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Use of Histidine pK_a Changes to Study Peptide–DNA Interactions

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Interactions between peptides and DNA can be followed by observing the chemical shift changes of the tyrosine and histidine side chain protons in the presence of DNA. These changes are often interpreted as arising from the diamagnetic anisotropy of the aromatic bases of DNA. In the case of histidine the change of the pK_a of the imidazole side chain caused by the proximity of the negatively charged DNA is an alternative explanation for the observed shifts. A distinction between these two effects in the complexes of histidinamide and Ac-His–Arg–Tyr–Arg–Pro-OH with DNA is made by recording the spectra over the complete titration curve. The observed shifts can be treated semiquantitatively using the Gouy–Chapman theory.

INTRODUCTION

Studies on protein–DNA complexes are of great interest in order to understand chromatin structure and regulatory systems. ^{1}H NMR has been extensively used in these studies with both complete proteins or model peptides. A number of these studies have focused attention on chemical shift changes induced by the diamagnetic anisotropy of the aromatic DNA bases. From these observations, insertion of aromatic molecules between the DNA bases has been demonstrated in several antibiotics (*1*) and in tryptophan-containing peptides (*2*). The possible stacking of the aromatic side chain of tyrosine residues has been the subject of great interest and controversy (*3*, *4*).

When histidine is involved, a second chemical shift effect caused by complexation of small ligands to DNA that has been largely overlooked is due to the electrostatic influence of DNA over the acid–base equilibria of the ligands. This phenomenon can be detected by measuring the spectra of the DNA–ligand complexes at different pH values. Furthermore, quantification of this effect can provide information on the distance between the ligand and DNA in the complex.

In this paper we present our studies of the DNA–complexes of tyrosine-containing pentapeptide **1** (*5*), Ac-His–Arg–Tyr–Arg–Pro-OH, that corresponds to residues 39–43 of histone H3.

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RESULTS AND DISCUSSION

Table 1 shows the chemical shifts of the aromatic side chains of peptide **1** in the presence of different molar ratios of DNA and polyadenylic acid.

The upfield shifts induced in the tyrosine resonances by the addition of polyadenylic acid can be interpreted by assuming the stacking of the aromatic residue between the adenine bases. The chemical shifts of the phenol ring protons of tyrosine do not change upon addition of double-stranded DNA. This indicates that the tyrosine residue in peptide **1** does not intercalate between the base pairs of native DNA. On the other hand, two different explanations are possible for the downfield shifts of the imidazole protons observed with DNA and polyadenylic acid. A first model had been proposed by Gourevich *et al.* on the basis of a similar observation made with histidinamide and DNA (*6*). In this model the induced shift arises from the in-plane approximation of the histidine side chain to a base pair. This approach would be stabilized by opening of the base pair and formation of a tertiary hydrogen-bonded complex involving the imidazole ring. The alternative explanation, already considered by the same authors, is a change in the pK_a of the imidazole side chain caused by the proximity of the negatively charged DNA. Discrimination between these two models for the peptide **1**–DNA system is possible by measuring the ^1H NMR spectra over the complete titration curve of the histidine side chain.

Figure 1 compares the pH-induced variations of the chemical shift of the H_2 proton in the histidine side chain of peptide **1** in the presence and in the absence of DNA. In both cases the curve shows a sigmoidal shape, reflecting the ionization of the imidazole ring. In the absence of DNA, the Henderson–Hasselbalch equation is fulfilled and gives a pK_a of 6.24 ± 0.06. Conversely, data obtained at low pH values in the presence of DNA show deviations from the above equation. In this situation the pK_a can be calculated by using Eq. [1] (*7*);

$$\delta = \delta_{AH} - K_a(\delta - \delta_A/h_O), \qquad [1]$$

TABLE 1

EFFECT OF NUCLEIC ACIDS ON THE CHEMICAL SHIFTS OF THE AROMATIC PROTONS OF Ac-His–Arg–Tyr–Arg–Pro–OH[a]

Peptide concentration (mM)	Nucleic acid[b]	R[c]	δ (Tyr)		δ (His)[d]	
			$H_{3,5}$	$H_{2,6}$	H_4	H_2
2	None	0.0	6.81	7.12	6.91	7.87
2	Poly A	3.7	6.76	7.07	6.92	7.92
2	Poly A	10.0	6.74	7.04	6.92	7.94
0.2	DNA	10.0	6.81	7.12	7.00	8.09
0.2	DNA	20.0	6.81	7.12	7.00	8.11

[a] Measured, in 1 mM phosphate in D_2O (pH* = 7.0), as ppm downfield from internal DSS.
[b] Poly A, polyadenylic acid; DNA is from calf thymus.
[c] R denotes the ratio of the concentration of nucleic acid (as phosphate) to that of peptide.
[d] Linewidths are 2 Hz (R = 0), 3 Hz (poly A, R = 10) and 4 Hz (DNA, R = 20).

