Biophysical Chemistry, 46 (1993) 207–215
Elsevier Science Publishers B.V., Amsterdam

BIOCHE 01739

Mechanism of hydroxylamine mutagenesis: Role of tautomerism, conformation and proton exchange on base pairing between the promutagen N^6-methoxyadenosine and uridine

Borys Kierdaszuk [a], Charlotta Johansson [b], Torbjorn Drakenberg [b], Ryszard Stolarski [a] and David Shugar [a,c,*]

[a] *Department of Biophysics, Institute of Experimental Physics, University of Warsaw, 93 Zwirki and Wigury St., PL-02089 Warszawa (Poland)*
[b] *Department of Physical Chemistry II, Chemical Center, University of Lund, P.O. Box 124, S-22100 Lund (Sweden)*
[c] *Institute of Biochemistry and Biophysics, Academy of Sciences, 36 Rakowiecka St., PL-02532 Warszawa (Poland)*

(Received 8 July 1992; accepted in revised form 4 November 1992)

Abstract

A study of the interaction of N^6-methoxyadenosine (OMe^6A, which exists as an equilibrium mixture of amino and imino tautomers) with the potentially complementary uridine in non-aqueous medium demonstrated that only the tautomer *amino*-OMe^6A base pairs with uridine. The association constant, determined by ^1H NMR spectroscopy, was 101 M^{-1} at +30°C, an order of magnitude greater than those for autoassociation of *amino*-OMe^6A or uridine. Base pair formation between *amino*-OMe^6A and uridine led to a shift of the amino–imino equilibrium of 10% in favour of the *amino* species which associates with uridine, with the concomitant decrease in population of the imino form. Base pairing was accompanied by intermolecular proton exchange between the N^6-H of *amino*-OMe^6A and the N(3)-H of uridine. The rate constants for these exchanges, as well as for tautomeric exchange of OMe^6A in the presence of uridine, were measured by means of the saturation transfer technique. The mechanism of proton exchange is compared with, and shown to be different from, that previously observed for base pair formation between the imino tautomer of OMe^6A and cytidine (Stolarski et al., *Biochemistry* 26 (1987), 4332 also linked with tautomeric exchange. The resulting proposed schemes of base pairing are dependent on the conformation of the exocyclic N^6-methoxy group, and are compared and discussed with reference to published data on base pairing at the oligonucleotide duplex level, as well as known data on base pairing of the analogous promutagen N^4-methoxycytidine. The overall findings further contribute to our understanding of the dual functionality of the promutagen OMe^6A at the molecular level, and furthermore, provide an excellent model system for studying the role of tautomerism on proton exchange in base pairs.

Keywords: Hydroxylamine mutagenesis; N^6-Methoxyadenosine; Base pairing; Tautomeric equilibrium; Conformation; Proton exchange

* Correspondence to this author at the University of Warsaw.

1. Introduction

The N^6-hydroxy and N^6-methoxy derivatives of adenosine, products of the reaction of adenosine with hydroxylamine (NH_2OH) and methoxyamine (NH_2OCH_3), respectively, are promutagens in a variety of prokaryotic and eukaryotic systems [1]. Their promutagenic properties are also reflected by their behaviour in *in vitro* replication and transcription systems with RNA and DNA polymerases [2,3], where they may behave like A or G, i.e. they possess dual functionality.

It was previously shown that the dual functional properties of OMe^6A derive from the fact that it exists as an equilibrium mixture of amino and imino tautomers in aqueous and low-polar aprotic media ($CHCl_3$, CCl_4). The tautomeric equilibrium depends strongly on solvent, as well as on planar hydrogen-bonded association (base pairing) with potentially complementary nucleosides such as cytidine and uridine [4–7]. Both tautomeric forms may be observed by optical [4] and NMR [5–7] spectroscopy because of the high barrier to interconversion (~ 80 kJ mol^{-1}) between the two forms. The appropriate thermodynamic parameters have been calculated from the solvent-, concentration- and temperature-dependence of the equilibrium. Both tautomeric species have also been observed in solution for a series of 9-substituted N^6-methoxyadenines, with the aid of optical and NMR spectroscopy [8].

