Multiple Structures for the 2-Aminopurine−Cytosine Mispair†

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ABSTRACT: The base pair formed between 2-aminopurine (2AP) and cytosine (C) is an intermediate in transition mutations generated by 2AP. To date, several structures have been proposed for the 2AP−C mispair, including those involving a rare tautomer, a protonated base pair, and a neutral wobble structure. In this paper, we describe a series of UV, fluorescence, and NMR studies which demonstrate that an equilibrium exists between the neutral wobble and the protonated Watson−Crick structures. The apparent p*K* value for the transition between the structures is 5.9−6.0. Formation of a Watson−Crick base pair is accomplished predominantly by protonation of the 2AP residue as indicated by UV spectral changes, fluorescence quenching, and changes in proton chemical shifts. Rapid transfer of the shared proton between the 2AP and cytosine residues is indicated by the rapid exchange of the cytosine amino protons of the protonated Watson−Crick configuration. The relative contribution of the neutral wobble and protonated Watson−Crick configurations to 2AP-induced transition mutations is discussed.

The purine analogue 2-aminopurine (2AP)[1] is a potent mutagen in bacteria, generating predominantly transition mutations (*1*, *2*). As an adenine analogue, 2AP is easily incorporated into DNA opposite thymine (*3*−*5*), forming a base pair with Watson−Crick geometry (*6*, *7*) and only slightly reduced thermal (*7*, *8*) and thermodynamic (*9*) stability. In contrast to adenine, however, 2AP is capable of forming a base pair with cytosine at a substantially greater frequency than adenine (*5*, *10*).

The base pair formed between 2AP and cytosine is the intermediate in the transition mutation pathway induced by 2AP (*1*−*5*). The configuration of this mutagenic mispair has been the object of several investigations. Initially, it was proposed that 2AP had an increased tendency to convert to the unfavored imino tautomer, capable of forming a base pair with cytosine in Watson−Crick geometry (*1*). This suggestion was based upon the original proposal presented by Watson and Crick (*11*) for the potential involvement of unfavored tautomeric forms as mutagenic intermediates. Studies published by Goodman and Ratliff, using UV spectrophotometric methods, presented data in support of the rare tautomer model (*12*).

Subsequently, it was proposed that ionized or protonated forms would likely be more favored than mispairs invoking rare tautomeric forms for specific mispairs (*13*). Theoretical studies have indicated that 2AP is less likely than adenine to populate the imino tautomer (*14*), arguing against the involvement of tautomerization in the mutagenesis induced by 2AP. In accord with the theoretical predictions, studies with ^{15}N-enriched 2-aminopurine-2′-deoxynucleoside have demonstrated that 2AP is predominantly in the amino tautomeric form in H_2O (*15*).

Proton NMR studies have established that the 2AP−T base pair is in a normal Watson−Crick geometry with both bases in the preferred tautomeric forms (*6*, *7*). An NMR study with the 2AP−C base pair considered rare tautomeric forms, a neutral wobble, and a protonated Watson−Crick structure. The data were considered to be inconsistent with rare tautomeric forms and more consistent with the protonated base pair (*6*).

Subsequently, however, in studies which examined the fluorescence anisotropy of 2AP-containing oligonucleotides of the same sequence, it was demonstrated that that system underwent a pH-dependent conformational change with formation of a duplex at high pH and a triple-stranded structure at neutral and lower pH (*16*). In an NMR study utilizing ^{15}N-enriched 2AP and C residues in the same base sequence at high pH, it was established that the 2AP and C were paired in a neutral wobble configuration (*17*). The ^{15}N resonances of both the C N3 and 2AP N1 ring nitrogens were observed as singlets whereas the ^{15}N resonances of the exocyclic amino groups of each base were observed as triplets, unequivocally demonstrating that both bases were in the preferred amino tautomeric forms.

Recently, Fagan et al. (*18*) revisited the 2AP−C mispair in a sequence context which was incapable of triple-stranded structures. They concluded that, in the sequence they studied, the 2AP−C base pair was neutral wobble at physiological pH. They further noted pH-dependent changes in the NMR

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[1] Abbreviations: 2AP, 2-aminopurine mononucleotide unit; A, C, G, T, mononucleotide units; DMT, 4,4′-dimethoxytrityl; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; HSQC, heteronuclear single quantum coherence spectra; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE spectroscopy; PRP, polystyrene reverse phase resin; TOCSY, total homonuclear correlated spectroscopy; UV, ultraviolet.

