR spectra with sufficient signal-to-noise ratio in a reasonable amount of time. Figure 2 shows spectra of FIII at various pH values. The total spectrum remains essentially unchanged down to pH 6.4. Further decrease of the pH causes an overall change of the NMR spectrum which is indicative for a general change of protein conformation.

As compared to the whole intensity of the aromatic region, seven singlet resonances, denoted A′, B′, C′, D′, B″, C″, and D″, have an intensity corresponding to about one proton each. They show the pH dependence of the chemical shift typical for the imidazole protons of histidyl residues (Figure 3).

The titration parameters are given in Table I. From the obtained pK values, line widths, and chemical shifts, it is possible to correlate the resonances as follows: lines B′ and B″, C′ and C″, and D′ and D″, respectively, correspond to C-2 and C-4 protons of histidyl residues designated as B, C, and D below, and A′ corresponds to the C-2 proton of a fourth histidyl residue A.

Some caution must be expressed concerning the assignments of line D′ and D″ to C-2 and C-4 protons of histidine D. D″ behaves like a C-4 proton resonance of histidine with respect to the chemical shift at alkaline pH whereas D′ is shifted about 1.6 ppm to a higher field as compared to the histidyl C-2 resonance in the model peptide (Table IV). It is unlikely that the signal D′ stems from an extremely downfield shifted

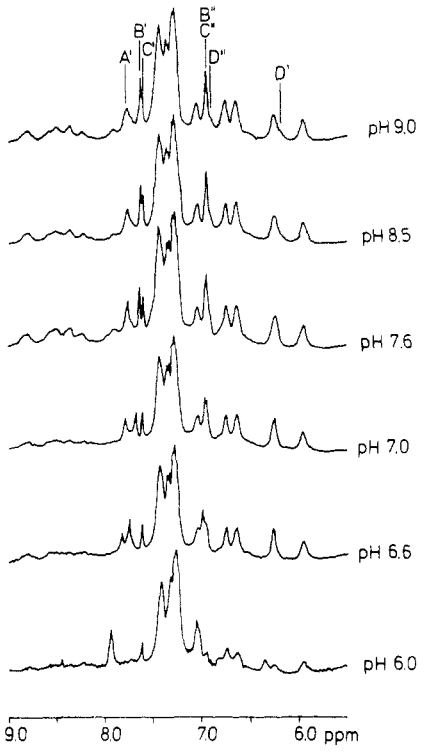

FIGURE 2: Changes in the downfield part of the spectrum of native FIII between pH 6.0 and 9.0. Same sample as in Figure 1.

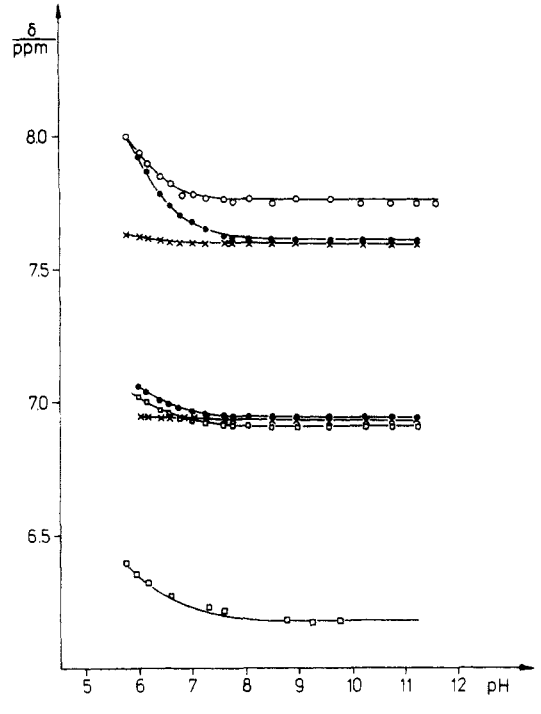

FIGURE 3: Titration of the C-2 and C-4 proton resonances of histidine residues in unphosphorylated FIII. Histidine A (○), histidine B (●), histidine C (×), and histidine D (□). The solid curves are computed with the parameters in Table I. Results of four different measurements under the same conditions as in Figure 1.

$C_\alpha H$ resonance because of its titration step of 0.3 ppm.

One of the resonances d_1, d_2, and d_3 in Figure 1, probably resulting from methyl resonances shifted to high field, also changes its chemical shift with the pH (resonance d_3). The pK value of 5.8 suggests that this change in chemical shift depends on the protonation of histidyl residue A or C.