Cytidine forms a planar associate only with the *imino*-OMe^6A, leading to a shift in the apparent amino/imino equilibrium in favour of this form, accompanied by exchange of a proton between the amino group of cytidine and the N^6-H of the *amino*-OMe^6A [7] coupled with tautomeric exchange. As previously pointed out [7], the conformation of the exocyclic N^6-methoxy group about the C(6)–N^6 bond is of fundamental importance in determining the structure of this complex, further discussed below (see Discussion).

Interaction of OMe^6A with cytidine accelerates tautomeric exchange, at a rate dependent on the concentrations of the two components, in chloroform solution. It was additionally noted that uridine forms base pairs only with the *amino*-OMe^6A, leading to an increase in population of this form and a corresponding decrease in population of the imino form.

We have now extended the foregoing observations to further confirm base pairing of uridine with the *amino*-OMe^6A, and show that this is also accompanied by proton exchange between the uridine N(3)-H and the N^6-H of the *amino*-OMe^6A, along with acceleration of tautomeric exchange.

2. Materials and methods

The 2′,3′,5′-tri-*O*-methyl derivatives of N^6-methoxyadenosine [4] and uridine [9], 1-cyclohexyluracil [10] and the 2′,3′,5′-tri-*O*-ethyl derivative of cytidine [9] were synthesized as described. The sole purpose of alkylation of sugar hydroxyls was to enhance solubility in apolar solvents, and will not be further discussed in this text.

^1H NMR spectra for compounds in C^2HCl_3 (Merck, Darmstadt, GFR, 99.5% mol ^2H, dried over A4 molecular sieves) were recorded on Brucker 270 AM and Nicolet 360 WB spectrometers, relative to internal Me_4Si to an accuracy of ± 0.005 ppm. Saturation transfer and T_1 measurements were performed as previously described [7]. The auto- and heteroassociation constants for OMe^6A and U, and an equimolar mixture of the two in chloroform solution, were calculated according to the procedure of Iwahashi and Kyogoku [11] with some modifications [7].

Abbreviations: OH^6A, N^6-hydroxy-2′,3′,5′-tri-*O*-methyladenosine; OMe^6A, N^6-methoxy-2′,3′,5′-tri-*O*-methyladenosine; OH^4C, N^4-hydroxycytidine; OMe^4C, N^4-methoxycytidine; U, 2′,3′,5′-tri-*O*-methyluridine; C, 2′,3′,5′-tri-*O*-ethylcytidine; c_1U, 1-cyclohexyluracil; *amino*-OMe^6A and *imino*-OMe^6A, the amino and imino tautomers of OMe^6A; *amino*-OMe^4C and *imino*-OMe^4C, the amino and imino tautomers of OMe^4C; $(Me)_4Si$, tetramethylsilane.

Rate constants for proton transfer in $OMe^6A:c_1U$ associates were determined from measurements of saturation transfer and of relaxation times T_1 [7] according to the theoretical treatment of Perrin and Johnson [12].

3. Results

3.1. Auto- and heteroassociation constants of OMe^6A and U

Previous qualitative observations demonstrated that addition of potentially complementary uridine to a chloroform solution of OMe^6A led to characteristic changes of the ^{13}C chemical shifts of the heterocyclic ring carbons, as well as of exchangeable N–H protons involved as donors in hydrogen bonding [6]. The resulting inference that only the amino form of OMe^6A base pairs with uridine, prompted the present more detailed investigation of this phenomenon.

The chemical shifts of N–H type protons in U, OMe^6A, and an equimolar solution of the two in chloroform depends on the concentrations of the individual components (Fig. 1), testifying to formation of hydrogen-bonded auto- and hetero-associates in which these are proton donors. An increase in concentration and/or decrease in temperature leads to an increase in the level of auto- and/or hetero-associates in an equimolar solution of U and OMe^6A, reflected in downfield shifts of the signals derived from the N–H protons involved in hydrogen bonding. This more directly confirms the previously proposed scheme for planar hetero-associates between U and *amino*-OMe^6A (Scheme 1) [7], taking into account the two rotamers, *anti* and *syn*, of the exocyclic N^6-OCH_3 relative to the adenine ring N(1). The *syn* rotamer is known to be predominant in solution [4,6] and only the *syn-imino* form is observed in the solid state for OMe^6A [13], as well as for 9-benzyl-N^6-methoxyadenine [14].