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spectrum below neutral pH, suggesting the presence of additional structures. However, additional forms were not investigated. Subsequent thermodynamic studies have been conducted on the system in which the neutral wobble structure predominates, and the results of these studies have been used to explain the mutagenesis induced by 2AP (*9*).

In contrast, however, the possible contribution of minor configurations to base mispair formation cannot as yet be discounted. Indeed, many aberrant base pairs studied to date are best described as a composite of structures in equilibrium with one another. In many of these cases, the equilibrium is pH dependent (*13*, *19*−*29*). On this basis, we have reinvestigated the 2AP−C mispair, searching for additional structures including a protonated Watson−Crick structure. We establish here that the 2AP−C base pair is indeed in a pH-dependent equilibrium between the neutral wobble and protonated Watson−Crick configurations. Based upon this composite structure, we discuss the potential involvement of the protonated Watson−Crick configuration in transition mutations induced by 2AP.

MATERIALS AND METHODS

Oligonucleotides containing 2AP were prepared by automated phosphoramidite synthesis using a method developed in this laboratory (*30*). The method for preparing a 2′-deoxycytidine residue in which the amino group was ^{15}N-enriched is described elsewhere (*31*). Preparation of the corresponding phosphoramidite of the ^{15}N-enriched cytosine residue followed established methods (*32*). The sequences of the two oligonucleotides were

strand A: 5′d(C1 A2 G3 C4 G5 G6 C7)

strand B: 3′d(G14 T13 C12 AP11 C10 C9 G8)

where C4 is ^{15}N-enriched in the exocyclic amino group and AP is the 2AP residue.

Oligonucleotides were synthesized with the 5′-DMT group on and purified by HPLC using a PRP column. Following detritylation in 80% acetic acid, the oligonucleotides were again purified by HPLC, using a semipreparative reverse phase column. The composition of each oligonucleotide was confirmed by analysis of the bases by GC/MS (*30*). The extinction coefficient for the 2AP-containing oligonucleotides was based upon the UV spectral characteristics previously reported by Fox et al. (*33*).

Equimolar amounts of the two strands were mixed in buffer containing 150 mM NaCl, 10 mM phosphate buffer, and 0.2 mM EDTA. The solution was 4 mM in strand concentration for NMR studies and 3×10^{-5} M for UV and fluorescence studies. NMR spectra were recorded in either 99.999% ^2H_2O or 90% H^2O/10% ^2H_2O. Chemical shifts were recorded relative to internal tetramethylammonium chloride (3.18 ppm).

NMR spectra were recorded with either a Bruker AMX600 or a Varian 500 Unity Plus NMR spectrometer. NOESY spectra were recorded in the phase sensitive mode (*34*) with a mixing time of 400 ms. A jump and return composite pulse sequence (*35*) was used to suppress the water signal in H_2O. A mixing time of 70 ms was used to record the total correlated spectroscopy (TOCSY) spectra (*36*).

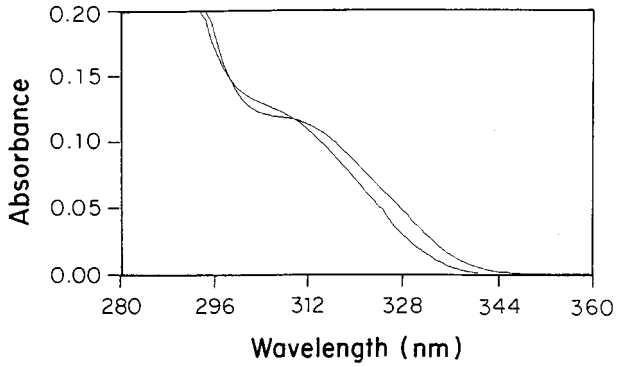

FIGURE 1: UV spectra of a solution containing the deoxynucleosides C, G, AP, and T in the molar ratio 3:2:1:1 (left at 320 nm), and a single-stranded 2AP-containing 7mer (right at 320 nm) of sequence 5′d(G C C AP C T G) in 150 mM NaCl, 10 mM phosphate buffer, pH 7.5, 25 °C.