Spectral Changes in the pH Range between pH 9 and 12.5. Figure 4 shows the spectral changes during titration in the alkaline pH range. The spectrum remains essentially unchanged up to pH 10.7, but changes significantly at higher pH values. Two new peaks, E′ and F′, become visible. Their titration parameters (peak E′, pK = 10.6, δ_{AH} = 7.12 ppm, δ_{A^-} = 6.95 ppm; peak F′, pK = 10.6, δ_{AH} = 6.81 ppm, δ_{A^-} =

Table I: pH Dependence of Chemical Shifts of Histidine C-2 and C-4 Protons and of a Few High Field Shifted Methyl Resonances in *Unphosphorylated* FIII[a]

residue		pK	chemical shift δ_{AH} in protonated form	chemical shift δ_{A^-} in deprotonated form	titration step $\delta_{AH} - \delta_{A^-}$
His-A	C-2 H	5.6	8.30	7.77	0.53
	C-4 H				
His-B	C-2 H	6.0	8.22	7.61	0.61
	C-4 H	6.1	7.15	6.94	0.21
His-C	C-2 H	5.7	7.67	7.60	0.07
	C-4 H	5.7	6.97	6.94	0.03
His-D	C-2 H	6.2	6.48	6.18	0.30
	C-4 H	6.2	7.10	6.91	0.19
d_1	CH_3			0.62	
d_2	CH_3			0.53	
d_3	CH_3	5.8	0.56	0.43	0.13

[a] Experimental conditions as described in Figure 1. δ_{AH}, δ_{A^-}, and pK values result from a computer fit of the data presented in Figure 3.

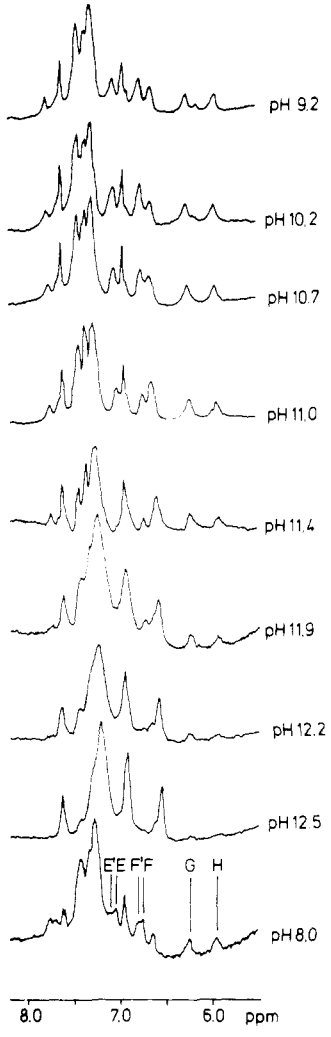

FIGURE 4: Changes in the downfield part of the spectrum of native FIII between pH 9.2 and 12.5. FIII (14 mg/mL) in 6 mM phosphate buffer and 0.5 mM EDTA; temperature 308 K. The bottom spectrum was obtained after backtitrating the sample from pH 12.5 to 8.0.

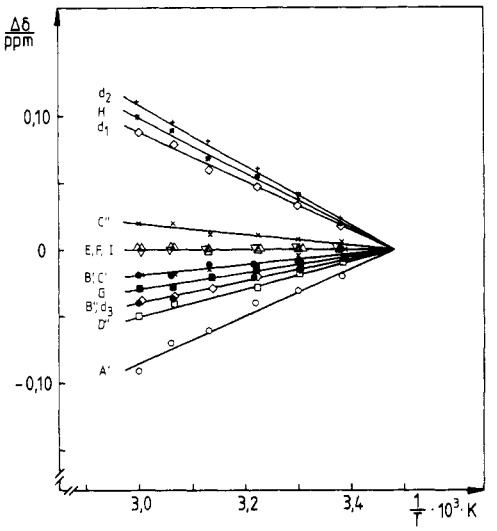

FIGURE 5: The change of chemical shift $\Delta\delta$ vs. the inverse of the temperature $1/T$ in unphosphorylated FIII. The resonance lines are denoted as in Figure 1, 2, and 4. Line I denotes the resonance line between line F and G in Figure 4. The chemical shift value at 287 K was chosen as zero. Experimental conditions: 14 mg/mL FIII in 2 mM phosphate buffer and 0.2 mM EDTA.

6.57 ppm) are close to the values of tyrosine measured in the tetrapeptide Gly-Gly-Tyr-Ala (Bundi & Wüthrich, 1979) (Table IV). The decrease in intensity of lines E and F at 7.03 and 6.74 ppm, respectively, parallels the growth of lines E′ and F′, finally leading to complete disappearence of lines E and F at pH 12.5. The spectral changes are not completely reversible, as may be seen from the last spectrum of Figure 4. The two additional lines E′ and F′ are still visible but with reduced intensity. In the native protein, the intensity of each of the resonance lines E and F corresponds to about two protons compared with the integral of the aromatic part of the spectrum. E and F as well as E′ and F′ may therefore be caused by the C-2/6 and C-3/5 protons of the same tyrosyl residue.

