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The role of the AT pairs in the acid denaturation of DNA

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ABSTRACT

It has been determined previously that the protonation of the GC pairs induces a DNA conformation change which leads to a "metastable" structure. The role of the AT pairs, however, is not well known because the protonation does not modify their spectral properties. By means of an indirect method based on the binding of proflavine, it has been determined that the AT pairs are protonated before the acid-induced denaturation and that they seem to be unable to assume a conformation change when protonated. These results would indicate that the protonated AT pairs may be responsible for the induction of the acid denaturation and not the GC pairs as it was thought previously.

INTRODUCTION

During the acid titration of DNA, the first protonated base pairs are the GC pairs (1-3). There is considerable evidence that the protonation takes place on guanine and not on cytosine although the latter has a higher pK in the monomeric form (3). This is probably due to the fact, that, in the Watson-Crick pairing, the N-7 of guanine lies in the major groove of the double helix and is thus accessible to the proton, while the N-3 of cytosine is implicated in one of the hydrogen bonds. When protonated, guanosine assumes a change from the anti to the syn conformation (4,5). A similar conformational change has been suggested in protonated DNA (3) leading to a stable acid structure containing protonated Hoogsteen GC pairs. As we have determined (6), the protonation occurs first in the GC rich regions, because several adjacent guanines participate cooperatively in the conformation change.

All the studies based on spectrophotometric titrations have demonstrated the role of the GC pairs in the protonation and the acid-induced denaturation of DNA (1-6). According to these studies, the instability of the protonated GC pairs is responsible for the DNA denaturation in acidic medium. The behaviour of the AT pairs is, however, not well known because the protonation does not induce any change in their spectral properties.

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It is impossible to follow their protonation directly by means of spectrophotometry, fluorescence or circular dichroism, for example.

In order to overcome this problem, we used a dye (proflavine) whose binding and spectral properties are sensitive to the structure modifications of the macromolecule. The data presented here indicate that the protonated AT pairs are -at least partly- responsible for the acid-induced denaturation and that the AT pairs, in contrast with the GC pairs, cannot assume any "intermediate" conformation before denaturation.

MATERIAL AND METHODS.

DNA from calf thymus was prepared according to the method of Kay et al.(7). Proflavine hemisulfate was obtained from the British Drug House and used without further purification. The concentrations of the solutions were calculated using the molar extinction coefficient $\varepsilon = 41000 M^{-1} cm^{-1}$ at $\lambda = 444$nm. All DNA-proflavine complexes (P/D=16) were made in NaCl 1mM and acidified by concentrated HCl.

Methods:

The absorbances were measured on a Shimadzu QV-50 spectrophotometer.
The fluorescence apparatus (Baird Atomic SF-100EE) and the calculations of (Φ/Φ_0), the ratio of the quantum yields, and of μ_{app}, the emission anisotropy, have been described previously (8). For proflavine, the excitation and emission wavelengths are 453 and 510 nm respectively.
The circular dichroic spectra were recorded on a Jasco ORD/CD/UV-5 instrument using cells of 1 cm pathlength.
The linear dichroism measurements were performed in our laboratory using the electro-optical apparatus previously described (9-10). The measurements were made at a field strength of 12 kV cm^{-1}.

RESULTS AND DISCUSSION.

In our experimental conditions (P/D=16), all the proflavine molecules are strongly bound into the DNA double helix at neutral pH. In order to estimate the stability of the complex, the absorbances were measured at 260 and 280 nm (fig.1a). At 260 nm, the hyperchromism is essentially due to the denaturation of DNA, while, at 280 nm, the absorbance is also sensitive to the protonation of the GC pairs. We observed that the absorbance at 280 nm increased up to pH 6 like in uncomplexed DNA at the same ionic strength(6). The protonation of the GC pairs was unaffected by the complexation with the dye. The acid-induced denaturation followed by the hyperchromism at 260 nm