The ^{15}N-edited proton spectra were recorded at an ^{15}N frequency of 50.65 MHz with a one-dimensional HSQC sequence (*37*, *38*). A total of 4K data points were obtained with a sweep width of 10 000 Hz. The delay used for acquisition of the edited spectra was based on a one-bond ^{1}H−^{15}N coupling constant of 90 Hz (*31*). A total of 128 transients were recorded for each spectrum at 4 or 10 °C as a function of pH between 9 and 4.5.

UV spectra of the single-stranded oligonucleotide and duplex were recorded with a Perkin-Elmer Lambda IIIB double beam spectrophotometer at a strand concentration of 3×10^{-5} M in 10 mM phosphate buffer, 150 mM NaCl. The sample cavity of the spectrophotometer was maintained at 9 °C. Spectra were recorded from 200 to 400 nm at increments of pH from pH 9 to 4. Fluorescence spectra were recorded with a Hitachi F-2000 fluorescence spectrophotometer. The excitation wavelength was at 303 nm, and emission was monitored at 340 nm. The oligonucleotide concentration was 3×10^{-5} M, and spectra were recorded at 9 °C as a function of solution pH.

RESULTS

Base Stacking Induces Changes in the UV Spectrum of 2-Aminopurine. Using standard phosphoramidite methods, we constructed a seven-base oligonucleotide sequence containing a 2-aminopurine residue in the central position. In contrast to the normal DNA bases, the UV absorbance maxima of 2AP is above 300 nm, allowing the UV spectral characteristics of 2AP to be monitored in DNA. In Figure 1, the UV spectrum of the seven-base oligonucleotide containing one 2AP residue is overlaid with the spectrum of a solution containing equimolar amounts of the corresponding deoxynucleosides. Significantly, the UV spectrum of the 2AP residue is red-shifted when incorporated into a single-stranded oligonucleotide, relative to the spectrum of the free deoxynucleoside. Previously, Goodman and Ratliff (*11*) ascribed similar UV spectral changes to formation of the rare tautomeric form induced by formation of a base pair with cytosine. The results obtained here with the 2AP-containing seven-base single-stranded oligonucleotide establish that the 2AP-associated UV spectral changes previously ascribed to rare tautomer formation more likely result from base-stacking induced changes.

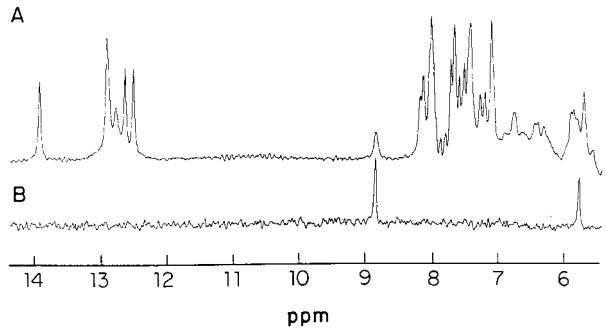

FIGURE 2: Exchangeable proton and aromatic regions of the AP−C-containing seven-base duplex in 150 mM NaCl, 10 mM phosphate buffer, pH 7.48, 5 °C. (A) ^{15}N-decoupled proton spectrum, (B) ^{15}N-decoupled and edited spectrum.

The 2AP−Cytosine Mispair Is Predominantly Neutral Wobble at Physiological pH. A duplex was prepared at neutral pH in which the 2AP residue was paired opposite cytosine. A one-dimensional NMR spectrum was then obtained and is shown in Figure 2A. In the strand complementary to the 2AP-containing strand, the cytosine residue paired with 2AP is ^{15}N-enriched in the amino group. The spectrum shown in Figure 2A was obtained with ^{15}N-decoupling.

Several resonances are observed at low-field which correspond to the imino protons of the normal AT and GC base pairs. The imino resonance of T13 is observed at 14 ppm. In the G-imino region, resonances corresponding to five protons are observed. At approximately 9 ppm, an additional exchangeable resonance is observed. In a normal DNA duplex, exchangeable resonances are not seen in this region, suggesting that the 9.0 ppm resonance might be one of the amino protons of the 2AP−cytosine mispair.

Resonance assignment of the imino protons of the normal bases was carried out by standard presaturation techniques as well as by examination of the two-dimensional NOESY spectrum recorded in H_2O as described below.

The assignment of the 9.0 ppm resonance to the amino group of the cytosine residue paired with 2AP is obtained directly from the decoupled ^{15}N-edited spectrum shown in Figure 2B. In such an edited spectrum, only resonances which have a strong coupling to ^{15}N are observed. The magnitude of the ^{15}N-proton coupling constant for the cytosine amino group is 90 Hz (*31*).