The concentration-dependence of the chemical shifts of N–H type protons permits calculations of auto-association constants of U and the individual tautomeric forms of OMe^6A, and hetero-association constants of U with each of the

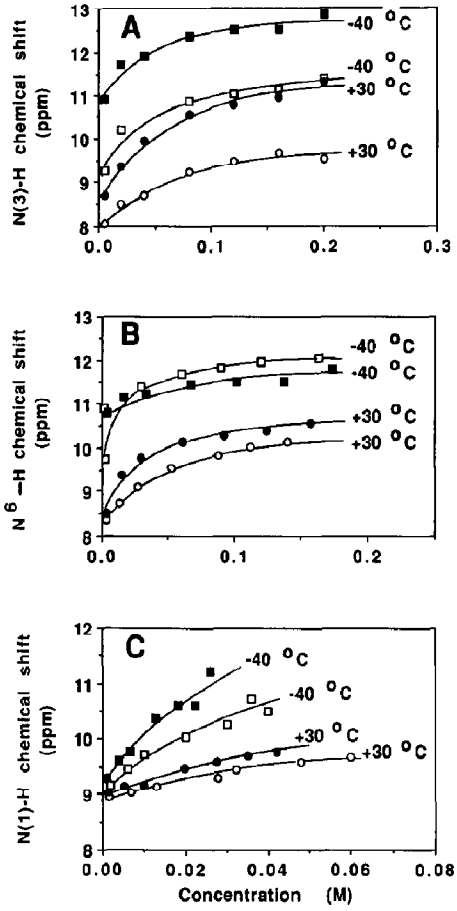
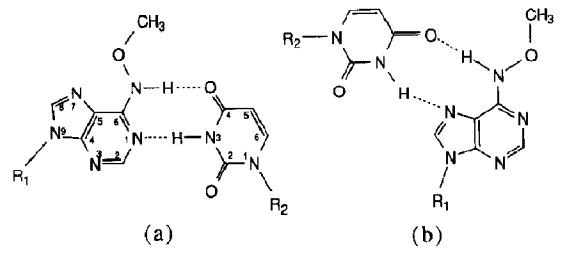


Fig. 1. Concentration-dependence, in chloroform solution, of the ^1H chemical shifts of: (A) the N(3)-H of U at −40°C (□) and +30°C (○), and its 1:1 mixture with OMe^6A at −40°C (■) and +30°C (●); (B) the N^6-H of *amino*-OMe^6A at −40°C (□) and +30°C (○), and its 1:1 mixture with U at −40°C (■) and +30°C (●); (C) the N(1)-H of *imino*-OMe^6A at −40°C (□) and +30°C (○), and its 1:1 mixture with U at −40°C (■) and +30°C (●). Note: Continuous lines denote the computer-fitted theoretical curves. In (A), concentrations refer to each of the components. In (B) and (C), concentrations refer to the amino and imino forms of OMe^6A.

OMe^6A tautomers, as earlier reported for C and OMe^6A, and an equimolar solution of the two [7]. As in the previous study, the calculated hetero-association constant values are apparent, since the proposed model does not take into account all possible planar associates, e.g. hetero-associates between the two tautomeric forms of OMe^6A. The calculated association constants hence account for the overall participation of a given



(a) (b)

Scheme 1. Proposed structures of hydrogen-bonded planar heteroassociates of uridine and the amino tautomer of OMe^{6}A with the N^6-OMe group of the latter in the *anti* (a) or *syn* (b) conformation relative to the adenine N(1). R_1 = 2′,3′,5′-tri-*O*-methylribose, R_2 = cyclohexyl or 2′,3′,5′-tri-*O*-methylribose (to increase solubility in non-aqueous media).

proton in hydrogen bonding, on the assumption that 1 : 1 complexes predominate (see also below).

The data in Table 1 show preferential hetero-association at 37°C of U with *amino*-OMe^{6}A (association constant $101 \pm 4\ M^{-1}$) relative to auto-association of this tautomeric species ($8.7 \pm 1.3\ M^{-1}$). This correlation is similar to that previously noted for association of C with *imino*-OMe^{6}A [7], and the frequently described preferential hetero-associates A : U and G : C relative to auto-associates of each component [15].

