Evidence of 2-Aminopurine-Cytosine Base Mispairs Involving Two Hydrogen Bonds*

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It is shown that the mutagen base analogue 2-aminopurine is hydrogen-bonded at its 1-ring position when annealed with cytosine in DNA. The presence of stable hydrogen-bonded regions proximal to the 2-aminopurine-cytosine base mispair is a prerequisite for the occurrence of two hydrogen bonds coupling the bases at their 1-3- and 2-2-positions. We consider the possibility that, in the resulting heteroduplex base mispair, 2-aminopurine or cytosine may be present as a disfavored imino tautomer.

The fidelity of DNA replication is determined by a set of base-pairing and base-stacking interactions mediated by the properties of a multienzyme replication complex. It is generally believed that efficient phosphodiestester bond formation catalyzed by DNA polymerase requires stabilization of an incoming deoxyribonucleoside triphosphate substrate on a polymerase-DNA template complex by at least two hydrogen bonds formed between the substrate and template bases. A molecular basis to explain spontaneous transition mutations in which two hydrogen bonds are made between base mispairing partners was first proposed by Watson and Crick (1, 2) in their classic papers on the structure of DNA.

The Watson and Crick proposal allowed that the four common nucleotides, if present in unfavored tautomeric forms, could form stable hydrogen bonds with incorrect partners. If, for example, the imino form of adenine were present at the replication site on DNA polymerase, then it could form two hydrogen bonds with a template cytosine resulting in an A-C mispair. Failure to remove the misinserted adenine deoxy-nucleotide prior to the next round of DNA replication would result in a G-C base pair being located at the original A-T site. A-T → G-C and G-C → A-T transition mutations involving all possible incorrect purine-pyrimidine partners could, in principle, be accounted for by base pairing via keto-enol (for Gua and Thy) and amino-imino (for Ade and Cyt) tautomers. As recently proposed by Topal and Fresco (3), transversions caused mainly by purine-purine base pairs could also be accommodated as disfavored tautomers in Watson-Crick base-pairing conformations provided that the purine residues can exist in the DNA double helix in a syn conformation.

Despite their logical simplicity, tautomer models for spontaneous mutagenesis have not yet been verified. One major difficulty in determining the biological significance of disfavored base tautomers is that they have not been detected free in solution; their equilibrium frequency has been estimated to be on the order of 10^-6 (4, 5). Based on a comparison of measured with theoretical UV difference spectra from a set of homopolymer-copolymer complexes, Fresco et al. (6) deduced that disfavored base tautomers are involved in A-C and I-U mispairs.

AmPur, a base analogue of adenine, is known to be a strong mutagen in Escherichia coli and bacteriophage T4 (for a review, see Ref. 7) and has recently been shown to be mutagenic in animal cells (8). AmPur stimulates bidirectional transition mutations. A-T → G-C and G-C → A-T. By analogy with tautomeric base-pairing schemes to account for spontaneous transition mutations, in 1959 Freese (9) proposed that AmPur-induced mutagenesis involved an ambiguity in the analogue's base-pairing properties derived from its supposed ability to exist as an imino tautomer more frequently than adenine. In its common amino form, AmPur can form two hydrogen bonds with thymine (Fig. 1a), while in its unfavored imino form (Fig. 1b) or proton-accepting amino form (Fig. 1d), AmPur can form two hydrogen bonds with cytosine. Two other AmPur-C structures (not shown) can also couple via two H-bonds at the 1-3- and 2-2-positions. 1) The imino tautomer of Cyt can pair with the amino tautomer of AmPur, and 2) Cyt instead of AmPur (Fig. 1d) may act as proton acceptor. It should not be possible to form a stable AmPur-C base pair having both AmPur and cytosine in their common tautomeric forms because only a single hydrogen bond is possible (Fig. 1c).

