Can a denaturant stabilize DNA? Pyridine reverses DNA denaturation in acidic pH **

Guillem Portella, Montserrat Terrazas, Núria Villegas, Carlos González and Modesto Orozco*

Abstract: The stability of DNA is highly dependent on its solvent environment, such as ionic strength, pH and the presence of denaturants and osmolytes. Addition of pyridine is known to unfold DNA by replacing π-π stacking interactions between bases, stabilizing conformations where the nucleotides are solvent exposed. We show here experimental and theoretical evidences that pyridine can change its role and in fact stabilize the DNA under acidic conditions. NMR and MD simulations demonstrate that the reversal in the denaturing role of pyridine is specific, and is related to its character as pseudo groove binder. The present study sheds light into the nature of DNA stability and on the relationship between DNA and solvent, with clear biotechnological implications.

The biological role of DNA is intimately related to its structure and stability in water solution. Full dehydration of DNA or the substitution of water by a solvent of lower polarity results in large changes in the structure of DNA [1]. Variations in ionic strength of the solution yield remarkable structural plasticity [2], and changes in the nature of counterions can even reverse the canonical rules of duplex DNA stability[3]. Some osmolytes such as urea, formamide, guanidinium chloride, dimethylsulfoxide or pyridine are known to chemical denaturants [4]. We recently used microsecond-long molecular dynamics (MD) simulations to demonstrate that the very strong denaturant properties of Pyr are related to its ability to capture microscopic unfolding events by stacking on open, solvent-exposed nucleobases [5]. Here we explore the denaturant properties of Pyr in the presence of another powerful denaturant agent: the pH. In this work we evaluate whether the effect of these two denaturants is additive, cooperative, or anti-cooperative.

We first explored the denaturing properties of pyridine at neutral pH for three DNA duplexes with different GC content (Table 1). Results shown in Figure 1(A,B) and Supplementary Table S1 demonstrate that addition of Pyr reduces the melting temperature of duplex DNA, even at low concentration (200 and 400 mM Pyr). At physiological ionic concentration (150 mM NaCl) the A·T pairings are more susceptible to the presence of Pyr than the G·C ones, in good agreement with earlier experimental findings [4]. Increasing the ionic concentration from 150 mM up to 550 mM NaCl in the presence of 400 mM Pyr practically counteracts the effect of Pyr (see Table S1), showing the protective action of Na^+. Very interestingly, the addition of NaCl protects DNA from Pyr addition especially well in AT-rich sequences, thus suggesting that Na^+ is competing with Pyr for the same DNA regions.

We next studied the effect of acidic pH in the stability of duplex DNA (see Suppl. Table S1). The same melting experiments done in the absence of Pyr at pH 4.2 and 3.8 revealed a dramatic decrease (up to 46 degrees) in the stability of the duplexes. The effect of pH is especially large as the percentage of GC increases; suggesting that protonation of cytosines (pK_a = 4.2) is the main mechanism for pH-dependent unfolding of DNA. To further support this hypothesis, we repeated the melting experiments substituting cytosine by 5-Mehtylcytosine (MetC), a modification that is known to stabilize the duplex at neutral pH [6]. Indeed, we observe a rise in melting temperature for MetC containing DNA, which as expected, correlates with the percentage of CG content (Suppl. Table S2). As MetC is more easily protonated (pK_a = 4.5) than cytosine, duplex containing MetC are more pH-dependent than wild-type DNA. For example, the melting temperature of wild-type Seq. 3 decreases by ~46 degrees when moving from pH=7.8 to 3.8; while the Tm difference is of ~58 degrees for the same sequence containing MetC.

Table 1. DNA sequences used in this work and their percentage of GC contents [a].

	Sequence (% of GC content)
Seq. 1	d(TATGTATATTTTGTAATTAA) (10%)
Seq. 2	d(CGTTTCCTTTGTTCTGGA) (44%)
Seq. 3	d(GTCCACGCCCGGTGCGACGG) (80%)

[a] Sequences given from 5' to 3', only the Watson strand is reported, the second strand is complementary in sequence.