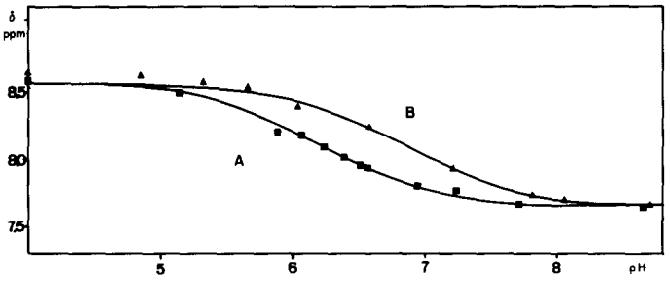

FIG. 1. pH dependence of the chemical shift of proton H_2 in the histidine side chain of Ac-His–Arg–Tyr–Arg–Pro-OH in the presence (B) and in the absence (A) of DNA. Solid lines represent theoretical fits of the data using the values presented in Table 2.

where δ is the observed chemical shift, $h_O = 10^{-pH}$, and δ_A is the measured chemical shift of the unprotonated form. The chemical shift of the acidic form (δ_{AH}) and the acidity constant (K_a) are determined from the intercept and the slope of the straight line obtained by plotting δ versus $\delta - \delta_A/h_O$.

This treatment is related to the Maroni and Calmon procedure for spectrophotometric pK_a determination using a single limiting value (*8*). The measured values are presented in Table 2.

From these data we can see that the chemical shifts of the fully protonated and neutral forms of the histidine residue do not change when peptide **1** interacts with DNA under our experimental conditions. The present results indicate that the observed shift effects can be explained by a change in the pK_a of the imidazole group and cannot be taken as an evidence for a in-plane hydrogen-bonded complex between the imidazole ring and the base pair framework of DNA.

The changes in the measured pK_a can be analysed by considering the different equilibria involved, namely the protonation equilibria of both free and complexed peptide and the dissociation constant of the complexes involving the peptide in the various ionization states (Fig. 2).

As the chemical shift of the histidine residue at pH values far from the pK_a is not affected by the presence of DNA (Table 2), we can assume that the measured chemical shift gives us the ratio of protonated to neutral form independent of the

TABLE 2

pK_a VALUES AND CHEMICAL SHIFTS OF THE PROTONATED AND NEUTRAL FORMS OF THE HISTIDINE SIDE CHAIN OF PEPTIDE **1** WITH AND WITHOUT DNA

Concentration (mM)		Chemical shifts (ppm)				
		Basic form		Acid form		
Peptide	DNA	H_2	H_4	H_2	H_4	pK_a
0.5	—	7.65	6.83	8.59	7.14	6.24
0.5	5	7.65	6.83	8.55[a]	7.13[a]	6.86

[a] Extrapolated using Eq. [1].

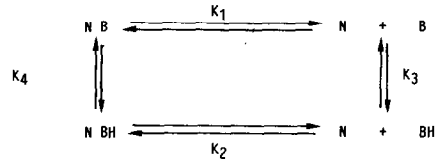

FIG. 2. Pattern of dissociation and protonation equilibria for a 1 : 1 complex of a basic peptide (B) and a nucleic acid (N).

complexation state of the peptide. Furthermore, as DNA is present in excess the concentration of uncomplexed DNA can be taken as a constant. Thus the K_a obtained from Eq. [1] is a composite constant that contains contributions from the acidity constants of the free (K_3) and bound (K_4) peptide, from the dissociation constant (K_1), and from the concentration of free DNA ([N]). From consideration of the equilibria in Fig. 2 K_a can be deduced to be

$$K_a = \frac{K_3K_4(K_1 + [N])}{K_1K_4 + K_3[N]}. \qquad [2]$$

Highly basic peptides form electrostatic complexes with DNA that present dissociation constants in the range from 10^{-4} to 10^{-6} M (*9*). As [N] is usually much larger than these values, the K_a values tend to the acidity constant of the complexed peptides (i.e., $K_a \simeq K_4$). Therefore, the pK_a values measured in the presence of DNA can be taken as the true pK_a of the complexed peptides. For less basic peptides, dissociation constants may be higher (up to 10^{-2} M for a monocation) and the pK_a of the complexed peptide must be calculated using Eq. [2].