A more comprehensive analysis is required to interpret the effect of addition of uridine to a chloroform solution of OMe^{6}A at −40°C on the chemical shifts of the uridine N(3)-H and the N^6-H and N(1)-H of the two OMe^{6}A tautomers. At this temperature the self-association constants of U, *amino*-OMe^{6}A and *imino*-OMe^{6}A are, respectively, 22-, 10- and 4-fold higher than at +30°C (Table 1). The hetero-association constant, calculated from the concentration-dependent chemical shift of the uridine N(3)-H, is not affected on addition of OMe^{6}A, although the N(3)-H undergoes a very marked downfield shift, as in the case of typical hydrogen bonds. In contrast to the situation at +30°C, the changes in chemical shifts of the N^6-H and N(1)-H of the OMe^{6}A tautomers are non-typical. The apparent association constant, calculated from the concentration-dependence of the chemical shift of N^6-H, is unaffected following addition of U, while the association constant derived from the N(1)-H increases about two-fold. It should also be noted that, simultaneously, the N^6-H signal undergoes an upfield shift, and not the downfield shift typical for hydrogen bonding. On the other hand, the N(1)-H signal is shifted downfield despite the absence of base pairing between *imino*-OMe^{6}A and U at +30°C (Fig. 1C, see also ref. [6]).

The foregoing underlines the more complex nature of the interaction of OMe^{6}A with U at −40°C, relative to that at +30°C. Because of the high auto-association constants of U and *amino*-OMe^{6}A at −40°C, one does not observe changes in the chemical shift of N^6-H typical for hetero-association (Fig. 1). By contrast, the changes in chemical shift of N(1)-H result from the decrease in the population of *imino*-OMe^{6}A due to hetero-association of *amino*-OMe^{6}A with U (Table 2), base pairing between the amino and imino tautomers of OMe^{6}A, and the strong auto-association of *amino*-OMe^{6}A, all of which are appre-

Table 1

Apparent association constants for auto-associates of U and OMe^{6}A and heteroassociates between them derived from the concentration-dependence of the chemical shifts of indicated protons. Accuracies (in brackets) were obtained from non-linear regression analysis of the data

Computed	Proton	Self-association constants (M^{-1})		Hetero-association constants (M^{-1}) of OMe^{6}A with U	
		−40°C	+30°C	−40°C	+30°C
U	N(3)-H	116 (6)	5.6 (0.2)	94 (6)	17.3 (0.4)
amino-OMe^{6}A	N^6-H	93 (7)	8.7 (1.3)	70 (4)	101 (4)
imino-OMe^{6}A	N(1)-H	13 (6)	3.0 (1.2)	32 (6)	7.5 (1.4)

Table 2

Percentage populations of amino (P_a) and imino (P_i) tautomers of N^6-methoxyadenosine in choroform solution at two concentrations and temperatures, and percentage populations following addition of an equimolar amount of the potentially complementary uridine [a]

Concentration (M)	−40°C				+30°C			
	free		mixt.		free		mixt.	
	P_a	P_i	P_a	P_i	P_a	P_i	P_a	P_i
0.04	75	25	83	17	67	33	75	25
0.20	82	18	87	13	70	30	79	21

[a] Population values accurate to 2%.

ciably enhanced when the temperature is reduced from +30°C to −40°C (Table 1). Bearing in mind the differences in concentrations of *imino*-OMe^{6}A in the presence and absence of U (Table 2), the appropriate curves representing the dependence of the chemical shift of N(1)-H on the concentration of *imino*-OMe^{6}A (Fig. 1C) are displaced with respect to each other.

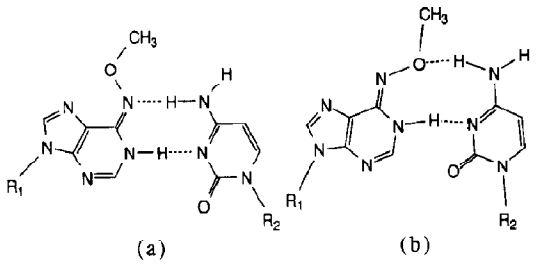
An additional effect accounting for the non-typical chemical shifts of N–H protons is formation of associates of more than two molecules, which may lead to involvement of *imino*-OMe^{6}A in association with U. Formation of higher associates will clearly be much more pronounced at −40°C relative to +30°C.