Based on these results, it could be extrapolated that addition of Pyr would decrease even further the stability of DNA in acidic conditions. Results show in Figure 1 and Suppl. Table S1 demonstrate that the opposite occurs. Adding 200 mM of Pyr, which destabilized duplexes at neutral pH by 2-5 degrees, stabilized the same duplexes by 6-7 degrees at acidic pH (Figure 1). Increasing the concentration of Pyr to 400 mM induces a decrease of up to 12.5 degrees at neutral pH, while the same amount of Pyr stabilizes the same duplex by more than 10 degrees at acidic pH (Figure 1). It is therefore clear that the effect of Pyr is drastically different when added to a neutral or to an acidic solution.

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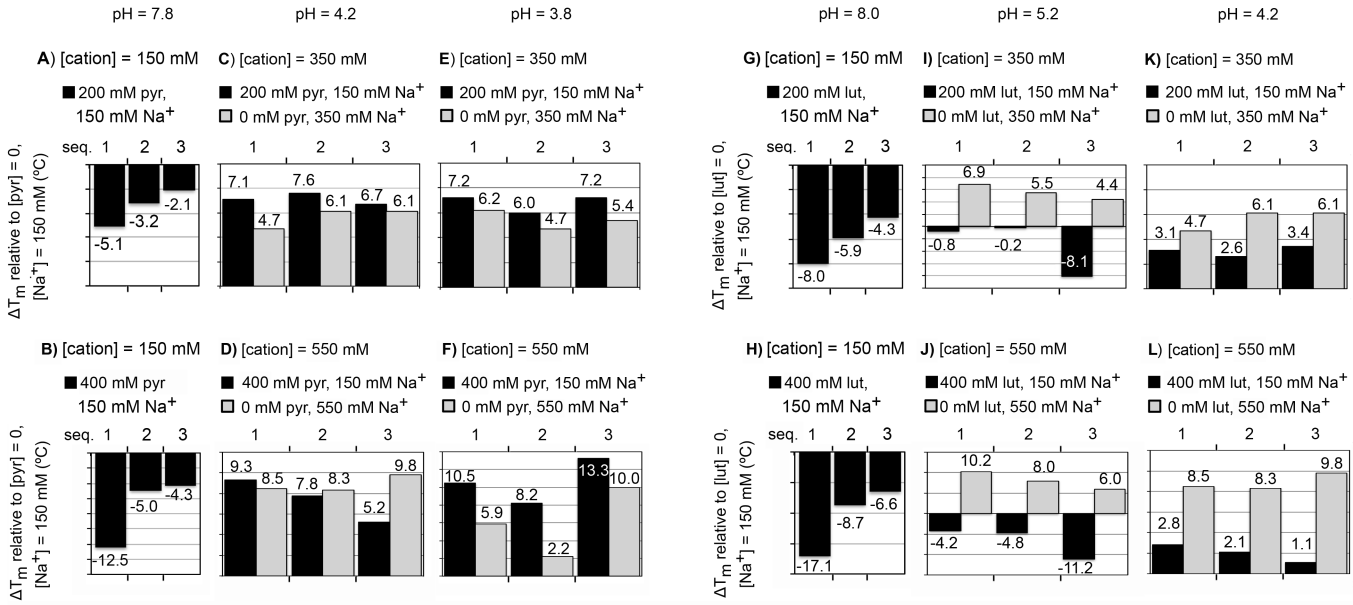

Figure 1. A-F) Effect of pyridine and Na^+ concentration on the thermal stability of DNA sequences of varying GC content at different pH values, measured as differences in melting temperature with respect to a solution containing no pyridine and 150 mM Na^+. The experiments in A, C and E contain 200 mM of pyridine, whereas in B, D and F the contain 400 mM of pyridine. G-L) Effect of 2,4-lutidine and Na^+ concentration on the thermal stability of DNA sequences of varying GC content at different pH values, measured as differences in melting temperature with respect to a solution containing no 2,4-lutidine and 150 mM Na^+.

The pH dependent denaturing/renaturing effect of Pyr is likely related to its ability to be protonated at acidic pH, since at the pH of the experiments 90-95% of all Pyr is in the cationic state. This generates an increase in the ionic strength of the environment, which should stabilize the folded state. However, a general increase in ionic strength does not explain the magnitude of the stabilization (see Figure 1), since the addition of NaCl to obtain the same cationic concentration (in the absence of Pyr) fails to substitute completely the stabilizing effect of Pyr^+. The same experiments performed with methylated DNA provide a very similar picture. Thus, at acidic pH the "denaturant" Pyr can stabilize up to 18 degrees the stability of the methylated version of Seq. 3, which compares with 13.5 found for unmodified DNA (see Suppl. Tables S1 and S2). Note again that adding equivalent concentration of NaCl does not match the stabilizing effect of Pyr^+ also for methylated DNA.