The H8/H6 to H1′/H5 region of the NOESY spectrum (400 ms mixing time, recorded at pH 7.9, 10 °C) of the AP-C duplex is shown in Figure 3. Reasonably well-resolved interbase cross-peaks are observed for both strands, through the location of the mispair, allowing assignment of all of the aromatic base proton resonances, and establishing that both the 2AP and cytosine residues of the mismatch are intrahelical. Additional interbase connectivities are observed, as indicated in Figure 3, establishing that the helix is predominantly B-form.

Relative assignments of H2′ and H2″ protons were made by analysis of cross-peak intensities observed in the NOESY spectrum recorded with a short mixing time (60 ms). The H3′ and H4′ proton resonance assignments were identified in a TOCSY spectrum.

The above results confirm that the 2AP and cytosine residues are intrahelical and in their neutral and preferred tautomeric forms. The ^{15}N-edited spectrum demonstrates two

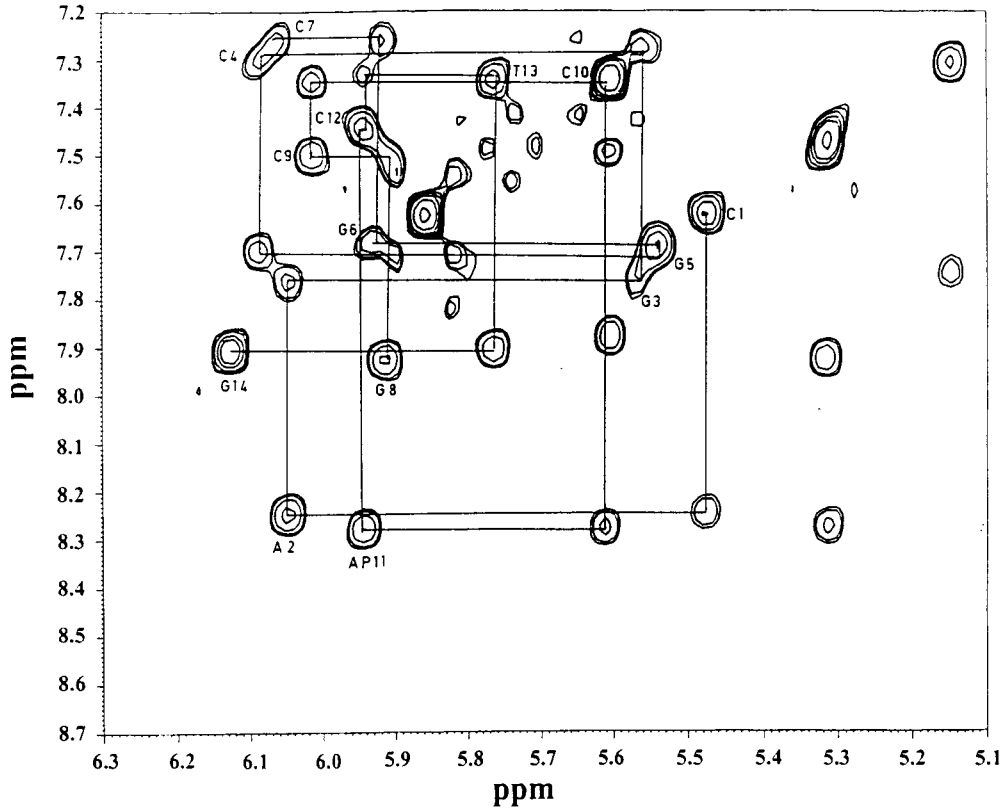

FIGURE 3: Aromatic region of the NOESY spectrum of the seven-base duplex in D_2O, 150 mM NaCl, 10 mM phosphate buffer, pH 7.9, 10 °C. The mixing time was 400 ms.