The change in the pK_a of the histidine side chain on complexation with DNA can be discussed by taking into account the polyanionic nature of DNA. Considering DNA as a negatively charged cylinder surrounded by a diffuse ionic layer, the potential (ψ) at any distance from the surface can be calculated from the charge density and the ionic conditions using Gouy–Chapman theory (*10, 11*). In this treatment the effect of the surrounding ions is included in the calculation of the potential at the DNA surface and in the equation that gives the variation of ψ with distance. The charge density of the cylinder can be estimated from the number of base pairs per turn of the DNA double helix in the B form. Therefore, the apparent pK_a can be calculated from the pK_a in the absence of DNA (pK_0) by using Eq. [3]

$$pK = pK_0 + 0.4343\,\frac{e\psi}{kT} \qquad [3]$$

which takes into account the electrostatic influence of DNA.

Figure 3 shows a plot of the calculated pK_a increment as a function of the distance of the ionizable group to the surface of DNA. Using this figure and the pK_a of the complexed peptide we can estimate the distance of the histidine residue to the surface of DNA in the electrostatic complex formed by peptide **1**. If the experimental pK_a is used directly a distance of 4–5 nm is estimated. Calculations

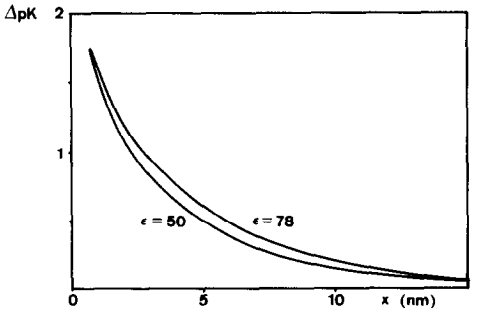

FIG. 3. pK_a variations caused by DNA as a function of the distance between an ionizable group and the nucleic acid. Curves for two values of the dielectric constant are presented.

assuming a higher dissociation constant (10^{-2} M) yield a shorter distance (ca. 2 nm) between the histidine side chain and DNA. This value is consistent with the assumption of the charged arginine side chain lying on the surface of DNA.

In the light of the above results we measured the pH effect on the chemical shift of the imidazole protons of histidinamide under the same conditions reported by Gourevitch *et al.* (*6*). Figure 4 shows a comparison of the results of two measurements at different histidinamide/DNA ratios.

Complete interpretation of the pH-induced chemical shift changes is difficult because of (i) the great variations in the ionic strength during the titration caused by the ionization of histidinamide present in the large concentrations needed for comparison with Ref. (*6*); (ii) the large dissociation constant of histidinamide–DNA complexes [reported to be ca. 10^{-2} (*6*) and that is probably pH dependent (*12*)]; and (iii) the overlapping ionization of the amino group, and thus cannot be described by a single sigmoidal curve.

When no DNA is present, the first effect dominates the titration curve. Therefore, the pK_a variations due to complexation were estimated by comparing samples containing different ratios of histidinamide to DNA. This is the same procedure used by Gourevitch *et al.* (*6*) to measure the chemical shift changes caused by the addition of DNA.

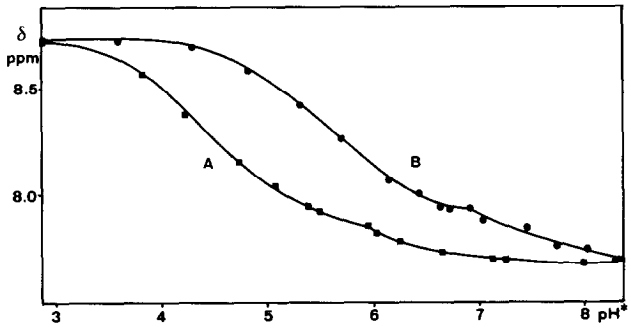

FIG. 4. Chemical shift of the H_2 protons of the imidazole ring of histidinamide at different pH* values. Curve A: [$HisNH_2$] = 107 mM, [DNA-P] = 10.7 mM. Curve B: [$HisNH_2$] = 11.8 mM, [DNA-P] = 11.8 mM.