We next confirmed that the canonical double helical structure of duplex DNA is indeed preserved both at neutral and low pH in presence of pyridine. To this end, we collected NMR spectra of AT and GC-rich duplexes (Seq. 1 and 3) at pH values of 4.2 and 7.8 in the presence of 400 mM deuterated pyridine (see Suppl. Info. Materials and Methods for details). At both pH conditions, the imino signals of the NMR spectra exhibit chemical shifts characteristic of AT and CG base pairs (Figure 2 A and B, and Fig. S2). In addition, NOESY spectra (Figure 2C) show the expected cross-peaks pattern for a canonical Watson-Crick duplex (i.e., AH2-TH3 contacts and medium or weak H1'-H8 cross-peaks). Although the size of these oligonucleotides is too large for a complete sequential assignment of the NMR spectra, the experimental information allows us to rule out the formation of structures different than a canonical Watson-Crick duplex in the presence of pyridine. In summary, results discussed above correspond to *bona fide* duplex B-DNA → single strand unfolding processes.

Results in Figure 1 and Suppl. Tables S1 and S2 strongly suggest that Pyr makes a rather specific stabilizing effect on DNA at acidic pH. To verify the specificity of the Pyr^+ stabilizing effect we repeated the melting experiments using 2-4-dimethylpyridine (2,4-Lutidine: Lut). This aromatic molecule is bulkier than Pyr, and has a pKa of 6.63, which implies that at it should be neutral at pH=8.0 and fully protonated at pH≤5.2. Results in Fig. 2 and Suppl. Table S3 demonstrate that neutral Lut is a strong denaturant (even better than Pyr). However, protonated Lut does not offer the same DNA stabilizing effect as protonated Pyr does, and in fact the stabilization found for Lut^+ at acidic pH is smaller than that obtained by adding equivalent quantity of NaCl (Figure 2). This confirms that the strong stabilizing effect of Pyr at acidic pH is related to short-ranged, specific duplex-Pyr^+ contacts, which are less likely to occur for the bulkier Lut molecule.

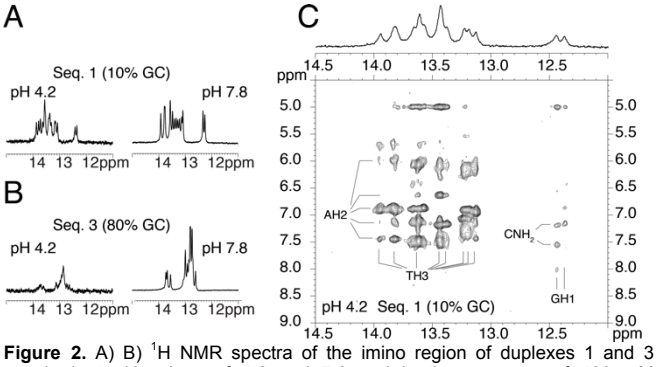

Figure 2. A) B) ^{1}H NMR spectra of the imino region of duplexes 1 and 3 acquired at pH values of 4.2 and 7.8 and in the presence of 400 mM deuterated pyridine. C) NOESY spectrum (100 ms mixing time) of duplex 1 acquired pH 4.2 and in the presence of 400 mM deuterated pyridine (T = 5ºC).