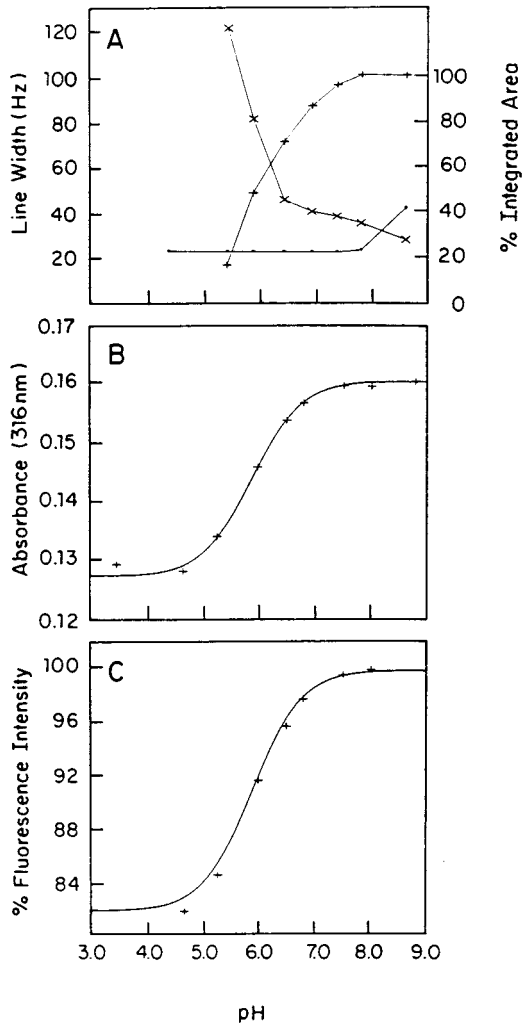

FIGURE 4: pH-dependent changes in the AP−C 7mer duplex. Panel A: Line width (Hz) of the C4 9 ppm amino resonance (×), line width of the T13 imino proton resonance (*), and relative integrated area of the C4 9 ppm resonance, normalized to the integrated area at pH 8.8, (+) as a function of pH at 5 °C. Panel B: Absorbance of the AP−C duplex at 316 nm as a function of pH at 9 °C. Panel C: Relative fluorescence intensity of the AP−C duplex monitored at 340 nm (excitation 303 nm) as a function of pH at 9 °C.

protons attached to the cytosine amino nitrogen, unequivocally eliminating the imino tautomeric form for cytosine in the 2AP−cytosine mispair. The low-field position of the cytosine amino proton and its narrow line width suggest that it is involved in an interbase hydrogen bond.

The NMR data further establish that the predominant sugar pucker of all nonterminal residues is C2′-endo and that all bases adopt an anti conformation (*39*). We conclude from these data that the 2AP−cytosine mispair is predominantly in a neutral wobble configuration at physiological pH, in the sequence examined here, in accord with previous studies (*17*, *18*).

The 2AP−Cytosine Mispair Undergoes a pH-Dependent Change. Substantial changes are observed in the NMR spectrum upon reduction of the solution pH, and these changes are localized to the region of the AP−C mispair. The resonance assigned to the hydrogen-bonded amino proton of the cytosine residue (C4) paired with 2AP broadens substantially with decreasing solution pH (Figure 4A). The relative integrated intensity of this resonance also diminishes

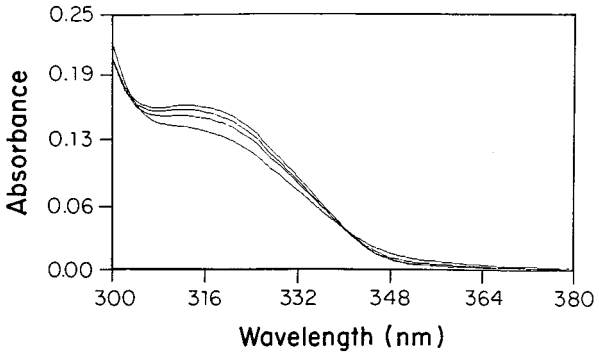

FIGURE 5: pH-dependent UV spectra of the seven-base duplex containing a central AP−C base mispair obtained in 150 mM NaCl, 10 mM phosphate buffer, 9 °C. Values of solution pH, with decreasing absorbance at 316 nm, are 6.84, 6.46, 5.96, and 5.32.

with decreasing pH (Figure 4A). By pH 6.0, neither of the C4 amino proton resonances are observed in the 1D proton or ^{15}N-edited proton spectra.