3.2. Effect of base pairing on tautomeric equilibrium of OMe^{6}A

As pointed out above, selective association of cytidine with only *imino*-OMe^{6}A is accompanied by a marked shift in the tautomeric equilibrium towards this form [7]. Under conditions favoring association, i.e. high concentration and/or low temperature, the population of the imino form increased from 56 to 82%. It was also noted that uridine base pairs preferentially only with *amino*-OMe^{6}A in an equimolar solution at 30°C [5,6]. We have now followed this interaction quantitatively at −40°C (Table 2), and find an up to 10% absolute increase in population of the amino form, with a concomitant equal decrease in population of the imino form.

3.3. Proton exchange in planar associates of OMe^{6}A with c_1U

In equimolar mixtures of OMe^{6}A and c_1U there is a characteristic exchange of protons similar to that previously noted in a 1:1 mixture of OMe^{6}A and C [7]. With the latter, exchange occurs between protons of the cytidine NH_2 and the N^6-H of *amino*-OMe^{6}A, which does not form planar associates with C (Scheme 2b). By contrast, in a 1:1 mixture of OMe^{6}A and c_1U (or U) exchange proceeds between the uracil N(3)-H and the N^6-H of the *amino*-OMe^{6}A, which associates directly with c_1U. The temperature-dependent first-order



Scheme 2. Proposed structure of the hydrogen-bonded planar heteroassociates of cytidine with the imino tautomer of OMe^{6}A with the N^6-OMe group of the latter in the *anti* (a) or *syn* (b) conformation relative to the adenine N(1). R_1 = 2′,3′,5′-tri-*O*-methylribose, R_2 = 2′,3′,5′-tri-*O*-ethylribose (to increase solubility in non-aqueous media).

rate constants for exchange, k_{AU} and k_{UA} (Table 3), range from ~1 s^{-1} at −20°C to ~10 s^{-1} at +30°C. Proton exchange between the c_1U N(3)-H

Table 3
First-order rate ronstants, k_{XY}, for exchange process of the type X ⇌ Y derived from measurements of saturation transfer and relaxation times T_1:X and Y = A (*amino*-OMe^{6}A), I (*imino*-OMe^{6}A), and c_1U. Errors of rate constants, if not specified, are ±20%

k_{XY} (s^{-1})	Analogue and condition of measurement			
	OMe^{6}A (0.04 *M*)	OMe^{6}A+c_1U (0.04 *M* each)	OMe^{6}A (0.2 *M*)	OMe^{6}A+c_1U (0.2 *M* each)
At +30°C				
k_{AI}	0.20	1.9	0.90	1.4
k_{IA}	0.38	[a]	1.70	5.0 [b]
k_{AU}		[c]		11.4 [b]
k_{UA}		[c]		12.8 [b]
k_{UI}		[c]		2.8 [b]
k_{IU}		[c]		3.1 [b]
At −20°C				
k_{AI}	10^{-4} [d]	0.1	10^{-4} [d]	0.1
k_{IA}	10^{-4} [d]	[a]	10^{-4} [d]	[a]
k_{AU}		1.3		2.0 [b]
k_{UA}		1.5		1.7
k_{UI}		0.2 [e]		0.2 [e]
k_{IU}		0.2 [e]		0.2 [e]

[a] Not determined because of inaccuracy of measurements of intensity changes in the case of weak *imino*-OMe^{6}A signals.
[b] Because of signal broadening, these values are of low accuracy (±50%).
[c] Not measureable because of overlapping *amino*-OMe^{6}A N^6H and c_1U N(1)H signals.
[d] Values estimated from the Eyring equation and the known value (80 kJ mol^{-1}) of the tautomeric exchange barrier.
[e] Approximate values, due to the low accuracy of T_1 and/or intensity measurements.

and the *imino*-OMe^{6}A N(1)-H is a second-order reaction with rate constants, k_{UI} and k_{IU}, five- to ten-fold lower than k_{UA} and k_{AU}, due to simultaneous exchange between the two tautomers of OMe^{6}A. The rate constants for the tautomeric exchange *amino* $\rightleftharpoons$ *imino*, k_{IA} and k_{AI}, are comparable to the values of k_{IU} and k_{UI}.