This implies that the interconversion between the native form of the protein and the form(s) at high pH values is slow on the NMR time scale. According to the relation for slow exchange, $\Delta\omega\tau_c \gg 1$, where $\Delta\omega$ is the difference in chemical shift and τ_c the exchange correlation time, one may estimate an upper limit for the exchange rate of 160 s^{-1}.

The steady state at pH values between pH 10.7 and 12 is reached very slowly (after several hours), and the amount of irreversibly denatured protein is dependent on the incubation time at high pH. The resonance lines G and H at 6.24 and 5.94 ppm also disappear with increasing pH without significant changes in their chemical shift, again indicating a slow exchange process.

Exchange of Amide Protons. Figure 1 shows clearly visible resonance lines of unexchanged amide protons between 7.5 and 9 ppm, although this spectrum was recorded after about 12 h of storage in D_2O at room temperature and pH 7.6. The rapidly exchanging protons could not be observed due to the poor signal-to-noise ratio, but for the slowly exchanging ones it is possible to calculate a half-life of about 15 h (36 °C, pH 7.6). Interpolation to time $t = 0$ results in an integral corresponding to about ten slowly exchanging protons.

CD measurements showed that the helix content at this pH should be about 45% (Deutscher, 1979). Therefore it may well be that these resonance lines correspond to hydrogen-bonded amide protons in helical regions.

Spectral Changes with Temperature. Many resonances in FIII change their chemical shift with the temperature, which

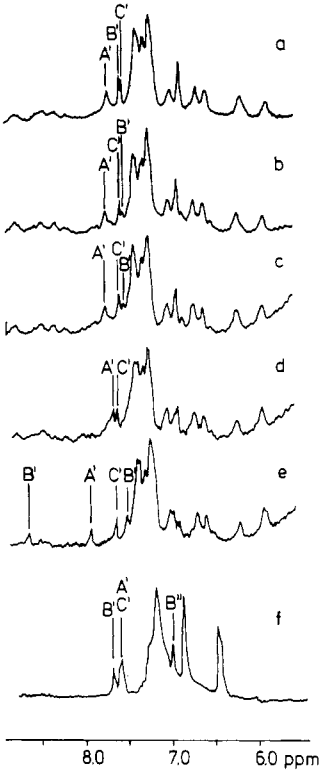

FIGURE 6: Enzymatic phosphorylation of FIII by HPr. The sample contained 8 mg/mL FIII in 5 mM phosphate buffer. (a) pH 8.4, temperature 308 K; (b) as (a), but HPr was added to a concentration of 1 mg/mL at pH 8.4 and temperature 308 K; (c) as (b), but $MgCl_2$ and PEP were added to a concentration of 5 mM and 20 mM, respectively, at pH 8.2 and temperature 308 K; (d) as (c), but enzyme I was added to a concentration of about 1 mg/mL at pH 8.1 and temperature 308 K; phosphorylation of FIII of more than 85%; (e) as (d), but pH 6.4 and temperature 287 K; phosphorylation of FIII more than 85%; (f) as (e), but pH 12.4; phosphorylation of FIII more than 85%.

may indicate that native FIII exists in at least two rapidly exchanging conformations. Their relative populations vary with temperature. The temperature dependence is represented for some well-resolved resonances in Figure 5.

FIII denatures slowly above 325 K, and native and denatured FIII coexist for several hours. At 341 K, it lasts about 3 h before denaturation is completed. Resonance lines E, F and E′, F′ do not change their positions significantly with temperature, indicating slow exchange even at this temperature. Their intensity changes in the same way as observed during alkaline denaturation.

Interaction of Unphosphorylated FIII with HPr. Figure 6b demonstrates the effect of adding a small amount of HPr protein to a solution of FIII. Because the resonances of the C-2 protons of histidyl residues A, B, and C do not overlap with any signals of the HPr protein as known from Schmidt-Aderjan et al. (1979), it is possible to follow precisely the dependence of their chemical shifts on the amount of HPr added. The results of these titration studies show a remarkable upfield shift of the C-2 resonance of histidine B with increasing HPr concentration. Additional signals of the HPr–FIII complex or changes in intensity of the imidazole resonance lines of FIII could not be observed even at high HPr concentrations. Therefore the condition of fast exchange between free FIII and FIII in the HPr–FIII complex may apply.

