Analysis of difference spectra of protonated DNA: determination of degree of protonation of nitrogen bases and the fractions of disordered nucleotide pairs

T.I.Smol'janinova, V.A.Zhidkov (deceased) and G.V.Sokolov

Institute of Biological Physics, USSR Academy of Sciences, 142292 Pushchino, Moscow Region, USSR

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ABSTRACT

The titration curves of nitrogen bases and fractions of disordered nucleotide pairs are obtained during DNA protonation. It is shown that purine bases are the first sites of the DNA double helix protonation. The cytosine protonation is due to proton-induced conformational transition within GC pairs with the sequence proton transfer from (N-7) of guanine to (N-3) of cytosine. Within DNA with unwound regions the bases are protonated in the following order : cytosine, adenine, guanine. It is shown that GC pairs are the primary centres in which the unwinding of protonated DNAs occurs.

INTRODUCTION

The problem of structural transformations in protonated DNA is intimately related to the elucidation of proton-acceptor centres in the macromolecule. There is no definite opinion in the literature on the problem of protonation of bases in the double helical DNA structure. In some works /I-IO/ cytosine /C/ and adenine /A/ are stated to be the primary sites of protonation. Other authors favour predominant guanine /G/ protonation /II-I4/. No data are available on the degrees of base protonation in the DNA structure. Conformation of protonated GC-pairs in the DNA double helix is a controversial problem /6,8,I3,I5,I6/. The question of pH-stability of base pairs is not yet clear /IO/. These questions can be approached only by a quantitative analysis of the protonated macromolecular states. This method was first used under the assumption that the change in the absorption of the acid DNA solutions at $\lambda = 280$ nm was determined by protonated cytidine /4, 6/. This allowed the calculation of the percentage of a protonated cytidine as a function of pH solution.

It is the purpose of the present investigation to carry out a quantitative analysis of the difference spectra of the protonated DNAs with different GC composition within a wide pH and temperature range in order to determine degrees of protonation of nitrogen bases, the fractions of disordered nucleotide pairs in the macromolecular and to find out DNA protonation mechanism.

MATERIALS AND METHODS

DNA samples. DNAs of different nucleotide composition were used. The preparations were obtained by phenol deproteinization methods /17,18/. Table I gives the characteristics of the DNAs. All DNA samples were melted in 0.15 M NaCl; *Micrococcus lysodeikticus* DNA - in 0.015 M NaCl. The melting curve for *Rhizobium lupini* DNA was not registered.

We investigated DNA preparations in a 0.15 M NaCl solution within the 20-25 mg/ml and 0.95 - 1 mg/ml concentration ranges for spectrophotometric and potentiometric titration, respectively. A 0.1 N HCl (+0.15 M NaCl) solution was employed as a titrant. The dilution of the DNA solutions during titration was taken into account.

Spectrophotometry. The spectrophotometric titration of the

Table I. The characteristics of DNA preparations.

DNA source	GC %	$M \cdot 10^{-6}$	n	A %	$T_{\frac{1}{2}}^{\circ}$	ΔT
Phage T2	35	98	2	43	82.7	1.9
Calf thymus	42	18	5	36	86.5	10.2
Hen erythrocyte nuclei	41	10	2	42	78.2	10.5
<i>Escherichia Coli</i>	50	33	6	36	87.7	6.3
<i>Rhizobium lupini</i>	65	11.7	3	32	-	-
<i>Micrococcus lysodeikticus</i>	72	25	6	30.6	83	5.7

M is the molecular weight (Dalton) determined from the characteristic viscosity of the DNA solution; n is the number of single-strand breaks per molecule; A, $T_{\frac{1}{2}}$, ΔT are the hyperchromic effect, DNA melting temperature, and melting temperature range, respectively.

DNA was achieved by the method of difference spectrophotometry on a Hitachi I24 spectrophotometer with a thermostated chamber ($\pm 0.1^\circ$). Titration and pH measurements were done directly inside a spectral cuvette. The pH values were recorded on a pH meter pH-262. Its measurement accuracy was ± 0.01 pH.