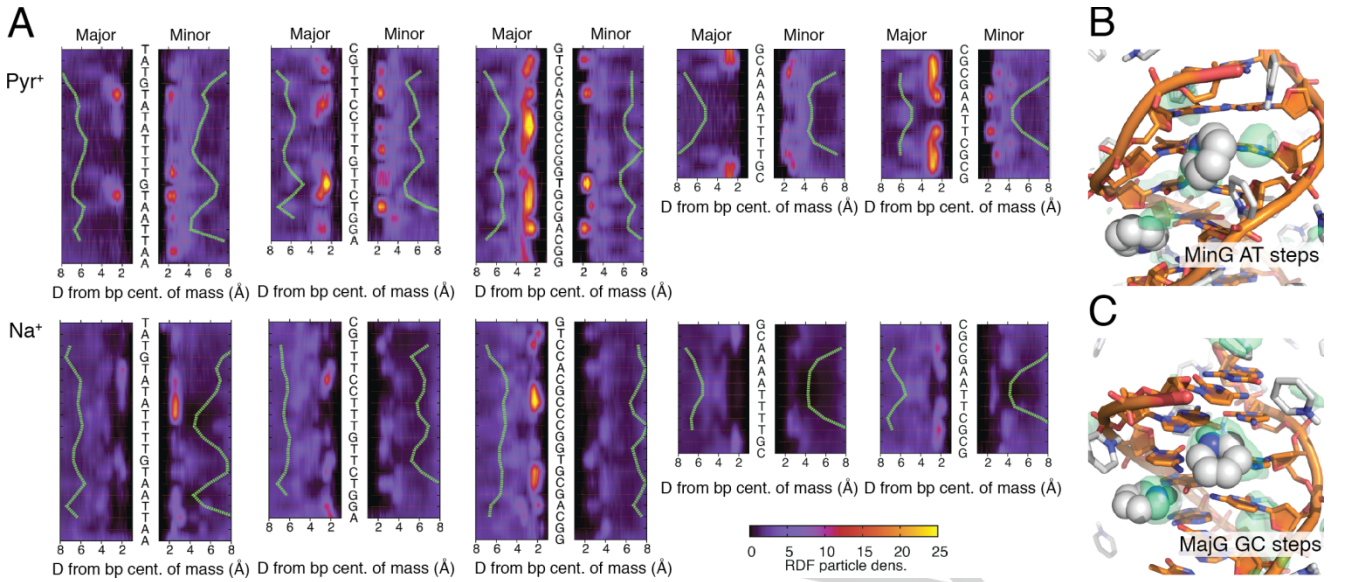

Figure 3. A) Comparison of protonated pyridine and sodium binding positions along the major and minor grooves five different DNA sequences: the three sequences experimentally studied, the Drew-Dickerson dodecamer and GCA_4T_4GC. The sequences are specified in a top-to-bottom, 5'-to-3', orientation for the Watson strand. The heat maps display the radial distribution function (RDF) of particle density with respect to the radial position in the major and minor grooves (plotted against the horizontal axis, reversed for the major groove) and the position along the duplex sequence (vertical axis). The reference of the RDF for both grooves is placed close to the center of the mass of the base pair. The density of solute was quantified as a function of the distance to the reference within a spherical section of 1 nm cutoff, using an angular cutoff of 2.35 rad. The green line overlaid on the maps displays the groove width (we subtracted 0.6 nm for the major groove curve). B) C) Protonated pyridine in the typical binding locations along (B) minor groove (MinG) of AT steps, and along (C) the major groove (MajG) of GC steps. The non-polar pyridine hydrogen atoms and all the DNA hydrogen atoms are omitted for clarity. The green transparent volumes contain regions of pyridine nitrogen density three times larger than the corresponding bulk density (2 M pyridine).

To gain detailed information on the nature of Pyr^+-DNA interactions we performed MD simulations of duplex DNA in 2 M pyridine at low pH (all Pyrs were considered protonated), as well as control simulations in 2 M NaCl (full details in Supp. Info). We performed 10 independent simulations of 1.5 microsecond long each for the sequences 5'-GCAAAATTTTGC-3' (A_4T_4) and 5'-CGCGAATTCGCG-3' (DDD), which could potentially lead to unfolding in the microsecond time scale [5]. To check for the generality of conclusion derived from these calculations additional 0.5 microsecond simulations were performed in 2 M NaCl, 2M Lut^+ and Pyr^+ for sequences 1 to 3 (Table 1), as well as for A_4T_4 and DDD. We did not observe unfolding events in any of the simulations performed (Suppl. Figures S3), consistent with our NMR spectra, whereas previous trajectories in the same time-scale and with the same force-field reported several unfolding events when simulated in neutral Pyr [5].