The change of chemical shift $\Delta\delta$ in the FIII–HPr complex and the association constant K_{assoc} (Table II) were calculated according to eq 6. No satisfying fit of the theoretical curve to the experimental data could be obtained for an association

Table II: Interaction of Unphosphorylated Factor III with Unphosphorylated HPr Protein[a]

Δδ (ppm) for the C-2 proton of			K_{assoc}	
His-A	His-B	His-C	(M^{-1})	k_{off} (s^{-1})
+0.20 ± 0.02	−1.01 ± 0.05	+0.14 ± 0.02	4.2×10^4	$>2.3 \times 10^3$

[a] Experimental conditions: 11 mg/mL unphosphorylated FIII in 5 mM phosphate buffer and 0.1 M KCl, pH 7.7; temperature 308 K. The HPr concentration was varied by addition of a concentrated solution of HPr protein.

Table III: pH Dependence of Chemical Shifts of Histidine C-2 and C-4 Protons in *Phosphorylated* FIII[a]

residue	proton	p*K*	chemical shift δ_{AH} in protonated form	chemical shift δ_{A^-} in deprotonated form	titration step $\delta_{AH} - \delta_{A^-}$
His-A	C-2 H	6.3	8.37	7.67	0.70
	C-4 H				
His-B	C-2 H	8.6	8.69	7.71	0.98
	C-4 H	8.6	7.56	7.06	0.50
His-C	C-2 H	7.5	7.70	7.61	0.09
	C-4 H			6.94	
His-D	C-2 H				
	C-4 H	6.2	7.19	6.93	0.26

[a] Experimental conditions as described in Figure 6. δ_{AH}, δ_{A^-}, and p*K* values are computed as described under Materials and Methods.

constant less than 5×10^3 M^{-1}. From the calculated change in chemical shift it is further possible to estimate a lower limit of the exchange rate (or k_{off}) of 2.3×10^3 s^{-1}. The phosphohistidyl residue of HPr must come close to a histidyl residue of FIII during the transfer of the phosphoryl group from the HPr protein to FIII. As the most pronounced change in the spectrum of FIII, an upfield shift of about 1 ppm in the deprotonated state could be observed for the C-2 proton of His-B. Therefore it seems reasonable to identify histidine B with the active-center histidine.

Phosphorylated FIII. The isolated P-FIII (see Materials and Methods) decomposed relatively fast at pH values below pH 9.5. To overcome these difficulties, we chose to phosphorylate FIII enzymatically by enzyme I, HPr, and PEP in the NMR tube. So it was possible to keep FIII phosphorylated to more than 85% over 12 h in the pH range 6–12.5. This has been tested by gel electrophoresis in urea before and after each measurement (Deutscher, 1979). Figure 6 shows the spectral changes when the components needed for the phosphorylation are added sequentially to a solution containing only FIII. The addition of a small amount of HPr to the solution results in spectral changes already discussed (Figure 6a,b). Addition of $MgCl_2$ and PEP at a final concentration of 5 and 20 mM, respectively, has no influence on the spectrum (Figure 6c). The phosphorylation has been started by addition of enzyme I in an amount which was tested before to have negligible resonance intensity in the downfield part of the FIII spectrum (Fig. 6d,e).

Figure 6f shows the spectrum of alkaline-denatured phosphorylated FIII. Compared to the spectrum of unphosphorylated FIII denatured by alkali shown in Figure 4, only two additional singlet lines in the aromatic part are visible at 7.71 and 7.06 ppm. These are the positions where the spectral lines of the C-2 and C-4 protons of 3-phosphohistidine in the model peptide Gly-Gly-His-Ala are observed (Kalbitzer & Rösch, 1981). Figure 7 shows the titration behavior of those

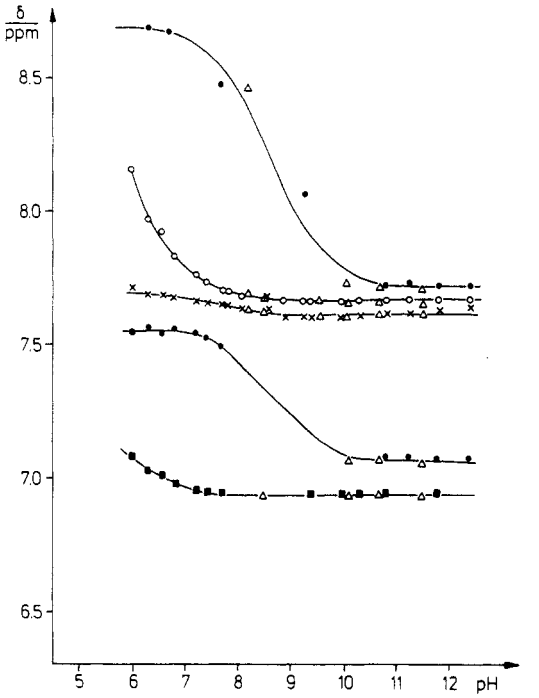

FIGURE 7: pH dependence of histidine resonances in phosphorylated FIII. FIII phosphorylated in the NMR tube (○, ●, ×, ■) and isolated P-FIII (△). Histidine A (○), histidine B (●), histidine C (×), and histidine D (■). The solid curves were drawn by using the parameters given in Table III.

histidine resonances which could be resolved. Table III summarizes the obtained parameters.