The rates for the tautomeric exchange *amino* $\rightleftharpoons$ *imino* at -20°C are increased by three orders of magnitude in the presence of c_1U (Table 3) as a result of the following process:

$$c_1\text{U-N(3)H } ^{*} + \textit{amino}\text{-OMe}^6\text{A-N}^6\text{H } ^{**} \rightleftharpoons c_1\text{U-N(3)H } ^{**} + \textit{amino}\text{-OMe}^6\text{A-N}^6\text{H } ^{*}$$

This effect is, however, less pronounced than for the base pair *imino*-OMe^{6}A : C [7], where the rate constants for the tautomeric exchange *amino* $\rightleftharpoons$ *imino* attain values comparable to those for proton exchange, as follows:

$$\text{C-HNH } ^{**} + \textit{amino}\text{-OMe}^6\text{A-N}^6\text{H } ^{*} \rightleftharpoons \text{C-HNH } ^{*} + \textit{amino}\text{-OMe}^6\text{A-N}^6\text{H } ^{**},$$

with a value $\sim 0.3\ s^{-1}$ at -40°C. In this instance tautomeric exchange dictates proton exchange between the cytidine NH_2 and the *amino*-OMe^{6}A N^6H.

4. Discussion

The present results on the tautomerism, accompanying proton exchange, and specific interaction (base pairing) of *amino*-OMe^{6}A with uridine, both confirm, and further extend, previous findings on the specific interaction of *imino*-OMe^{6}A with cytidine.

4.1. Relation between base pairing and tautomeric equilibrium

OMe^{6}A is clearly one of a limited number of nucleoside analogues which, in striking contrast to the natural nucleosides found in nucleic acids, exhibits appreciable tautomerism in solution at room temperature. Furthermore, the amino–imino tautomeric equilibrium of OMe^{6}A is dependent on the nature of the solvent, and is also modified on interaction with potentially complementary nucleoside [5,7].

Additional examples of nucleoside analogues which display pronounced tautomerism in solution at room temperature include, e.g. the natural nucleoside antibiotics formycins A and B [17], which exhibit prototropic tautomerism; and the naturally occurring isoguanosine with keto–enol tautomerism [18]. But none of these are found in nucleic acids. Isoguanosine, initially isolated from croton seeds, was subsequently shown to be a significant constituent of some marine organisms [19,20], but not of their nucleic acids, obviously because their presence would result in marked degeneracy of the genetic code [21].

By contrast, OMe^{6}A, like OMe^{4}C [22,23], is "incorporated" into nucleic acids by reaction with the potent mutagen methoxyamine (NH_2OCH_3), which converts cytosine residues to OMe^{4}C, and adenosine residues to OMe^{6}A. The corresponding OH^{6}A, and OH^{4}C, are introduced into nucleic acids by reaction with hydroxylamine (NH_2OH).

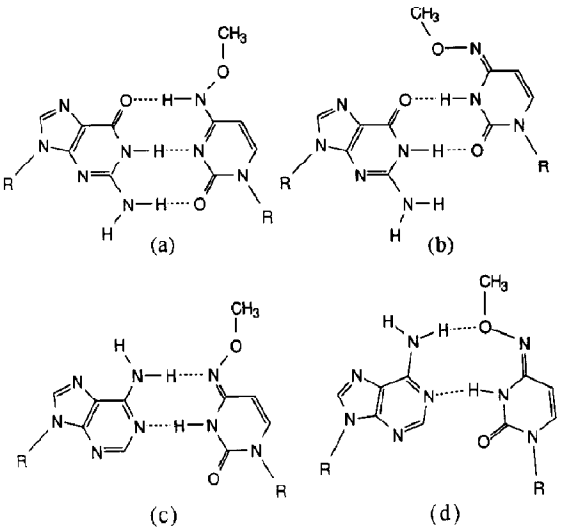
The observed significant tautomerism of OMe^{6}A should be reflected by the behaviour of such residues in replication and transcription. It was, in fact, demonstrated by Singer and Spengler [2] that *in vitro* transcription on a template containing OMe^{6}A residues leads to incorporation at sites occupied by this analogue, of both U (90%) and C (10%). And Thabit Abdul-Masih and Bessman [3] found that OH^{6}dATP exhibits similar dual functionality in base pairing, in that it could replace both dATP and dGTP in a DNA replicating system. It was further shown, using five DNA polymerases from various sources, that each enzyme distinguishes OH^{6}dATP from the canonical dATP and dGTP to differing extents.