In a previous paper (10), we compared the rate of AmPur-C to A-C mispair formation by purified DNA polymerase. It was shown that AmPur substitution for Ade in a synthetic DNA template results in an increase in dCMP misincorporation. Base stacking strongly influences this increase which is 35-fold when the 5'-nearest neighbor is G-C and 240-fold when the nearest neighbor is A-T. In the complementary experiment where AmPur and Ade were present as deoxyribonucleoside triphosphate substrates, AmPur is incorporated opposite a template Cyt at least 40 times more frequently than Ade. These in vitro measurements strongly support the

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1The abbreviation used is AmPur, 2-aminopurine.
2The imino tautomer of Cyt occurs by the transfer of a proton from the exocyclic amino group located at the 4-position to the 3-position on the pyrimidine ring.
3D. Mhaskar and M. F. Goodman, manuscript in preparation.
Aminopurine pairing of unfavored imino form with cytosine, a result of tautomeric shift of a hydrogen to the N-1-position; c, pairing of the normal amino form with cytosine by means of a single hydrogen bond; d, pairing of 2-aminopurine acting as a proton acceptor with cytosine.

In this paper, we will present direct evidence showing that AmPur-C base pairs in DNA are indeed stabilized by two hydrogen bonds. This observation bears directly on the specific mechanism for mutagenesis by 2-aminopurine and indirectly on the general mechanisms for spontaneous mutagenesis. Although the observation that AmPur-C base pairs are stabilized by two hydrogen bonds does not, by itself, constitute a direct observation or “proof” of the existence of disfavored tautomers in forming base mispairs, we will discuss the possibility that the imino tautomer of 2-aminopurine or cytosine may be present.

EXPERIMENTAL PROCEDURES

Synthetic polynucleotides containing random mixtures of AmPur and Gua, poly[d(AmPur,G)], and AmPur and Ade, poly[d(AmPur,A)], were synthesized using terminal deoxynucleotidyltransferase purified from calf thymus gland (11, 12). Primers used to initiate synthesis were prepared synthetically either as pdC or pdA. Poly(dT) and poly(dC) strands were synthesized to an average length of about 200 nucleotides from initiators of pdT and pdC, respectively.

Analyses of polymer compositions were performed by degrading a portion of each polymer enzymatically to deoxynucleoside monophosphates followed by separation using CsCl reverse phase high performance liquid chromatography and isotopic elution with 0.2 M ammonium phosphate, pH 5.5.

Thermal melting profiles and UV absorption spectra (1-cm path length cells) were run on a spectrophotometer having a temperature controller unit with programmed temperature increase of 0.5 or 2 °C/min beginning at about 2 °C; the melting profiles were similar at the two heating rates. Melting profiles were run under the following conditions: d(AmPur,A) (dT), d(AmPur,A-T), and (dA) (dT) polymers, λ = 260 nm, 0.05 M Na+ concentration (in sodium citrate buffer), pH 7; d(AmPur,G) (dC) polymer, λ = 265 nm, 0.5 M Na+ concentration, pH 10; (dG) (dC) polymer, λ = 263 nm, 20 mM Na+ concentration, pH 7. The alternating copolymer d(AmPur,A-T) was synthesized with Micrococcus luteus DNA polymerase (13) and contained about 1000 nucleotides.

RESULTS

Relating Acidic and Basic Spectroscopic Properties of 2-Aminopurine to a Protonated and Possibly Imino Tautomeric Form of AmPur in DNA—2-Aminopurine has a pH-dependent UV absorption maximum in the wavelength range 303 to 315 nm. This wavelength range is displaced far enough to the red by comparison with the common nucleotides to allow an unambiguous identification of the AmPur peak for a broad set of conditions when AmPur is present along with other bases in a DNA copolymer. In Fig. 2, UV spectra are presented which show a clearly resolved AmPur peak at about 305 nm for synthetic DNA copolymers containing AmPur alone or in random mixtures with guanine and adenine.

A change from basic or neutral to acidic pH is accompanied by a shift in AmPur’s absorption peak from 303 to 313 nm (Fig. 3). Janion and Shugar (14) showed that AmPur in acid is protonated at the 1-position on the purine ring. The pK for protonation at the 1-position is 3.4 for an AmPur monomer (15) and about 4.8 for AmPur in a DNA polymer (14).

The 1-position is precisely the position at which a hydrogen bond is shared with thymine when AmPur is in its common amino form (Fig. 1a); it is also the ring position where AmPur could hydrogen bond to cytosine if the imino form of AmPur (Fig. 1b) or of cytosine2 (not shown) occurred or if the presence of a proton at the 1-position came about by some mechanism other than tautomer formation (Fig. 1d). The key point is that the presence of a proton associated with the central H-binding position on 2-aminopurine gives rise to an absorption maximum at about 313 nm.