We traced the locations of cations (Pyr^+, Na^+ or Lut^+) in our MD simulations to explore the existence of cations placed at stable positions along the DNA, which might stabilize the entire duplex [3, 7]. Analysis of cation distribution functions (Figure 3 and Figure S4) show that protonated pyridine and sodium cation interact in a non-sequence-specific way with the phosphate groups along the DNA backbone, as can be observed the slight increase in density at distances of ~7 Å from the base pair center of mass in Figure 3. Furthermore, we detected regions of high Pyr^+ density along the minor (A·T) and major (G·C) grooves of DNA. When placed in the minor groove (see Figure 3B) the Pyr^+ ring is positioned parallel to the groove with the acidic proton interacting directly with T(O2) and A(N3). When placed in the major groove the Pyr^+ plane is mainly oriented perpendicular to the groove with the acidic proton placed in the electronegative patch created by the N7 nitrogen and O6 oxygen of guanine (Figure 3C). Overall, the highest concentration of Pyr^+ is found in A_nT_n (n >= 2) tracts where the minor groove width is widest, e.g. around the extremes of A_4T_4 sequence (5-ApA and the 3'-TpT), mimicking the situation found for choline and TMA and in agreement with previously described binding modes for a large number of cations [7a, 8]. Binding specificity of sodium cations is qualitatively similar to that of Pyr^+, but the binding free energy is significantly smaller (~-1.4 kcal/mol for Na+, ~-2.4 kcal/mol for Pyr^+; see methods section of SI material). Ion uptake experiments (SI material and Table S4) show that the number of pyridine cations that condense with Seq. 3 approximately double the number of sodium cations, in good agreement with the computed binding free energy and cation densities in Figure 3. Lut^+ is bulkier than Pyr^+ and therefore does not penetrate the DNA grooves as efficiently: few Lut+ are bound to the major groove, and while present in minor groove they are always placed at more distant locations from the bottom of the groove (Figure S4), which justifies its smaller stabilizing effect.

Thermodynamic analysis of the melting of duplex Seq. 3 in Pyr^+ and NaCl solutions confirmed the differential stabilization of DNA in these two solutions (Table S5), which seems to be due to enthalpic origin related to the strong interactions of well-positioned Pyr^+ in the minor grooves. As expected due to its size, Pyr^+ distorts the minor groove hydration pattern found in NaCl solutions. Thus, our simulations reveal that solvents sites in the presence of Na^+ are organized in a pattern reminiscent of a fused hexagonal motif[9] (Figure S5A and S5D), and that Pyr^+ disrupts somewhat such a pattern of hydration in the minor groove (Figure S5B, S5E and differential plots S5C and S5F).

Our study reveals that one of the most powerful denaturants of DNA can become structure protective when combined with another strong denaturant: acidic pH. This effect appears to be specific, as neither Na^+, nor the closely related DNA denaturant 2,4-lutidine exhibit the same behaviour. Our MD simulations suggest that Pyr denaturant properties can be explained considering its ability to stack nucleobases[5], while the protective properties of Pyr^+ are related to sequence-specific interactions of protonated pyridine molecules along the grooves of DNA. Overall, our results highlight the tuneable nature of pyridine as denaturant and open new means to alter the thermal

stability of nucleic acids. The switchable behavior of the Pyr^+/Pyr pair might find applications in the field of nanobiotechnology. For example, it has been shown that a mixture of glycerol and choline chloride allows for a more efficient folding of DNA nanostructures such as DNA origamis[10]. The similar mode of interaction between choline and Pyr^+ cations with DNA suggests an avenue for the design of new DNA agents for nanobiotechnological applications, such as nanodevices with applications in photonics, litography and electronics. Also, the tunable denaturing/renaturing effect of Pyr could aid the development of programmable fluorophore-quencher DNA-based nanoswitches such as pH nanosensors with ability to respond to pH changes of their localized environment[11]. The structural origin of Pyr^+ stabilization could be also exploited in experiments where DNA should be kept stable while other macromolecules are unfolded. Finally, our results could be exploited to design new cationic pyridine derivatives for the delivery of genes which should act on acidic media [12]. These potential applications of Pyr will be explored in future works.