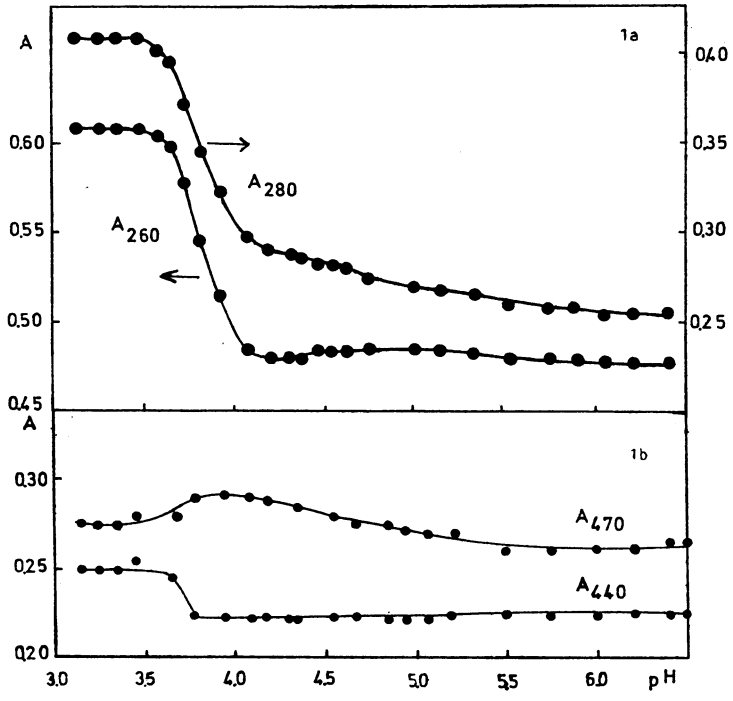

Fig.1: Effect of pH on DNA-proflavine (P/D=16) absorption in 1 mM NaCl. Fig.1a: λ= 260 and 280 nm; fig.1b: λ=440 and 470 nm.

began at pH 4. In the same experimental conditions, the acid-induced denaturation of the uncomplexed DNA began at pH 5 (6). The bound dye thus stabilized the DNA double helix against acid denaturation.

In fig.1b, the absorbances at 440 and 470 nm are plotted versus pH. This test is highly sensitive to the modification of the dye binding. The only small variation observed indicated that the proflavine molecules remained intercalated in DNA until the beginning of the denaturation (pH 4). We may conclude that the degree of binding was unaffected by protonation of DNA.

This assertion is supported by the electric dichroism measurements presented in fig.2. The linear dichroism measured in the visible absorption band of the dye (λ= 453 nm) remained almost constant and highly negative ((ΔA/A)=-0.4) down to pH 4. This dichroism has almost the same magnitude compared with the value observed with the DNA bases ((ΔA/A)=-0.5). The dye molecules had thus almost the same orientation as the DNA bases. Therefore all the dye molecules remained intercalated in the double helix

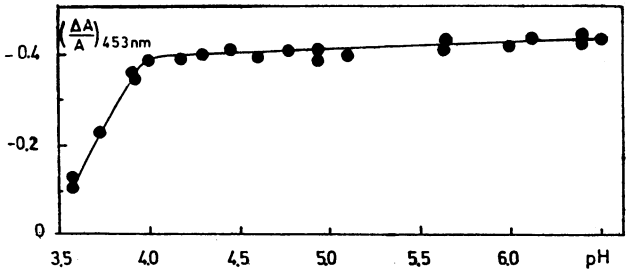

Fig.2: DNA-proflavine complex (P/D=16) in 1mM NaCl. Electric dichroism measured in the dye absorption band at 12 kV cm^{-1}.

until the acid-induced denaturation. It is also interesting to point out that the DNA regions where the dyes are bound, must have a fairly negative dichroism down to pH 4 , because that of the dye remains strongly negative in spite of the protonation of the DNA bases.

Fluorescence of the complex in acidic medium:

The fluorescence depolarization is caused by energy transfers between adjacent dye molecules; the emission anisotropy is lowered by these transfers, the probability of which falls off with the reciprocal of the sixth power of the distance between the donor and the acceptor. The probability of these transfers is also related to the relative orientation of the two molecules. The emission anisotropy is also lowered by the Brownian movements of the fluorescent molecules. In our case, if the dye is not tightly bound to the DNA (only partial intercalation or external binding), it can rotate between excitation and emission, this rapid motion provoking a fluorescence depolarization.

Fig.3. shows that, at neutral pH, the emission anisotropy was relatively high (μ= 0.21), because at P/D 16 the dye molecules are bound far away from each other along the DNA helix (about one dye every eight base pairs). In acidic medium, μ decreased and reached a value around 0.12 at pH=4 at the beginning of the acid denaturation. This depolarization may result from the increase of the rotational motions of the dye in its intercalation site when this site contains a protonated base. It is impossible to explain the large decrease of μ taking only in account these rotational motions. One could also suppose that this enhancement of energy transfers results mainly from the shortening of the mean distance between two adjacent dyes.This would imply that an important fraction of the proflavine