2-Aminopurine-Thymine base pairs in DNA—Let us now consider the properties of synthetic DNA polymers containing AmPur alone or randomly interspersed with Ade along a single polynucleotide chain. Single-stranded DNA polymers containing AmPur at neutral or alkaline pH, where the 1-position of AmPur is virtually free of protons, have absorption maxima at 305 nm (Fig. 2). However, when (dAmPur) homopolymers or d(AmPur,A) copolymers are annealed in 1:1 ratios with poly(dT), the AmPur absorption peak shifts to 315 nm (Fig. 4a). Thus, in the double-stranded (dAmPur)-dT and (dAmPur,A)-(dT) copolymers, AmPur acts as a proton acceptor at its 1-position. The acceptance of a proton by AmPur is indeed expected since a proton is available from the 3-position on Thy for sharing in a 1–3 normal Watson-Crick hydrogen bond between 2-aminopurine and thymine (Fig. 1a).

A shift in the AmPur peak for d(AmPur,A) copolymers when going from an annealed to melted out state is shown in Fig. 4a. Spectra for an alternating d(AmPur,A-T) copolymer have been included in Fig. 4b because the presence of two peaks, one at 315 nm in the annealed state and one at 305 nm in the melted state, are clearly visible. Poly(dAmPur,A)-poly(dT) in the annealed state shows a peak at 315 nm (Fig. 4a). In the melted state, a shift of the 315 nm peak toward the blue results in the appearance of a shoulder at about 305 nm. It is important to emphasize that the “protonated” form of AmPur is clearly present when either polymer is in its annealed state.

In thermal melting profiles for the d(AmPur,A)-(dT) polymers, a sharp transition is observed having a melting temperature which is inversely proportional to the amount of AmPur present (Fig. 4c; see also Refs. 15 and 16). In the annealed, melted, and re-annealed states, the AmPur absorption peak shifts from 315 nm to a shoulder at about 305 nm and back to 315 nm, respectively, similar to the data shown in Fig. 4a.

2-Aminopurine-Cytosine Base Pairs in DNA—The spectrum of a mixed random copolymer of AmPur and Gua annealed to poly(dC) shows an absorption peak at 315 nm (Fig. 5a). This spectrum was taken at an alkaline pH. It is the presence of this peak at 315 nm which we believe is direct evidence for the presence of a hydrogen bond shared between
the 1-position of AmPur and the 3-position of Cyt, and may perhaps also serve as a signature of the imino tautomers of 2-aminopurine or cytosine (see "Discussion"). Once the duplex is melted, the absorption peak shifts toward the blue, becoming a shoulder at about 305 nm (Fig. 5a). The 315 nm peak appears once again when the two strands are re-annealed.

For the mixed d(AMpur,G) copolymer in the presence of poly(dC), an absorption peak is no longer apparent at 305 nm in the melted state because of significant absorption from the tail of the (dC) polymer. However, in the presence of a sufficiently large amount of AmPur, the 305 nm peak can be observed in the presence of poly(dC). In Fig. 5b, a peak at 305 nm is present for poly(dAmPur) in the presence of poly(dC) both at low and high temperatures. Thus, in the absence of guanine, AmPur-C base pairs are not stable enough to permit annealing of the (dAmPur) and (dC) polymers. We have verified that the small increase in hyperchromicity associated with an increase in temperature for poly(dAmPur) in the presence of poly(dC) (Fig. 5b) can be attributed entirely to unstacking of the homopolymer (dAmPur) strand (14) since the same hyperchromic shift is observed in the absence of poly(dC).

Evidence that the mixed d(AMpur,G) copolymer anneals to poly(dC) follows from a typical cooperative melting profile (Fig. 5c) where the melting temperature increases with sodium ion concentration (data not shown). By comparison with the sharp transition associated with the poly(dG)-poly(dC) melt, the presence of increasing proportions of AmPur in the mixed d(AMpur,G) polymer causes a significant reduction in the Tm and a decrease in the degree of cooperativity in the melt (Fig.
between 2-aminopurine and cytosine is the amino group located with 2-aminopurine. Thus, it would appear that a possible source of protons available to form a 1–3 hydrogen bond between 2-aminopurine and cytosine is the amino group located at the 2-position of AmPur, i.e., 2-aminopurine tautomerizes to its disfavored imino form when base-paired with cytosine or, alternatively, cytosine could tautomerase to its disfavored imino form to base pair with AmPur.