We emphasize that the chemical shifts of these lines are not affected by the interaction with the remaining components in the phosphorylation system. This has been shown by comparison with the few spectra of the at least partially phosphorylated isolated P-FIII. No deviations of the shifts could be detected. Moreover, an increase of the HPr concentration by a factor of 5 did not influence the chemical shifts of the lines whose titration behavior is depicted in Figure 7. Because the hydrolysis of PEP led to a small but significant change of the pH value, we could not obtain sufficient data in the p*K* range of phosphohistidine B to determine the p*K* value accurately.

Comparison of the chemical shift values of the other histidyl residues in phosphorylated and unphosphorylated FIII shows the following effects. Histidyl residue A changes its chemical shift in the deprotonated form but not in the protonated form within the experimental error. The p*K* values of histidines A and C are increased by about 0.7 and 1.8 units. In contrast, histidine D exhibits no significant changes in p*K* value or in chemical shift.

Tryptic Peptide T-2. Figure 8 shows the low-field part of the ^1H NMR spectrum of phosphorylated and unphosphorylated peptide T-2 which was prepared as described under Materials and Methods. Phosphorylation leads to a downfield shift of the C-2 and C-4 proton resonances of one of the histidyl residues. The titration parameters obtained by observation of the pH dependence of chemical shifts in the pH range 2–12 are given in Table IV.

The spectra of unphosphorylated peptide T-2 could easily be correlated with those of phosphorylated peptide by comparison of spectra with varying degrees of phosphorylation. Whereas p*K* values and chemical shifts of the resonances of one histidyl residue (His-I) are identical in phosphorylated and unphosphorylated fragment T-2, the phosphorylation results in distinct changes of the titration parameters of the other

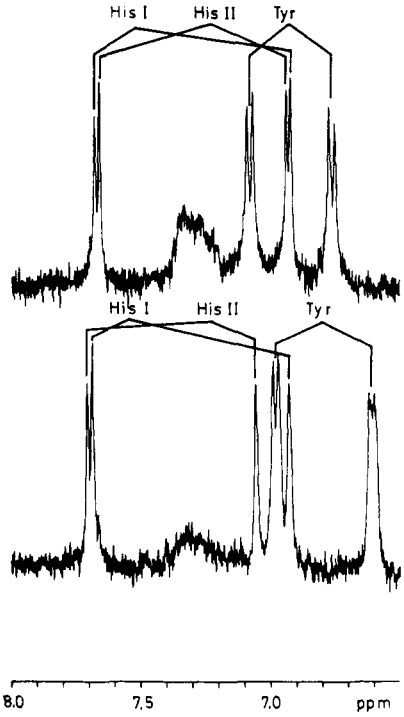

FIGURE 8: Downfield part of the spectrum of phosphorylated and unphosphorylated peptide T-2. (Upper spectrum) Unphosphorylated peptide T-2 (2 mg/mL) in D_2O, pH 10.7. (Lower spectrum) Phosphorylated peptide T-2 (2.2 mg/mL) in D_2O, pH 11.1.

histidyl residue (His-II). These agree very well with the changes found in the model peptide Gly-Gly-His-Ala upon phosphorylation of the histidyl residue at its N-3 position (pK = 7.4; C-2 H, δ_{AH} = 8.44 ppm, δ_{A^-} = 7.74 ppm; C-4 H, δ_{AH} = 7.29 ppm, δ_{A^-} = 7.05 ppm). Since phosphorylation at the N-1 position causes different changes of the pK value and of the chemical shift (Kalbitzer & Rösch, 1981), peptide T-2 must be phosphorylated at the N-3 position of one of its two histidines.

The only tyrosyl residue, Tyr-I, in both phosphorylated and unphosphorylated peptide T-2, exhibits chemical shifts for the C-2/6 and C-3/5 protons at neutral pH, which resemble very closely those obtained in model peptides. Its pK value is about 0.5 unit higher (Bundi & Wüthrich, 1979). The chemical shift values at high pH change significantly upon phosphorylation. The titration steps are 0.10 and 0.16 ppm for the unphosphorylated peptide and 0.16 and 0.25 ppm for the phosphorylated peptide (Table IV).