Potentiometry. Potentiometric titration was done in a thermostated cell ($\pm 0.1^\circ$) of pH-meter PHM-26 (Radiometer Co.) with a ABU-I2 autoburette, a TTA-3I titration system with stirring and a TTT-II titrator. The pH meter accuracy was ± 0.05 pH.

Analysis of difference spectra of protonated DNA. A method of mathematical modelling DNA protonation was based on a regression analysis of the dispersion of spectrophotometric changes induced by protonation of the macromolecule /19/. The linear equation of multiple regression without the constant member was analysed. Plausible results were achieved by minimizing of the sum of square deviations between the experimental and the calculated values. Regression coefficients were determined from a system of normal equations by methods of the matrix algebra.

The model was based on the following assumptions:

1. The changes in optical density due to DNA base protonation and perturbed stacking-interactions in the macromolecule are additive.
2. The change in optical density due to base protonation is proportional to the difference of the extinctions for deoxynucleosides in molecular and ionized forms. The extinction coefficients of the non-protonated and protonated nucleosides present in the polymer structure and in a free state were assumed to be similar.
3. The change in optical density due to perturbed stacking-interaction is proportional to the difference of extinctions for base pairs in an ordered double helical and in a disordered single-stranded DNA.

The model was constructed in this way to solve two tasks:

1. Determination of the spectral characteristics of the processes inducing the optical density changes during DNA protonation;
2. Determination of the contributions of these processes into the optical density change.

The solution of the first task was based on the analysis of difference spectra of protonated DNAs characterized by the different nucleotide composition and by the complete conformational transition. The latter was judged by the maximum hyperchromism. It was assumed that all bases were protonated. The matrix of the mathematical model is presented as follows:

$$\| \Delta D_{\infty} \| = \| \Theta \| \cdot \| \varepsilon \| \quad (I)$$

where:

$\| \varepsilon \|$ is the matrix of dimension $r \times n$. This matrix includes the values of spectral parameters for n wave-lengths and r processes inducing the optical density change during DNA protonation.

$\| \Theta \|$ is the matrix of the nucleotide composition, its dimension is $l \times r$. l is the number of DNA samples; r is the content of various bases and base pairs.

$\| \Delta D_{\infty} \|$ is the matrix of optical density changes during DNA protonation. Its dimension is $l \times n$. The parameters of this matrix were estimated experimentally.

The required matrix of spectral parameters was calculated from the equation (I):

$$\| \varepsilon \| = \| \Theta^* \Theta \|^{-1} \cdot \| \Theta^* \Delta D_{\infty} \| \quad (2)$$

The solution of the second task allowed the determination of fractions of protonated bases and disordered nucleotide pairs during DNA protonation. For this purpose difference spectra of DNAs with various protonation degree were analysed. The matrix is expressed in this way:

$$\| \Delta D \| = \| \beta \| \cdot \| K \| \quad (3)$$

where:

$\| \beta \|$ is the matrix of the spectral parameters similar to $\| \varepsilon \|$. Its dimension is $n \times r$;

$\| K \|$ is the matrix of contributions of r processes characterizing optical density changes for m degrees of DNA protonation.

Its dimension is $r \times m$;

$\| \Delta D \|$ is the matrix of optical density changes for m degrees of DNA protonation and n wave-lengths. Dimension is $n \times m$. The

parameters of this matrix were estimated experimentally.

The required matrix was calculated from the equation (3):

$$\|K\| = \|\beta^* \beta\|^{-1} \cdot \|\beta^* \Delta D\| \quad (4)$$

Modelling of protonated DNA states was carried out on a Minsk-22 computer.