TABLE 3

CHEMICAL SHIFTS OF THE IMIDAZOLE PROTONS OF FULLY PROTONATED AND UNPROTONATED HISTIDINAMIDE

Concentration (mM)		Chemical shifts (ppm)			
		Basic form		Acid form	
$HisNH_2$	DNA	H_2	H_4	H_2	H_4
107	—	7.68	6.96	8.74	7.49
107	10.7	7.69	6.96	8.71	7.47
11.8	11.8	7.68	6.97	8.73	7.48

The estimated pK_a values of histidinamide measured by NMR in the presence of DNA are 5.4 and 7.6 (11.8 mM $HisNH_2$, 11.8 mM DNA) and they can be compared to the pK_a of pure histidinamide determined by standard potentiometric titration, which are of 5.13 and 7.29 (6.3 mM). If the correction for the isotopic effect (*7*) is applied to the pK_a determined in D_2O the two sets of values agree within the experimental error. The observed shifts of the histidinamide protons in the presence of DNA probably only reflect the change in the activity coefficients that affect mainly the doubly protonated form. The low pK_a values of the imidazole ring of histidinamide can be explained by the effect of the positive charge of the protonated amino group (*13*).

Especially relevant with regard to the problem of the possible interaction between the histidine side chain and DNA is the fact that, as found in peptide **1**, the chemical shifts at pH values far from the pK_a are not affected by addition of DNA (Table 3). Therefore, the NMR data indicating changes in chemical shifts of the imidazole protons of histidinamide in the presence of DNA cannot be interpreted in terms of a hydrogen-bonded complex.

CONCLUSIONS

The interaction between the pentapeptide histone model, Ac-His–Arg–Tyr–Arg–Pro-OH, and nucleic acids has been monitored by ^1H NMR. The stacking of the phenol ring of tyrosine only takes place with polyadenylic acid but not with calf thymus DNA. This result is consistent with previous observations of Hélène *et al.* for tyrosine-containing peptides (*3*).

A chemical shift change is observed for the imidazole protons of the histidine side chain when DNA is added. This effect is also found in histidinamide (*6*) and could be interpreted as an evidence for a specific hydrogen-bonded complex between the imidazole group of histidine and DNA. Our results demonstrate that in the two cases the observed shifts of the NMR signals of the histidine side chain protons can be accounted for by changes in the pK_a due to the polyelectrolyte nature of DNA.

Chemical shift measurements as a function of the pH value afford an apparent pK_a that contains contributions from the dissociation constant of the electrostatic complex between a basic peptide and DNA and from the true pK_a values of the free and bound peptide. Nevertheless, under suitable limit conditions the measured pK_a can be taken as the pK_a of the complexed peptide. In these cases, the pK_a increment can be interpreted using the Gouy–Chapman model to give valuable information on the distance between the ionizable group and the DNA surface.

MATERIALS AND METHODS

The synthesis of Ac-His–Arg–Tyr–Arg–Pro-OH using a chloromethyl-Pab resin has been described elsewhere (*14*). Histidinamide was from Serva. Polyadenylic acid was from Sigma. Calf thymus DNA was prepared by the method of Zamenhof (*15*), exhaustively dialyzed against 1 mM EDTA, pH 7, and for 2 h against 0.2 M NaCl. For the NMR experiments DNA was sonicated three times with a Branson B-12 sonicator for 10 min at 0°C and recovered by ethanol precipitation.

Peptide solutions were prepared in 1 mM phosphate buffer, containing 0.02 mM EDTA. The concentration was determined from the uv absorbance of the tyrosine side chain and subsequently adjusted with buffer to 0.5 mM. Transfer to D_2O was carried out by lyophilizing the solution and adding D_2O until the initial weight was recovered.

The sonicated DNA was dissolved in H_2O and the concentration was determined spectrophotometrically. Samples containing 25 μmol of DNA were lyophilized and dissolved in deuterated buffer containing the peptide or histidinamide. In a control experiment without added ligand, thermal denaturation of a DNA sample caused a hyperchromicity of 35%.

Immediately before recording the NMR spectra the solutions containing DNA and the ligand were lyophilized and redissolved in the same amount (by weight) of D_2O to reduce the signal from HDO coming from the exchangeable protons of DNA and the ligands. 4,4,-Dimethyl-4-silapentanesulfonate (DSS) was added as an internal standard. The sample pH was varied by adding NaOD or DCl with a microburette and was measured inside the NMR tube with a microelectrode (Ingold) connected to a Radiometer Model 51 pH meter. The electrode was soaked in D_2O for 1 h prior to the experiment and an equilibration time of 5 min was allowed before reading each pH value. NMR spectra were recorded on a Varian XL-200 instrument operating at 200 MHz in the Fourier transform mode, using the D_2O signal for the field-frequency lock. Chemical shifts were measured downfield from DSS.

The pK_a values of histidinamide were also determined potentiometrically in CO_2-free water and an inert atmosphere following the method described by Albert and Serjeant (*16*). Corrections due to variations in the activity function and dilution during the titration were introduced using the program described by the same authors.

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