There are, however, sources other than the exocyclic amino groups on AmPur and Cyt which could act as proton donors. These include the NH lactam at the 1-position of guanine and also the solvent water. One can, with confidence, rule out guanine as a proton source since the 1-proton is itself involved in forming a hydrogen bond with cytosine and would therefore not be available for transfer to AmPur.

One cannot eliminate the possibility that the proton at the 1-position of AmPur originates from water, resulting in a net positive charge on the purine ring and an amino group resident at the 2-position (Fig. 1d). Although the number of protons free in solution in the pH range 8.5 to 10 would be insufficient to donate a proton to AmPur at its 1-position (pK = 4.8 in the polymer form (14)), generation of additional protons from the solvent could occur as a result of base-sacrificing interactions during the formation of a poly(dAmPur,G)–poly(dC) double helix. Although it may be somewhat less likely that a charged base would exist in a hydrophobic base-pairing environment in preference to the electrically neutral imino tautomers of AmPur or Cyt, there is no implied dichotomy concerning the origin of the 1–3 charged proton. An equilibrium exists between the various structures capable of forming one or two hydrogen bonds between AmPur–C base mispairs (Fig. 1). The relative abundance of each of these structures in the AmPur–C population would depend on polynucleotide and surrounding solvent parameters.

The specific question which we have addressed is whether an AmPur–C base mispair contains a 1–3 hydrogen bond. The appearance of an absorption peak at 315 nm (Fig. 5a, solid curve) not at some value intermediate between 305 and 315 nm suggests that almost all the AmPur–C base mispairs share a hydrogen bond at the 1–3 position. We have additional evidence to suggest that the presence of surrounding G–C base pairs is primarily responsible for stabilizing this hydrogen bond. When the amount of AmPur is increased beyond about 75% in a (dAmPur,G) mixed single-stranded polymer, hybridization to poly(dC) can still be observed as a weakly cooperative melting profile, but an absorption peak is no longer present at 315 nm. As the amount of AmPur is increased relative to Gua, the location of the peak moves to-

**Fig. 5.** Spectra and corresponding thermal melting profiles of DNA polymers containing random mixtures of 2-aminopurine and guanine mixed with an equimolar concentration of homopolymer containing cytosine. a, 40% double-stranded polymer poly[d(AmPur)68%, G 32%]–poly(dC). Temperature: 4°C (—) and 70°C (—). b, 45 μM poly[d(AmPur)68%, G 32%]–poly(dC). Temperature: 4°C (——) and 70°C (——). c, melting profiles for 58 μM poly(dG)–poly(dC) (——), 25 μM poly[d(AmPur)54%, G 46%]–poly(dC) (——), 40 μM poly[d(AmPur)68%, G 32%]–poly(dC) (——), and 63 μM poly(dAmPur) or 63 μM poly[d(AmPur)–poly(dC)] (——). The single-stranded polymers contain an average of 200 nucleotides. The melting profiles for the polymers containing AmPur were run at λ = 260 nm, 0.5 M Na+ concentration, pH 10. The profile of the (dG)–(dC) polymer was run at λ = 256 nm, pH 7, 20 mM Na+ concentration (at 0.5 M Na+ concentration, Tm of polymer (dG)–(dC) is above 100°C).
wards 305 nm, characteristic of the non-hydrogen bonded (nonprotonated) form of poly(dAmPur) (Fig. 2). In the absence of guanine, poly(dAmPur) and poly(dC) do not appear to anneal (Fig. 5c), and an absorption peak is again observed at 305 nm (Fig. 5b).

The original Watson and Crick hypothesis that tautomers are involved in base transition mutagenesis (1, 2) followed from the supposition that two hydrogen bonds are required to stabilize A-C and G-T normal base mispairs. To explain the mutagenic properties of 2-aminopurine, Freese (9) invoked a similar two H bond requirement for AmPur-C base mispairs. We believe that our data provide a direct measurement showing that base mispairs in DNA can be stabilized by two hydrogen bonds. The precise origin of the 1–3 hydrogen-bonding proton remains an open question.

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