Matrix equation (I) was concretized by a system of linear equations (5) for sixteen wave-lengths within the range of 230-300 nm:

$$\Delta D_{\infty}^i(\lambda) = c^i \cdot \left\{ \theta_{AT}^i \Delta \epsilon_{AT}^{\lambda} + \theta_{GC}^i \Delta \epsilon_{GC}^{\lambda} + \theta_A^i \Delta \epsilon_{dA}^{\lambda} + \theta_G^i \Delta \epsilon_{dG}^{\lambda} + \theta_C^i \Delta \epsilon_{dC}^{\lambda} \right\} \quad (5)$$

where:

$\Delta D_{\infty}^i(\lambda)$ is the observed change of optical density due to the complete acid-induced conformational transition of the i -th DNA preparation, $i = 1, 2, \dots, 6$;

c^i is the molar concentration of the i -th DNA sample (on a nucleotide base);

θ^i is the mole fractions of bases A, G, C or base pairs AT, GC for the i -th DNA sample;

$\Delta \epsilon_{AT}^{\lambda}$, $\Delta \epsilon_{GC}^{\lambda}$ are the differences in extinction coefficients due to perturbed pairing within AT and GC - pairs during DNA protonation;

$\Delta \epsilon_{dA}^{\lambda}$, $\Delta \epsilon_{dG}^{\lambda}$, $\Delta \epsilon_{dC}^{\lambda}$ are the differences between the extinction coefficients of protonated and non-protonated deoxynucleosides dA, dG, dC, respectively. These values were obtained in special experiments with spectrophotometric titration of deoxynucleosides.

In the equation (5) $\Delta \epsilon_{AT}^{\lambda}$ and $\Delta \epsilon_{GC}^{\lambda}$ are the only unknown parameters. The data are given in Table 2. The nature of the observed differences of spectra at 12.5°C and 30°C is not yet clear.

Matrix equation (3) was concretized by a system of linear equations (6) for sixteen wave-lengths within the range of 230-300 nm:

$$\Delta D(\lambda, \text{pH}) = C \cdot \left\{ \Delta \epsilon_{\text{AT}}^{\lambda} \theta_{\text{AT}} K_{\text{AT}}(\text{pH}) + \Delta \epsilon_{\text{GC}}^{\lambda} \theta_{\text{GC}} K_{\text{GC}}(\text{pH}) + \Delta \epsilon_{\text{dA}}^{\lambda} \theta_{\text{A}} K_{\text{A}}^{+}(\text{pH}) + \Delta \epsilon_{\text{dG}}^{\lambda} \theta_{\text{G}} K_{\text{G}}^{+}(\text{pH}) + \Delta \epsilon_{\text{dC}}^{\lambda} \theta_{\text{C}} K_{\text{C}}^{+}(\text{pH}) \right\} \quad (6)$$

where:

$\Delta D(\lambda, \text{pH})$ is the observed change of optical density induced by DNA protonation;

$K_{\text{AT}}, K_{\text{GC}}$ are the fractions of disordered base pairs within the protonated DNA;

$K_{\text{A}}^{+}, K_{\text{G}}^{+}, K_{\text{C}}^{+}$ are the fractions of protonated bases within the DNA structure;

The fractions of disordered nucleotide pairs and protonated bases are the unknown elements in the equations (6). The data are illustrated by the Figures in the Results.

Table 2. The differences in extinction coefficients due to perturbed pairing within adenine-thymine and guanine-cytosine pairs during DNA protonation ($\times 10^{-3}$).