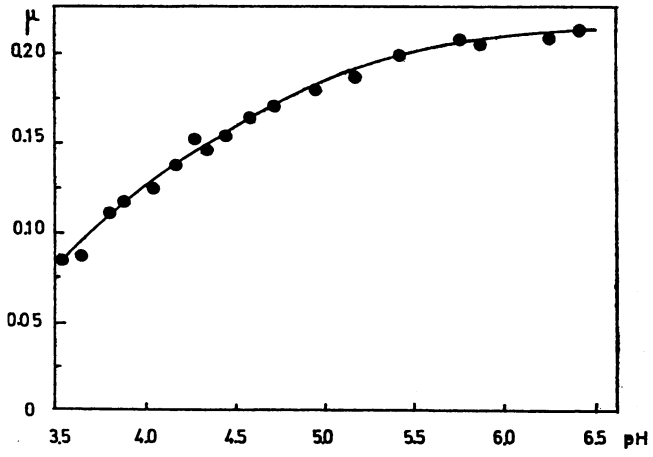

Fig.3: DNA-proflavine complex (P/D=16) in 1 mM NaCl;
Fluorescence anisotropy: λ_{exc}= 453 nm, λ_{em}= 510 nm

would be concentrated in small regions of the DNA and no more statistically bound along the DNA helix. These dye molecules remain intercalated in the double helix, since, as shown before, the linear dichroism remains constant down to pH 4. A quite important fraction of the dye could leave its intercalation site, perhaps if one of the adjacent bases was a protonated guanosine; the dye would migrate then to an unaffected region of DNA. In these regions the concentration would increase which would explain the fluorescence depolarization by a lowering of the mean distance between adjacent dye molecules.

In order to ascertain this "migration" hypothesis, we measured the variation of the relative fluorescence intensity of the proflavine. If the dye is intercalated between two AT pairs, its fluorescence intensity is enhanced, and is quenched if at least one of the adjacent base pairs is a GC pair (11). In fig.4, the ratio of the quantum yields(Φ/Φ_0) of the proflavine is presented as a function of pH. The increase of the fluorescence intensity may be explained as follows:a proflavine molecule bound near a GC pair -and thus non-fluorescent- could leave its binding site and migrate to a site formed by two AT pairs where the dye becomes fluorescent.

Below pH 4.6, (Φ/Φ_0) decreased (fig.4). It is difficult to justify this fact because there are many possible origins for this variation. Since the absorbance and the linear dichroism of the dye remain constant down to pH=4, this decrease of (Φ/Φ_0) should be due to a modification of the bin-

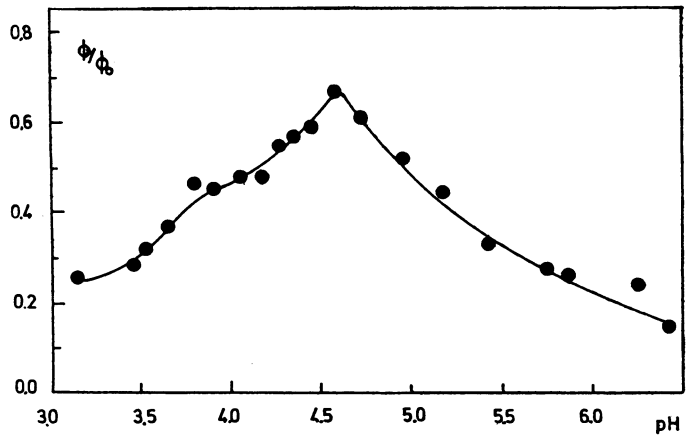

Fig.4: ratio of the fluorescence quantum yields same conditions as in fig.3.

ding site and not to an ejection of the bound dye. The variations of the quantum yield concern only the proflavine intercalated between two AT pairs, because only these dyes are fluorescent. The decrease of the dye fluorescence will denote a modification of the intercalation properties of the proflavine molecules bound between two AT pairs and thus a modification in the geometry of the binding site itself.

At pH 4.6, the first AT pairs would become protonated and the repulsion between the positive charge of the dye and that of the protonated base would provoke a partial ejection of the dye which would be less tightly bound in the double helix. In this way, the positive charge of the proflavine would be as far as possible from the positively charged ring nitrogen of the base and as close as possible to the negatively charged phosphate of the DNA backbone. In this new geometry, the dye would be more in contact with the solvent which quenches partially the proflavine fluorescence.

Circular dichroism of the complex.

The free dye does not show any optical activity in any of its ultraviolet or visible absorption bands. When fully bound to DNA, the proflavine shows a typical circular dichroism spectrum. In the visible absorption band it has a maximum at 467 nm and a minimum at 437 nm. It results essentially from exciton interactions showing a highly non-conservative pattern. In the UV region, the induced CD spectrum has a maximum at 275 nm and a minimum at 247 nm. It is the superposition of the CD spectrum of the DNA and that of the dye.