Experimental Section

Analysis of the thermal stability of DNA duplexes. Ultraviolet (UV) absorbance was measured on a JASCO V-650 spectrophotometer equipped with thermoprogrammer. UV melting curves at 290 nm were measured at 1 μM strand concentration in buffers containing different amounts of protonated/deprotonated pyridine and in the corresponding control buffers, containing no pyridine (see the Supporting Information). Experiments were performed in Teflon-stoppered quartz cells of 1 cm path length. The samples were heated to 95 ºC, allowed to cool slowly to 4 ºC, and then warmed during the denaturation experiments at a rate of 1 ºC min-1 to 90 ºC. Melting curves were determined by computer fit of the first derivative of absorbance with respect to 1/T[13]. Further details are described in SI material.

NMR spectroscopy. Buffers for NMR experiments were: (a) 100 mM citric acid (2,2,4,4-d4), 150 mM NaCl and 400 mM pyridine-d5, pH 4.2, and (b) 69mM Na_2HPO_4, 6.3 mM NaH_2PO_4, 6.3 mM NaCl and 400 mM pyridine-d5, pH 7.8. The pH was adjusted by adding small amounts of concentrated deuterium chloride. DNA duplexes (sequences **1** or **3**) were dissolved in 500 μL of buffers a or b. The equimolar concentration of each strand was 0.5 mM. The solutions were heated to 95 ºC, allowed to cool slowly to room temperature and stored at 4 ºC until NMR was acquired. NMR spectra were acquired in Bruker spectrometers operating at 600 and 800 MHz, equipped with cryoprobes and processed with the TOPSPIN software. A jump-and-return pulse sequence[14][14][14][14] was employed to observe the rapidly exchanging protons in 1D H_2O experiments. NOESY spectra 9:1 H_2O/D_2O were acquired with mixing times of 100, and 250 ms. In these experiments, water suppression was achieved by including a WATERGATE module in the pulse sequence prior to acquisition.

Molecular Dynamics simulations The DNA duplexes were immersed in an octahedral box of previously equilibrated mixtures of water and solutes (protonated pyridine, protonated 2,4-lutidine or NaCl) at a 2 M concentration. Systems containing 5'-GCAAAATTTTGC-3' (A_4T_4) sequence and the Drew-Dickerson dodecamer sequence (5'-CGCGAATTCGCG-3', DDD) in a 2 M protonated pyridine solution were simulated independently 10 times for 1.5 microseconds, and sequences **1** to **3** were simulated for 1.5 microseconds each. The systems A_4T_4 and DDD were also simulated in 2 M NaCl and 2 M protonated 2,4-lutidine solution for 500 ns. The simulations were carried out using the Gromacs-4.5 software[15] and the parmbsc0 force-field[16], further details in SI material. Cation binding free energies were computed from the densities of ions in the binding sites with respect to their densities in the bulk, corrected by the free energy required for transferring a solute molecule from a given standard volume to the binding site. Full details can be found in the SI material.

Keywords: Nucleic acid thermodynamics • ion-DNA interactions • denaturant • Molecular Dynamics • NMR

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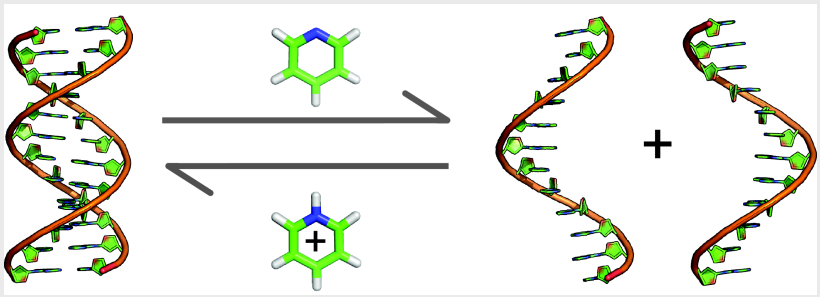

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Can a denaturant stabilize DNA? Pyridine reverses DNA denaturation in acidic pH

The thermal stability of duplex DNA can be decreased by addition of pyridine, a well-known denaturant. In acidic solutions, pyridine is found protonated and displays its role as DNA renaturant, which increases the melting temperature of the duplex further than a solution of NaCl at the same concentration. This orthogonal behaviour is rather specific to pyridine, e.g. the effect is not observed in bulkier derivatives, such as 2,4 dimethylpyridine.