λ nm	12.5 - 25°C		30°C	
	$\Delta \epsilon_{\text{AT}}$	$\Delta \epsilon_{\text{GC}}$	$\Delta \epsilon_{\text{AT}}$	$\Delta \epsilon_{\text{GC}}$
230.0	0.698 $\pm$ 0.18	2.476 $\pm$ 0.17	1.209 $\pm$ 0.10	1.472 $\pm$ 0.09
235.0	0.951 $\pm$ 0.16	2.418 $\pm$ 0.15	1.296 $\pm$ 0.04	1.682 $\pm$ 0.04
240.0	1.374 $\pm$ 0.13	2.227 $\pm$ 0.13	1.701 $\pm$ 0.07	1.656 $\pm$ 0.06
245.0	1.873 $\pm$ 0.13	1.996 $\pm$ 0.13	2.328 $\pm$ 0.15	1.489 $\pm$ 0.14
250.0	2.440 $\pm$ 0.16	1.593 $\pm$ 0.16	3.096 $\pm$ 0.21	0.960 $\pm$ 0.19
255.0	2.932 $\pm$ 0.20	1.453 $\pm$ 0.19	3.969 $\pm$ 0.31	0.519 $\pm$ 0.28
260.0	3.350 $\pm$ 0.22	1.456 $\pm$ 0.21	4.632 $\pm$ 0.29	0.293 $\pm$ 0.26
262.5	3.305 $\pm$ 0.23	1.694 $\pm$ 0.22	4.678 $\pm$ 0.23	0.398 $\pm$ 0.21
265.0	3.173 $\pm$ 0.23	2.008 $\pm$ 0.22	2.594 $\pm$ 0.23	0.749 $\pm$ 0.21
267.5	2.953 $\pm$ 0.24	2.429 $\pm$ 0.23	4.474 $\pm$ 0.13	1.029 $\pm$ 0.12
270.0	2.516 $\pm$ 0.24	2.764 $\pm$ 0.23	4.086 $\pm$ 0.16	1.320 $\pm$ 0.14
275.0	1.575 $\pm$ 0.22	2.933 $\pm$ 0.21	3.140 $\pm$ 0.08	1.415 $\pm$ 0.07
280.0	0.668 $\pm$ 0.21	2.465 $\pm$ 0.20	2.154 $\pm$ 0.04	0.938 $\pm$ 0.04
285.0	0.337 $\pm$ 0.17	1.521 $\pm$ 0.16	1.459 $\pm$ 0.08	0.046 $\pm$ 0.07
290.0	0.380 $\pm$ 0.15	0.426 $\pm$ 0.15	0.877 $\pm$ 0.16	-0.448 $\pm$ 0.14

RESULTS

The pH dependences of the fractions of protonated bases within the DNA structure are shown in Figs I and 2. Fig. I is an illustration of these dependences for DNAs with different nucleotide composition from 35 to 72% GC pairs at 17.5°C. The analogous data for *Micrococcus lysodeikticus* DNA within the 12.5–30°C temperature range are presented in Fig. 2. Adenines are proto-

Figure I. pH-dependences of the fractions of protonated bases (A, G, C) within the DNA at 17.5°C (0.15 M NaCl). DNA potentiometric titration curves are at the right. --- computed curve from degrees of base protonation; ○- T2 Phage; ●- Calf thymus; ▲- *Micrococcus lysodeikticus*; ■- *Rhizobium lupini*.

nated within the double helix of this DNA at all temperatures given and they are not shown in Fig. 2. Potentiometric titration curves of the investigated DNAs are given in the right part of Fig. 1. The potentiometric titration curves for *Rhizobium lupini* DNA was not registered. The dotted lines indicate the titration curves obtained from computed degrees of base protonation. Vertical arrows separate pH intervals corresponding to the DNA double helix and the structure with perturbed base pairing.

From Fig. 1 it is seen that purine bases are the primary sites of the protonation within the DNA double helix. Cytosine protonation either takes place at more acidic pH or is not observed at all as in the case of T2 Phage DNA. Within the 12.5–20°C temperature range the guanine titration curves for GC-rich preparations pass through maximum (Figs 1 and 2). The higher temperature, the lower the maximum and in a range of 25–30°C

Figure 2. pH dependences of the fractions of protonated bases (G,C) within *Micrococcus lysodeikticus* DNA at 0.15 M NaCl. —○— 12.5°; —●— 17.5°; —△— 20°; —■— 25°; —□— 30°.

it drops to zero (Fig. 2). There is no maximum in the guanine titration profiles for AT-rich DNAs. When comparing the titration curves of guanine and cytosine for GC-rich DNAs it should be noted that the decrease of guanine protonation (the right portion of the bell-like curve) is predominantly observed within the pH range corresponding to the increase of cytosine protonation. If pH is moved to the range of perturbed base pairing (below pH ≈ 2.8), a sharp increase of cytosine protonation degree is observed. The bases of the macromolecule with perturbed pairing can be arranged according to the protonation efficiency in the order: cytosine, adenine, guanine (Figs 1 and 2).