Table IV: pH Dependence of Chemical Shift of the Aromatic Protons of Histidyl and Tyrosyl Residues in *Phosphorylated* and *Unphosphorylated Peptide* T-2[a]

residue	proton	pK	chemical shift δ_{AH} in protonated form	chemical shift δ_{A^-} in deprotonated form	titration step
His-I of unphosphorylated T-2	C-2 H	6.7	8.62	7.70	0.92
	C-4 H	6.7	7.31	6.92	0.39
His-I of phosphorylated T-2	C-2 H	6.7	8.61	7.69	0.92
	C-4 H	6.7	7.30	6.93	0.37
His-II of unphosphorylated T-2	C-2 H	6.6	8.62	7.68	0.94
	C-4 H	6.6	7.29	6.94	0.35
His-II of phosphorylated T-2	C-2 H	7.2	8.47	7.71	0.76
	C-4 H	7.2	7.32	7.05	0.27
His of Gly-Gly-His-Ala	C-2 H	7.0	8.61	7.75	0.86[b]
	C-4 H	7.0	7.35	6.97	0.38[b]
Tyr-I of unphosphorylated T-2	C-2/6 H	10.8	7.11	7.01	0.10
	C-3/5 H	10.8	6.81	6.65	0.16
Tyr-I of phosphorylated T-2	C-2/6 H	10.9	7.11	6.93	0.18
	C-3/5 H	10.9	6.81	6.56	0.25
Tyr of Gly-Gly-Tyr-Ala	C-2/6 H	10.3	7.15	6.98	0.17[b]
	C-3/5 H	10.3	6.86	6.57	0.29[b]

[a] δ_{AH}, δ_{A^-}, and pK values are computed as described under Materials and Methods. [b] Bundi & Wüthrich (1979).

Discussion

Conformational Changes. The alkaline and thermal denaturation proceeds in a cooperative manner very similar to the acidic and thermal denaturation, extensively studied on lysozyme by McDonald et al. (1971). Denatured and native FIII coexist and interconvert with a rate of less than 160 s^{-1} [lysozyme, less than 200 s^{-1} (McDonald et al., 1971)]. Prolonged exposition to denaturing conditions leads to a partially irreversible denaturation.

This process can be formally described by N $\rightleftarrows$ C $\rightarrow$ D where N symbolizes the native conformation(s), C the random-coil form, and D the irreversibly denatured form of the protein. Under denaturing conditions forms C and D cannot be distinguished in the ^{1}H NMR spectrum. If the transition from C to D follows from a chemical alteration of the protein molecule or if form C represents a molecule in which some local conformation has been maintained cannot be decided from our results.

The ^{1}H NMR spectrum of globular proteins like lysozyme or HPr protein (Maurer et al., 1977) remains essentially unchanged upon variation of the temperature in a large range. In contrast, the resonance lines of FIII shift significantly with the temperature (Figure 5). This suggests the existence of more than one conformation N_i of native FIII which exchange rapidly on the NMR time scale. The relative populations vary with the temperature. This is not unexpected in light of the fact that FIII has to react with two different proteins in two different environments.

The phosphorylation of peptide T-2 causes an additional change of the titration step of the resonances of the ring protons of Tyr-I. A mutual direct interaction of the phosphohistidine with the tyrosyl residue is not likely because its chemical shift at neutral pH and the pK value are not significantly influenced by the phosphorylation of His-II. The most probable explanation for the increase of the titration step in the phosphorylated peptide T-2 seems to be that the transition to the random-coil form parallels the increase in pH whereas in unphosphorylated peptide some tertiary structure remains preserved even at high pK values. This is supported by the observation that the titration step in the phosphorylated peptide is very close to that obtained in a random-coil peptide (Bundi & Wüthrich, 1979). The change of the local environment of this tyrosyl residue may be characteristic for the irreversible denaturation in the peptide as well as in FIII.

The irreversible denaturation as observed by ^{1}H NMR proceeds much faster in phosphorylated FIII than in unphosphorylated FIII. This is in line with CD measurements which indicated a decrease of the helix content from 42% of unphosphorylated FIII to 25% of phosphorylated FIII at pH 8.7, a value identical with that found in the denatured unphosphorylated FIII at pH 12.8.

Interaction of FIII with HPr. Binding of HPr to factor III results in a remarkable upfield shift of the C-2 H resonance line of histidine B (which carries the phosphoryl group in P-FIII).