The UV spectra of oligonucleotides containing 2AP have a characteristic shoulder above 300 nm attributed to the absorbance of 2AP (Figure 5). We observe that, upon lowering the solution pH, the UV spectrum of the duplex containing the 2AP−cytosine mispair undergoes a substantial decrease at 316 nm, with an isosbestic point at 340 nm, as shown in Figure 5. The apparent midpoint in the titration curve is 5.9−6.0 (Figure 4B). The fluorescence of 2AP may also be measured in oligonucleotides with excitation at 303 nm and emission at 340 nm (*40*). The fluorescence of 2AP is diminished by protonation (*40*), and we observe here that the fluorescence from 2AP in the AP−C mispair also diminishes with decreasing pH due to protonation of the 2AP residue (Figure 4C). The titration midpoint in the fluorescence titration is similarly observed to be 5.9−6.0.

The 2AP−Cytosine Mispair Is Protonated at Low pH. The H8/H6 to H1′/H5 region of the NOESY spectrum of the 7mer duplex containing the AP−C mispair, recorded at pH 4.5 and 10 °C, is shown in Figure 6. The NOESY spectra of the 7mer duplex (Figures 3 and 6) change little upon lowering the solution pH, with the exception of the central region containing the AP−C mispair. Proton−proton connectivities are observed on both strands, as with the neutral species, indicating that the helix is still predominantly B-form. The AP and C residues are still intrahelical when protonated. The H6 of the C residue is moved upfield slightly; however, the H8 resonance of the AP residue has moved significantly downfield by 0.3 ppm. The remainder of the spectrum remains unchanged. Upon protonation of the 2AP monomer in solution, the H8 proton is observed to move downfield by 0.35 ppm.

DISCUSSION

Multiple Structures for the AP−C Mispair. Our results with the seven-base duplex containing a central AP−C base mispair indicate that the predominant configuration at neutral pH is the neutral wobble configuration (Figure 7) which has been described previously (*17*, *18*) in other DNA sequence contexts. The results of NOESY spectra demonstrate that the helix is predominantly B-form, and both the AP and C residues are intrahelical. The use of selective ^{15}N-enrichment of the C paired with 2AP allows unambiguous assignment

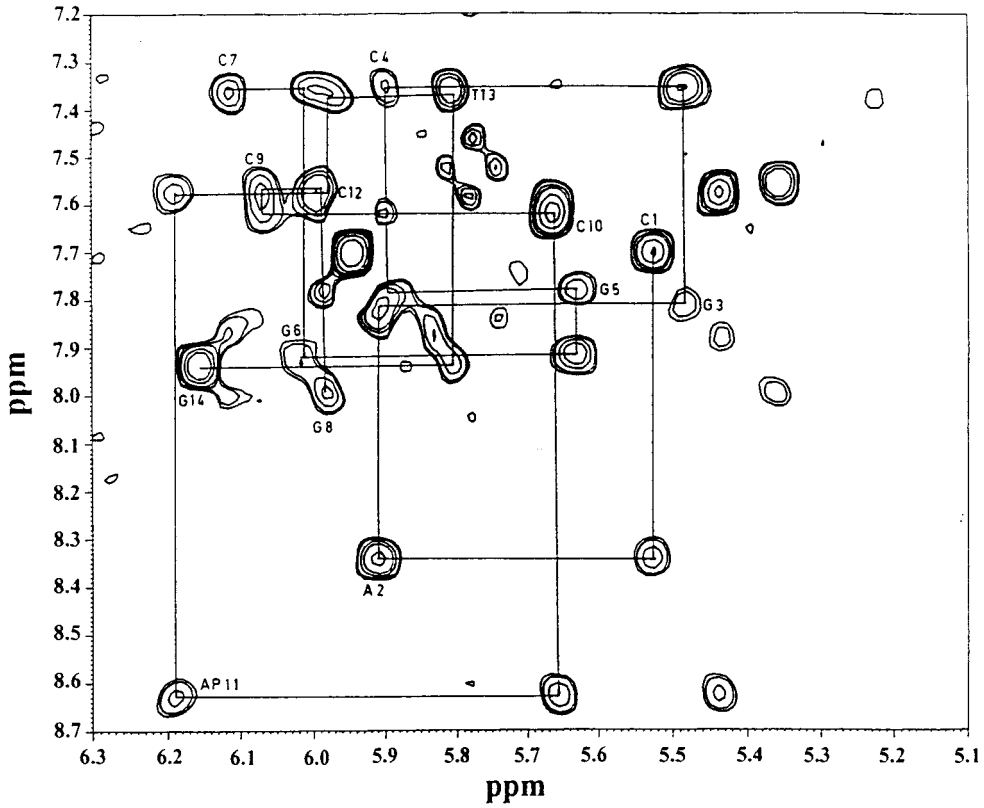

FIGURE 6: Aromatic region of the NOESY spectrum of the seven-base duplex in D_2O, 150 mM NaCl, 10 mM phosphate buffer, pH 4.5, 10 °C. The mixing time was 400 ms.