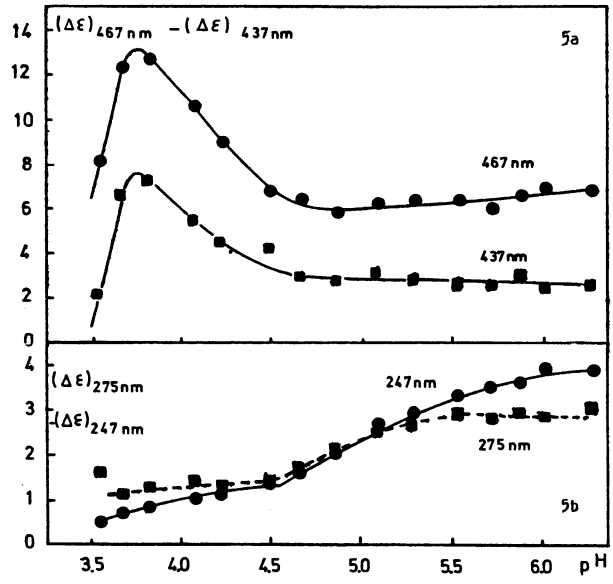

Fig.5: DNA-proflavine complex (P/D=16) in 1mM NaCl. Circular dichroism measured in the visible absorption band of the dye (fig.5a) and in the UV region (fig.5b).

The results in term of molar dichroism ($\Delta\varepsilon$) are given in fig.5. The observed amplitude of the ultravtolet CD spectrum (fig.5b) decreased when the pH decreased, while in the visible region (fig.5a) the amplitude of the signal remained constant down to pH 4.8 and then increased strongly down to pH 3.7.

The fluorescence measurements (fig.3 and 4) suggested that above pH 4.8, the dyes migrated from GC-containing binding sites to "pure AT" binding sites where the geometry of the binding remained constant. If the CD signals are interpreted in terms of exciton interactions, it should be normal that the visible CD signal, essentially sensitive to the dye-dye interactions, remained almost constant, if one considers that these interactions are unaffected by the nature of the binding site.

CONCLUSIONS.

In the conditions of low ionic strength (1mM NaCl) used in this study, the protonation and the acid-induced denaturation of DNA are less cooperative

and are spread over a larger pH range than at higher ionic strength. The protonation begins at about pH=6 and the acid-induced denaturation at pH 5, when DNA is not complexed with proflavine (6). If DNA is complexed with the dye, the protonation begins also around pH 6, but the beginning of the denaturation is shifted down to pH 4. The dye stabilizes DNA against the acid-induced denaturation as it does against heat denaturation. It seems that the dye binding does not interfere with the protonation process itself. This fact is easy to understand considering the behaviour of the bound dye in acidic medium.

It is well established (11) that the fluorescence emission of proflavine intercalated between two AT pairs is enhanced compared with that of the free dye. On the contrary, when at least one of the adjacent base pairs is a GC pair, the dye does not fluoresce anymore. Between pH 6 and 4,6, the observed fluorescence depolarization and fluorescence intensity enhancement result from the migration of the proflavine molecules from non-fluorescent binding sites to fluorescent ones; these fluorescent sites being located in specific regions of DNA, the dyes are bound closer to each other. The non-fluorescent dye is ejected from its site when an adjacent GC pair is protonated and migrates then to an unaffected intercalation site, i.e, between two AT pairs. This ejection means that the intercalation site is greatly modified by the protonation of guanine. This fact supports the hypothesis (3) according to which the protonated guanine can rotate around the glycosidic linkage into a syn conformation in order to form a Hoogsteen pair with cytosine. This new pair is less stable (two instead of three H-bonds), but the secondary structure is not destroyed by the protonation.

Below pH 4.6, there is a second step in the acid titration of the DNA-proflavine complex. The fluorescence intensity of the dye decreases and its circular dichroism in the visible region increases. Since the linear dichroism of the dye remains constant down to pH 4, we assert that the dye is still fully bound in the DNA double helix. These variations in fluorescence intensity and in circular dichroism can be accounted for in the following way. The protonation of adenine induces repulsion between the positive charge of the dye and that of the protonated base. These repulsions provoke a partial ejection of the proflavine. The dye is more in contact with the solvent which quenches its fluorescence. This partial ejection may explain the increase of the circular dichroism signal below pH 4.6, because, when the dye is partially intercalated, the dye stacking configuration is quite different. It affects the exciton interactions between adjacent dyes.

In all previous studies, one considered that the instability of the protonated GC pairs was responsible for the induction of the acid denaturation of DNA. Our results show that the AT pairs are protonated just before the denaturation and that their behaviour seems to be different from that of protonated GC pairs. These AT pairs seem to be unable to assume a new pairing when protonated. Only one H-bond remains between A^+ and T; i.e, between the 6-NH_2 of adenine and the 4-C=O of thymine. This pairing must be more unstable than that of the protonated GC pairs in which two H-bonds remain. The protonation of the AT pairs just before the acid induced denaturation may be responsible for this denaturation. Thermal denaturation experiments at different pH (6) confirm this hypothesis. They show that, just before the acid denaturation, the most unstable regions in DNA are the AT-richest ones.

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