The calculation of the chemical shift in the HPr–FIII complex was based on the assumption of fast-exchange conditions. Because of the relatively poor signal-to-noise ratio, it is impossible to verify that this assumption holds strictly by line shape analysis. But as demonstrated by Feeney et al. (1979), slower exchange rates on the borderline of fast exchange to intermediate exchange where only $\Delta\omega\tau_c \leq 1$ applies lead to an underestimation of the chemical shift difference between the bound and the unbound state of the protein; i.e., the calculated values can be considered as the lower limits of the real chemical shift differences. The upfield shift of histidine B may be a ring current effect from one of the aromatic residues in the active center of HPr. Since the ring current intensity is less for histidine and tyrosine than for phenylalanine (Giessner-Prettre & Pullman, 1969), one can estimate an upper limit for the distance of the center of this aromatic ring to the C-2 proton of histidine B as 0.38 nm (Perkins & Dwek, 1980). Because of the direction of the change in chemical shift, the proton has to be inside of the shielding conus above and below the ring plane. Sterochemical considerations, however, postulate a planar arrangement of the histidyl residue transferring the phosphoryl group and of the acceptor histidine to promote the nucleophilic attack of the free electron pair of N-3 of histidine B to phosphorus of N-1 of histidine-15 in HPr.

Consequently, if the phosphorylation of histidine-15 does not lead to a local rearrangement of the histidyl residues in the HPr–FIII complex, then histidine-15 of HPr probably does not cause the upfield shift since this would require a stacked arrangement of these aromatic rings. Another candidate for this stacking interaction could be tyrosine-56, which is supposed to be in the near neighborhood of histidine-15 in HPr (Rösch et al., 1981).

The strong effect of binding of HPr to FIII on the ^{1}H NMR spectrum in contrast to the lack of evidence for an interaction between P-FIII and P-HPr suggests that the association constant is strongly decreased by phosphorylation of both proteins. This seems reasonable from the mechanistic point of view, because a strong complex of P-HPr with P-FIII would decrease the rate of phosphoryl transfer.

Position of the Phosphoryl Group in FIII. The chemical shift of P-histidine vs. histidine at alkaline pH is known to change in a characteristic way, depending on whether N-1 or N-3 of the histidine ring is phosphorylated (Gassner et al., 1977; Kalbitzer & Rösch, 1981). Consequently, phosphorylated peptide T-2 carries its phosphoryl group at the N-3 position of one of its histidyl residues. Since phosphopeptide T-2 has been prepared by tryptic cleavage of phosphorylated factor III, the phospho group in intact P-factor III is likely to be attached also to the N-3 position.

Phosphorylation of FIII leads to two additional resonance lines in the alkaline-denatured FIII very close to the positions of the spectral lines of the phosphohistidine in peptide T-2. In contrast, the changes of the titration step and of the pK value of FIII do not agree with the changes observed in the model peptide phosphorylated either at the N-3 position or at the N-1 position. This is not surprising because the negatively charged phospho group is very likely to interact strongly with the molecular environment in the native structure.

Factor III is also phosphorylated at the N-3 position in at least its alkaline-denatured form. Since the transition from native P-FIII to alkaline-denatured P-FIII and vice versa

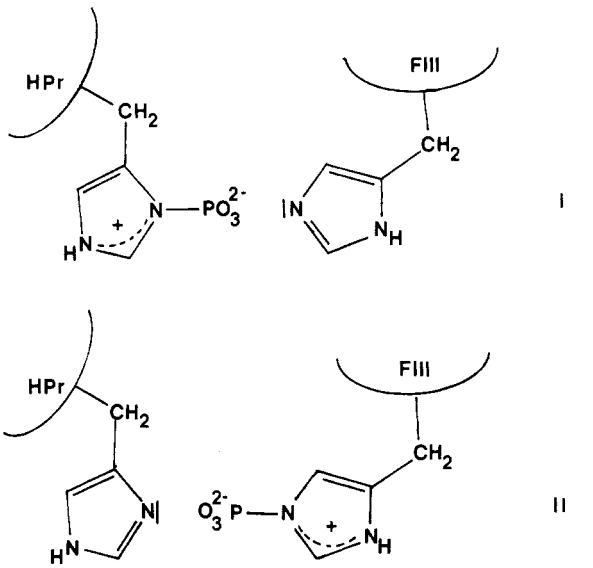

FIGURE 9: Ionization state of the histidyl residues in the active center of HPr protein and factor III before (I) and after (II) the phosphoryl transfer at pH 7.4.

reveals no discontinuity in the titration curves of the phosphohistidyl residue, it may be concluded that native FIII also carries its phosphoryl group at the N-3 position of the histidine ring.

One significant advantage of the determination of the position of the phosphoryl group at the histidyl residue by NMR methods is the fact that the procedure can be carried out with intact protein at physiological pH values. Complete alkaline hydrolysis of phosphorylated factor III also resulted in isolation of 3-phosphohistidine, which means that during the alkaline hydrolysis no migration of the phospho group had occurred (Hays et al., 1973).