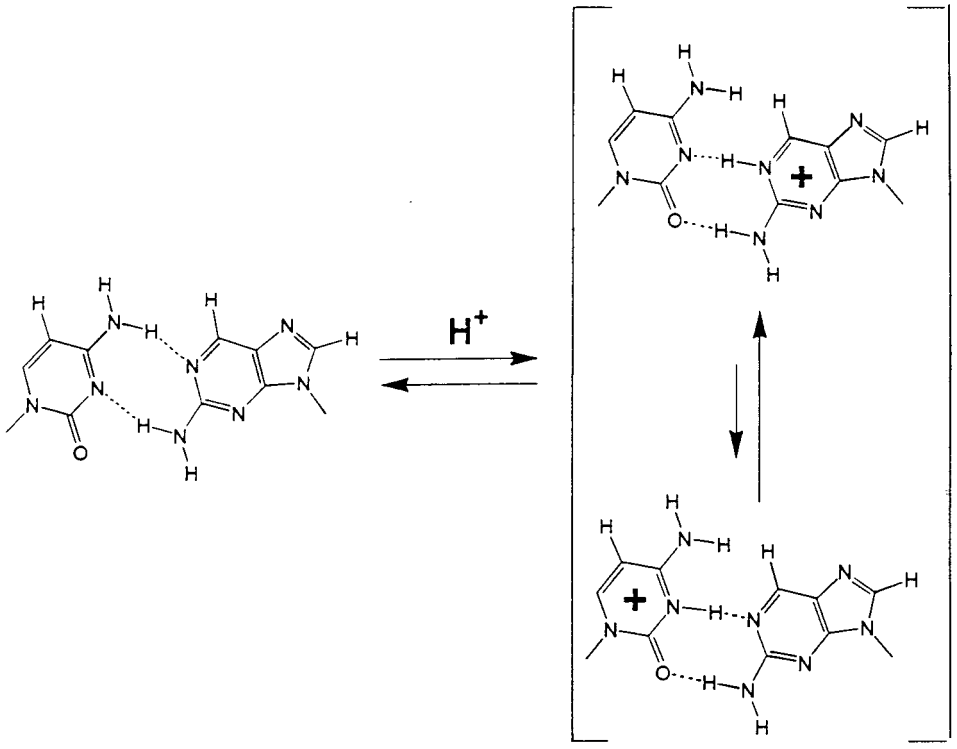

FIGURE 7: Structures of the possible AP−C mispair hydrogen-bonded configurations. Left: neutral wobble; right-upper, ionized Watson−Crick configuration, protonated on the AP residue; right-lower, ionized Watson−Crick configuration, protonated on the C residue.

of the amino resonances and confirms that the C residue is in the preferred amino configuration. The amino proton of the cytosine residue is shifted downfield due to formation of a hydrogen bond with the N1 position of the 2AP residue.

With decreasing solution pH, substantial changes are observed in the NMR spectra which are localized to the region of AP−C base mispair. Based on pH-dependent changes in the NMR, UV, and fluorescence spectra (Figure 4), the apparent p*K* value for the transition is 5.9−6.0. Two possible structures can be drawn for the protonated base pair as indicated in Figure 7, in which either the AP or the C residues may be protonated. We note that Fagan et al. (*18*)

similarly reported pH-dependent changes in the C amino resonance of the AP−C base pair; however, additional structures were not investigated.

The protonation of the 2AP−C mispair at lower pH is not unexpected. Many other aberrant base pairs have also been demonstrated to exist as a composite of different structures in pH-dependent equilibria (*13*, *19*−*29*). Of particular interest is the mutagenic base pair formed between C and O^6-methylguanine (O^6G) which similarly exists in an equilibrium between neutral wobble and protonated Watson−Crick configurations (*13*, *26*, *41*, *42*). The apparent p*K* for the transition between the two species is 5.5 (26), slightly lower than for the 2AP−C system reported here. The adenine−cytosine mispair is also described as at least two structures in pH-dependent equilibrium with a p*K* of 7.4 (*24*, *43*).

