Direct Monitoring of Cytosine Protonation in an Intramolecular DNA Triple Helix

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Potential applications in molecular biology and medicine have sparked renewed interest in DNA triple helices recently. Binding of a single stranded DNA or RNA to the major groove of a double helical target results in the formation of specific Hoogsteen hydrogen bonds with the Watson−Crick purine bases.[1] Thus, in triple helices of the pyrimidine motif a third pyrimidine strand interacts with the double helix forming C^+GC and TAT base triplets. However, the requirement for protonation of the cytosine bases in the third strand results in decreased stabilities of the triplex with increasing pH. Studies on the pH dependence of triple helix formation with a third pyrimidine strand by ethidium bromide fluorescence,[2] by UV absorption spectroscopy,[3] and by CD spectroscopy[2,4,5] yielded semiprotonation points for oligonucleotide triplexes. However, the determination of such apparent pK_a values by the above methods is based on a two-state model with the cytosine protonation being the only pH-dependent process for the duplex−triplex transition. Moreover, analysis is restricted to the influence of pH on a global equilibrium process. Protonation of individual bases within a sequence is not resolved.

In a recent NMR study a uniformly ^{13}C- and ^{15}N-labeled RNA third strand was used for the unambiguous structural characterization of the triplex formed with a DNA hairpin.[6] To assess the effect of cytosine protonation on triple helix formation at single sites we decided to perform heteronuclear NMR experiments on a specifically ^{15}N-labeled oligonucleotide. The oligonucleotide was designed to form an intramolecular triple helix with two $(T)_4$ loops and seven base triplets by folding back on itself under appropriate conditions. Except for the 3′-terminal cytosine all cytosine bases within the Hoogsteen bound "third strand" were specifically ^{15}N labeled at the 4-amino group (Figure 1).[7a,b] Although this group is not directly protonated, studies on monomers indicate a downfield shift of the ^{15}N amino resonance by more than 10 ppm upon cytidine protonation.[8] The specifically isotope-labeled cytidine was synthesized according to published procedures,[9] protected, and subsequently used as its phosphoramidite in the automated DNA synthesis.

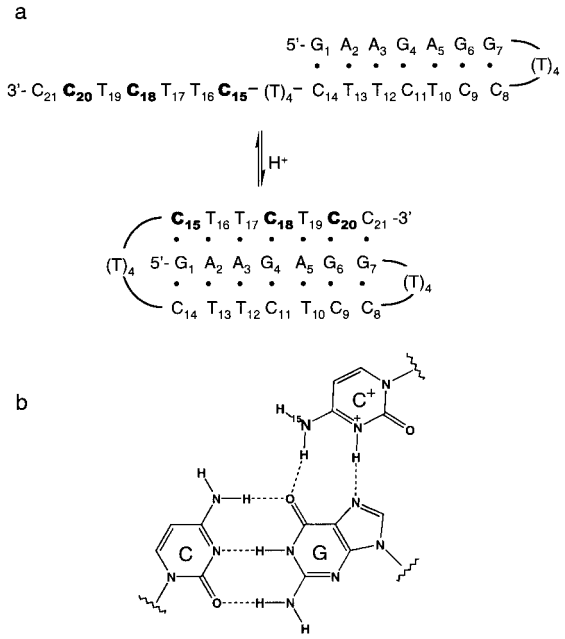

Figure 1. (a) Sequence and numbering scheme of the 29 base oligonucleotide and its folding into an intramolecular duplex and triplex structure. ^{15}N labeled cytidines are shown in bold. (b) C^+GC base triplet with Hoogsteen bound cytosine specifically ^{15}N labeled at the amino group.

^1H NMR spectra showing imino resonances of the oligonucleotide at 5 °C are plotted as a function of pH in Figure 2a. Below pH 5.80 formation of an intramolecular triplex is indicated by the signals of Watson−Crick and Hoogsteen hydrogen bonded imino protons between 12 and 16 ppm. 2D NOE measurements at pH 5.0 confirm the formation of a triple helical structure and also allow the unambiguous assignment of all imino as well as amino resonances (data not shown). With increasing pH additional signals of a duplex emerge which at pH 6.65 are of similar intensity compared to those of the triplex. Above pH 6.90 resonances of the triple helix disappear and only the reduced set of imino signals expected for a duplex remains observable. The presence of triplex and partially unfolded duplex structures slowly exchanging on the NMR time scale has been observed before during intramolecular triple helix formation.[10]

To follow protonation of the cytidine nucleobases we have performed $^1H-^{15}N$ HSQC experiments over a pH range of 4.80−8.25. Representative spectra are shown in Figure 2b. Cross-peaks connect the amino nitrogens of the three labeled cytosines with their attached amino protons. Downfield- and upfield-shifted ^1H resonances are associated with hydrogen-bonded and non-hydrogen-bonded protons, respectively. From the assignment of the amino protons at low pH the three nitrogen signals with chemical shifts of 83.9, 84.5, and 85.9 ppm at pH 4.80 can be unambiguously associated with cytidines C20, C15, and C18. The significantly downfield-shifted ^1H and ^{15}N amino resonances indicate that at pH 4.80 all of the three labeled cytidines are in their protonated state. As seen from Figure 2b, cross-peaks due to protonated cytidine 15 disappear first upon raising the pH. At pH 6.35 only resonances of protonated cytidine 18 remain observable but have also disappeared at pH 7.45. At the same time increasingly strong cross-peaks at higher field typical of nonprotonated cytosine bases appear with ^1H and ^{15}N chemical shifts clustered at 7.3, 6.6, and 72.2 ppm.[11]

A closer inspection of the cross-peaks for protonated C15 reveals that intensities start decreasing appreciably above pH 5.30