Histidyl Residues. All four histidyl residues exhibit pK values much lower than 7, as found in the random-coil model Gly-Gly-His-Ala (Bundi & Wüthrich, 1979).

Especially for the active-center histidine B, this means that the aromatic ring is almost completely deprotonated under physiological conditions and therefore a good candidate for a nucleophilic attack to the phosphoryl group of P-HPr. The phosphohistidyl residue of HPr protein is completely protonated at this point; i.e., the histidyl ring carries a positive charge (step I, Figure 9).

Phosphorylation of FIII as well as of HPr protein (Gassner et al., 1977; Dooijewaard et al., 1979) results in an increase of the pK value at the histidine ring remarkably higher than that expected from the model peptide. Therefore, the histidyl residue in the active center of HPr protein and factor III changes its protonation state under physiological conditions from the deprotonated to the completely protonated form when it becomes phosphorylated.

The phosphorylation of histidine B leads to remarkable changes in the titration parameters of histidine A and C, which are also influenced to a small extent by the binding of HPr to FIII. Histidine C shows no major changes of the chemical shift value at alkaline pH but an increase of the pK of about 1.8 units. This could be the direct influence of the negative charge of the phosphoryl group on the protonation state of the neighboring histidine.

The chemical shift of the C-2 proton of histidine D in the neutral form is 1.57 ppm upfield of the normal position for a histidine C-2 proton (Bundi & Wüthrich, 1979) whereas the resonance line of the C-4 proton is shifted only 0.06 ppm to higher field. This suggests a close contact of the C-2 proton

of histidine D to another aromatic residue in FIII similar to the interaction of His-48 and Tyr-52 in pancreatic phospholipase A2. Aguiar et al. (1979) observed an upfield shift of 1.57 and 0.4 ppm for the C-2 H and the C-4 H resonances of His-48, respectively, which could be explained on the basis of the X-ray structure as the ring current effect of Tyr-52.

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Thermal Denaturation of *Streptomyces* Subtilisin Inhibitor, Subtilisin BPN′, and the Inhibitor–Subtilisin Complex†

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ABSTRACT: The thermal unfolding of the microbial proteinase inhibitor *Streptomyces* subtilisin inhibitor (SSI) [Sato, S., & Murao, S. (1973) *Agric. Biol. Chem. 37*, 1067–1074), the bacterial proteinase subtilisin BPN′ (EC 3.4.21.14), and the complex formed by these two proteins has been studied by differential scanning calorimetry (DSC). The thermal denaturation of SSI at pH 7.00 is fully reversible while those of subtilisin BPN′ and its complex with SSI are not. The DSC data show that dimeric SSI remains dimeric as the temperature is raised until it unfolds and that it then dissociates during the unfolding process. The apparent specific heat of denatured SSI decreases rapidly with increasing temperature, a behavior not previously observed for proteins. The shape of the DSC curves observed with the enzyme–inhibitor complex suggests that the two components of the complex undergo their unfolding transitions more or less independently. The enthalpies of unfolding of mixtures of enzyme and inhibitor in various molar ratios indicate a substantially larger enthapy of interaction than that deduced from fluorescence titrations (Uehara, Y., Tonomura, B., & Hiromi, K. (1978) *J. Biochem. (Tokyo) 84*, 1195–1202).

Streptomyces subtilisin inhibitor (SSI), isolated from *Streptomyces albogriseolus*, strongly inhibits bacterial proteinases such as subtilisin BPN′, with inhibition constants as high as 10^{10} M. The inhibitor consists of two identical subunits, each containing a polypeptide chain of 113 amino acid residues with 2 intrachain disulfide bridges. Each subunit, of molecular weight 11 483, binds to one molecule of subtilisin to form a complex E_2I_2 (Inouye et al., 1978). The amino acid sequence of the inhibitor was determined by Ikenaka et al. (1974), and its three-dimensional structure has been recently determined at 2.3-Å resolution, along with that of the inhibitor–protein complex E_2I_2 at 4.3-Å resolution, by Mitsui et al. (1979).

The detailed characterization available for SSI and for its complex with subtilisin makes this system an attractive one for investigation by DSC. The results of such a study are presented in this paper.

Materials and Methods

Materials. Partially purified SSI was a gift from Professor Keitaro Hiromi of Kyoto University, Japan. The protein was further purified by the method of Sato & Murao (1973). Its concentration was determined from the absorbance at 280 nm by using a value for the absorptivity of 0.82 cm^2 mg^{-1}. Crystalline subtilisin BPN′ (molecular weight 27 500), purchased from Nagase Sangyo Co., Osaka, Japan, was also a gift from Professor Hiromi. Its concentration was determined

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