Of the two possible forms of the protonated AP−C mispair shown in Figure 7, the data reported here indicate that the AP residue is most likely protonated. The UV spectrum of the AP-containing duplex displays a significant decrease at 316 nm upon reduction of the solution pH (Figures 4B and 5). A similar pH-dependent change is observed upon protonation of 2-aminopurine-2′-deoxynucleoside (APdR, ref *15*). It has also been reported that the fluorescence intensity of 2AP is diminished upon protonation (*40*). We observe here that the fluorescence for the 2AP residue in the duplex is reduced upon lowering the solution pH.

It has been previously demonstrated that downfield shifts of selected proton resonances are a marker of base protonation (*43*). The H8 proton of APdR in solution moves downfield by 0.35 ppm upon protonation. Protonation of the AP−C mispair causes the chemical shift of the H8 proton of AP11 to move downfield by 0.3 ppm, whereas the H6 proton resonance of C4 moves slightly upfield.

The amino protons of C4 paired with AP are also observed to undergo pH-dependent changes in both line width and intensity. In the protonated structure, the C4 amino protons are no longer hydrogen-bonded to the AP residue and are therefore accessible for exchange with solvent. Although the data suggest that the protonated AP structure predominates, rapid proton transfer would be expected between AP and C (Figure 7). Transient protonation of the C residue would result in rapid exchange of the unpaired amino protons (*44*).

In the analogous O^6G−C mispair, the cytosine residue is reported to be protonated (*26*). The p*K* of monomeric O^6-methyl-2′-deoxyguanosine is 2.5 (*45*), substantially lower than the p*K* of 4.6 reported for 2′-deoxycytidine (*43*). The p*K* of monomeric 2-aminopurine-2′-deoxynucleoside is 3.8 (*15*). However, this value increases to 4.5 in oligonucleotides (*46*), perhaps due to enhanced stacking promoted by base protonation (*47*) and the negative electrostatic potential of the DNA helix (*48*). The similarity in the basicity of 2AP and C is consistent with a shared proton between the two bases, and the distribution of the proton charge density may be highly base sequence-dependent.

Which Configuration Explains the Mutagenicity of 2AP? The as yet unanswered biological question is then: Which of the possible forms of the 2AP−C base pair is involved in mutagenesis? Previously, Goodman and co-workers suggested a correlation between thermodynamic properties of the neutral AP−C wobble with polymerase insertion data (*9*, *49*). The neutral wobble form of the 2AP−C mispair is less thermally and thermodynamically stable than the corresponding Watson−Crick AP−T base pair (*9*). The free energy difference between the two is approximately 1.8 kcal/mol, which, according to the thermodynamic model, would predict a misinsertion frequency of roughly 1/20. The DNA polymerase preference for insertion of AP opposite C versus T is approximately 1/25 (*5*). Therefore, Goodman and coworkers have concluded that the neutral wobble configuration of the AP−C mispair, which predominates at physiological pH, is the configuration incorporated by DNA polymerase (*9*).

The thermodynamic model has been widely applied in examining the mispairing potential for other aberrant base pairs. The prevailing view is that stability, as opposed to base pair configuration, dictates base selection (*49*−*51*). More recently, however, it has been recognized that, in some cases important to mutagenesis discussed below, free energy differences measured between the correct Watson−Crick base pair and the predominant configuration of the aberrant base pair do not predict correctly the observed preference for polymerase base selection. In cases where a correlation does exist, the free energy differences are often too small to account for the observed polymerase preferences. Recognition of these discrepancies has recently resulted in a reconsideration of the thermodynamic selection model and the acknowledgment that base pair configuration and geometry must play an important role in base selection (*52*).

Within this context then, we propose another model to account for the mutagenic mispairing of 2AP. We have demonstrated here that neutral wobble and protonated Watson−Crick configurations are in equilibrium with one another with an apparent p*K* of 5.9−6.0. At physiological pH, the ratio of the geometrically correct, protonated Watson−Crick structure to the neutral wobble configuration would also be approximately 1/25. The difference in the polymerase insertion frequency for G versus AP pairing with C is also roughly the same as discussed above. We therefore propose that the existence of a "minor" configuration, which is geometrically correct (isosteric with Watson−Crick), could also account for the mispairing of 2AP.

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