When comparing experimental and computed curves of DNA titration it can be seen that there is a sufficiently good correlation between these two types of the curves. The deviation of the computed curve from the experimental one for *M. lysodeikticus* DNA is possibly due to the insignificant spectral contribution of protonated guanines and adenines. However, the character of the dependence, namely, the presence of bends both on the computed and experimental curves is similar. All this enables us to assume that the chosen mathematical model of the protonated equilibrium state of DNA is valid and the maximum on the guanine titration curve is trustworthy.

The change of the fraction of disordered GC and AT pairs on the degree of DNA protonation is presented in Fig. 3. Fig. 4 illustrates the change of contribution of perturbed nucleotide pairs with the degree of DNA protonation. In Figs 3 and 4 the data for DNAs with different nucleotide composition from 35 to 72% GC pairs at 17.5°C are given. The pH dependences of the fraction of disordered nucleotide pairs in DNA from *Micrococcus lysodeikticus* within the 12.5 - 30°C temperature range are shown in Fig. 5. From Figs 3 and 5 it is seen that GC pairs are the primary centres in which the unwinding of protonated DNA occurs independently on its nucleotide content and temperature. The value of contributions of disordered GC and AT pairs into the unwinding of DNAs is determined by its protonation degree and by its base composition (Fig. 4). GC pairs provide the main contribution into the total number of unwound pairs of the protonated GC-rich DNAs. For the protonated AT-rich DNAs only the beginning

Figure 3. Change of the fraction of disordered guanine-cytosine (GC) and adenine-thymine (AT) pairs with the degree of DNA protonation in 0.15 M NaCl at 17.5°C.

K, φ are fractions of disordered nucleotide pairs and percentage content of nucleotide pairs within DNA, respectively.

-○- T2 Phage; -●- Calf thymus; -▲- Micrococcus lysodeikticus.

Figure 4. Change of the difference between fractions of disordered GC and AT pairs with the degree of DNA protonation in 0.15 M NaCl at 17.5°C.

K, φ and other symbols are as in Fig. 3.

of the melting is predominantly determined by GC pairs until the degree of DNA protonation ≈ 0.47 . With the increase of the DNA protonation degree the contribution of perturbed AT pairs rises.

DISCUSSION

From Fig. 1 it is seen that purine bases are the primary sites of the protonation in the DNA double helix. This is probably governed by a sterical accessibility of nitrogen atoms (N-7) of guanine, (N-3) and (N-7) of adenine with excessive electron density. These considerations are supported by the data on the DNA alkylation /20/ according to which the reaction occurs on (N-7) of guanine and (N-3) of adenine. An important role

Figure 5. pH dependence of the fraction of perturbed AT and GC pairs within DNA from *Micrococcus lysodeikticus* at 0.15 M NaCl.

K, ψ are as in Fig. 3.

-○- 12.5°; -●- 17.5°;

-△- 20°; -■- 25°;

-□- 30°.

of (N-7) of guanine in DNA acid titration is shown by polarography /II/ and by optical rotatory dispersion /I3/. However, it is difficult to understand the presence of the protonated cytosine in the DNA double helix because the proton accepting centre (N-3) of this base contributes to the hydrogen bond formation and is little accessible to protons from the solution. The further discussion deals primarily with this problem.