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(7) (a) The 3′-terminal cytosine was not isotope labeled due to the protocol of DNA solid phase synthesis. (b) Although being directly protonated, ^{15}N isotopes at cytosine N-3 are less suited as NMR probes due to the absence or fast exchange of scalar coupled protons.
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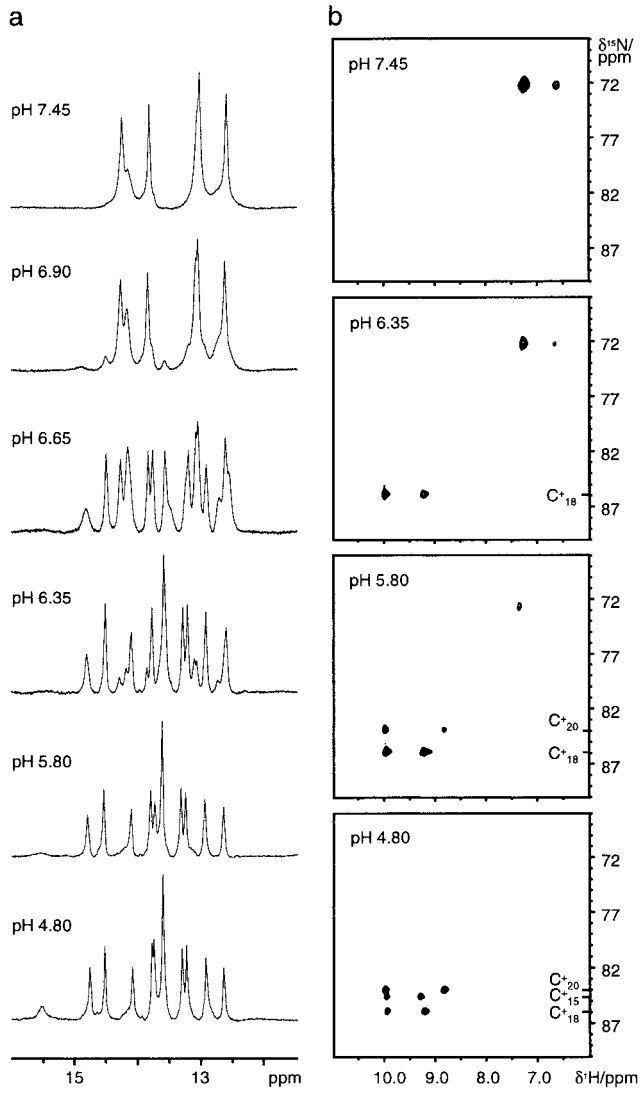

Figure 2. (a) ^{1}H NMR spectra showing imino protons of the specifically ^{15}N labeled oligomer in 90% H_2O/10% D_2O, 100 mM NaCl, 10 mM $MgCl_2$ at 5 °C as a function of pH. The spectra were acquired on a Bruker AMX500 spectrometer using a $1\bar{3}3\bar{1}$ pulse sequence with the excitation maximum set to the imino proton region at 14 ppm. The pH was adjusted by addition of NaOH or HCl and measured before and after the experiment. Spectra were obtained at increasing and decreasing pH and were identical in each case. (b) Phase-sensitive ^{1}H−^{15}N gradient selected HSQC spectra as a function of pH. Sample and other experimental conditions correspond to those of (a). Assignments to the protonated cytosines are indicated at the right of the spectra.

and cross-peaks finally disappear in the spectra at pH >5.70. Hence a pH value of 5.5 ± 0.1 for semiprotonation can be derived. Correspondingly, semiprotonation points for C20 and C18 are found to be 6.0 ± 0.1 and 6.7 ± 0.1. Deprotonation is known to occur in two steps, namely local opening of the base pair followed by proton abstraction from the open state.[12a,b] With most of the protonated cytosines in a closed state the determined apparent pK_a values will depend on both the pK_a of non-base-paired cytosines as well as on the base pair dissociation constant K_D. Assuming similar acidities for the various cytosine bases, higher apparent pK_a values are therefore indicative of smaller base pair dissociation constants.[13]

Taken together, a detailed picture of the pH-dependent duplex−triplex equilibria emerges. Obviously, deprotonation of C15 next to the loop region has no significant influence on triplex formation. Even with C20 fully deprotonated at about pH 6.20 the triplex predominates over the duplex under the given solution conditions. Thus, triplex formation does not require the protonation of all cytidines in the third strand, and formation of the central C^+GC base triplet which is flanked by two TAT triplets sufficiently stabilizes the triple helical structure. However, with the onset of deprotonation for cytosine 18 triplex stability strongly decreases. Interestingly, pH-dependent CD measurements on a triple helix with the same sequence except for two $(T)_5$ loops yielded an apparent pK_a value for the duplex−triplex transition of about 6.5.[4] In accord with our data, this value is close to the pH of semiprotonation for C18 whose protonation mostly determines triple helix formation but not to the corresponding value for cytosine 20 or 15. In the same report an estimation based on calorimetric data yielded an uptake of only 1.5 protons upon triple helix formation in excellent agreement with our results on cytosine protonation. Further studies on the protonation of cytosine or other base analogues are expected to contribute to a better understanding of the pH dependence of triple helix stability under various conditions.

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Supporting Information Available: Portions of a 2D NOE spectrum of the triplex showing connectivities which can be used for the unambiguous assignment of the third strand cytosine amino protons (3 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(11) The weaker cross-peaks for nonprotonated bases are mostly due to the amino proton chemical shifts being closer to the water resonance.

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(13) The observed slow exchange between protonated and nonprotonated bases sets an upper limit of 500 s^{-1} for the overall exchange rate based on chemical shift differences. However, the rate limiting process cannot be unambiguously identified with the available data.