It can be seen from Figs I and 2 that in one and the same pH interval corresponding to DNA double helix a decrease of protonation of guanine (the right portion of the bell-like curve) and an increase of that of cytosine are observed. The analysis of the circular dichroism data together with kinetic investigations of protonated deoxynucleosides and DNAs showed that in the same pH range one can observe both the change of the sign of Cotton effect /I2,I3,23/ and the formation of the stable protonated structure "S" with the fully ordered nitrogen bases /2I, 22/. According to /I2,I3/, the alteration of the sign of Cotton

effect is due to pH-induced reversible transitions between *anti* and *syn* conformations of guanine. Within DNA double helix such transitions can probably take place when the protonated molecule is present in an unstable "L" state characterized by the Watson-Crick double helix with the locally unwound regions so called "loops" /21,22/. It should be noted that it is the stage of loop formation that induces the conformational transition of the protonated macromolecule into the stable "S" state /21,22/. The nature of this state is not yet clear. But there are data /13/ permitting to suggest that the "S" state corresponds to Hoogsteen's scheme of double helical pairing between the protonated guanine in *syn* conformation and cytosine (Fig. 6).

As it was mentioned above, "S" structure forms in the same pH interval where the decrease of guanine protonation and the increase of cytosine one is observed. It seems the differences in the titration of these bases can be attributed to the proton transfer within Hoogsteen protonated GC pairs. Therefore it is assumed that in the stable "S" structure the guanine serves as a proton carrier from (N-7) to (N-3) of cytosine.

Thus, the scheme of the protonation of guanine-cytosine pairs within the DNA double helix may be represented as follows:

Protonation of the bases within DNA double helix induces the perturbation of stacking-interaction and the formation of loops (the unstable "L" state). The number and the mean size of loops increases with the increase of the temperature and the degree of DNA protonation. Under these conditions it should be ex-

Figure 6. The scheme of protonated guanine-cytosine pair with Hoogsteen pairing.

pected the enhancement of the probability of the transition of the protonated guanine from *anti* to *syn* conformation with subsequent formation of Hoogsteen pairing in protonated GC pairs and the proton transfer within them. Indeed, with the rise of the temperature the fraction of the protonated guanine drops to zero, whereas the cytosine protonation sharply grows (Fig. 2).

Thus, the presence of the maximum in the guanine titration curve for GC-rich DNAs is explained by diminution of the guanine protonation due to the proton transfer within protonated GC pairs with Hoogsteen pairing. The absence of the peak in the guanine titration profile for AT-rich DNAs enables one to put a question if the proposed scheme of the GC pair protonation is valid. However, it may be considered that the appearance of the maximum is the result of the cooperativity of proton-induced conformational transitions within GC pairs. Evidently, the cooperativity of these transitions is determined by the DNA primary structure, in particular by GC clusters along polynucleotide chain. Since, *Micrococcus lysodeikticus* and *Rhizobium lupini* DNAs contain more GC clusters than calf thymus DNA the maximum in the guanine titration curves for the first two types of DNAs is more pronounced. The absence of protonated cytosine within the double helix of Phage T2 DNA is probably the result of the minimization of the conformational transitions within protonated GC pairs due to the presence of AT clusters along macromolecule.

The presented results can be summarized. Purine bases are the primary points of the DNA double helix protonation. Nitrogen atoms (N-7) and (N-3) are proton acceptor centres of guanine and adenine, respectively. Cytosine protonation is the result of the proton-induced conformational changes within GC pairs with subsequent the proton transfer from (N-7) of guanine to (N-3) of cytosine. The cytosine protonation cannot be realized within the DNA double helix if the blocking (N-7) of guanine or the reduction of basicity (N-3) of cytosine takes place. The efficiency of the cytosine protonation also depends on the number of GC clusters along nucleotide sequence.

It should be noted that the given scheme of the GC pair protonation within the DNA double helix is like a scheme proposed by Y.Courtois *et al.* /13/ though each of them bases on diffe-

rent experimental data.

In DNAs with unwound regions bases are protonated in the following sequence: cytosine, adenine, guanine (Fig. I). The protonation probably occurs mainly on the most efficient proton acceptor centres: (N-3) of cytosine, (N-1) of adenine and (N-7) of guanine.

It is shown that GC pairs are the first sites in which the unwinding of protonated DNAs takes place (Figs 3-5). This fact can probably indicate for the decrease of DNA helix stability in the regions of the formation of Hoogsteen type protonated GC pairs.

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