Furthermore, during the course of this study, Nishio et al. [24,25] prepared the synthetic oligomer d(CCTGGTA*X*CAGGTCC), with *X* = OMe^{6}A or OH^{6}A. Using these as templates with the Klenow DNA polymerase, they found that both C and T, but not G or A, were incorporated at the site opposite OMe^{6}A or OH^{6}A. In addition, helical complexes of the foregoing oligomers with a complementary chain containing various base residues opposite the OMe^{6}A, or OH^{6}A,

demonstrated that the duplexes containing the base pairs $OMe^6A:C$ and $OMe^6A:T$, were more stable than a duplex containing a mismatched A:C pair.

Furthermore, although duplexes prepared with the base pairs $OMe^6A:A$ and $OMe^6A:G$ were more stable than those containing $OMe^6A:C$ and $OMe^6A:T$, neither A or G were incorporated by the polymerase in opposition to OMe^6A (see above). It follows that the type of base pairing, and not the stability of the base pairs (due most likely to stacking interactions), is dictated by the polymerase.

An analogous situation prevails with the corresponding N^4-methoxy (or hydroxy) cytidine. OH^4C may base pair with A or G, a property long ago utilized in the first-reported demonstration of site-directed mutagenesis in the Qβ replicase system [26]. The dual functionality of OH^4C was interpreted as due to its existence in aqueous medium as a mixture of the imino (90%) and amino (10%) forms, calculated by means of the basicity method [27]. Experimentally only the imino form is observed in the crystal structure [28,29], in non-polar solvents [30], and in the crystal structure of oligodeoxynucleotides [31]. Hence, whereas *amino*-OMe^4C may pair with G, as shown in Scheme 3a, the observed pairing of *imino*-OMe^4C with G in the crystal [31] is of the wobble type (Scheme 3b).



Scheme 3. Proposed structures of hydrogen-bonded planar heteroassociates of guanosine (a, b), and adenosine (c, d) and the amino (a) or imino (b–d) tautomer of OMe^4C with the N^4-OMe group of the latter in the *anti* (a, c) and *syn* (b, d) conformation relative to the cytosine N(3). R = 2′-deoxyribose.

4.2. Role of conformation of N^6-methoxy group

Whereas NMR data point to the conformation *anti* of the methoxy group in the complex (Scheme 1a) of *amino*-OMe^6A with uridine [6], only the *syn* conformation is observed for *amino*-OMe^6A in the solid state [13] and its complex with cytidine in solution [6]. This indicates that the *imino*-$OMe^6A:C$ associate is as in Scheme 2b. Whether this holds at the oligonucleotide level remains to be resolved by X-ray crystallography or NMR.

Considerably more information is available for the analogous OMe^4C. As in the case of OMe^6A, the N^4-methoxy group in an oligonucleotide duplex adopts the conformation *syn* in the solid state, as shown in Scheme 3b [31]. The *anti* conformation proposed by Morozov et al. [32] was based in large part on a misinterpretation of the crystallographic data for OH^4C [28,29].

One of the possible structures for the base pair $OMe^4C:A$ in DNA is shown in Scheme 3d, analogous to that proposed for $OMe^6A:C$ in Scheme 2b. An NMR study of the octamer $[d(CGAATOMe^4CCG)]_2$ led to the proposal that the base pair $OMe^4C:A$ is as in Scheme 3c with the methoxy group in the *anti* conformation [33]. This proposal is based solely on a *qualitative* analysis of the NOE effect, and differs from the experimentally observed $OMe^4C:G$ pair in the same oligonucleotide in the crystal (Scheme 3b), where the conformation is *syn* [31]. Furthermore, in accord with the known energetic preference for the conformation *syn* in OMe^4C, Stone et al. [33] noted the presence of helical or partially helical structures, in equilibrium with the predominant duplex structure, and in which the methoxy group is in the *syn* conformation, leading to the postulate that $OMe^4C:A$ pairing is of the wobble type. However, an alternative equally plausible pair is that shown in Scheme 3d, analogous to that proposed by us for the pair $OMe^6A:C$ (Scheme 2b).

4.3. Proton exchange in base pairs

The preferential association of *imino*-OMe^{6}A only with cytidine, and of *amino*-OMe^{6}A only with uridine, is in each case accompanied by a marked shift in the tautomeric equilibrium in favour of these tautomeric species.

By contrast, the accompanying observed proton exchanges in the two types of hetero-associates proceed by quite different pathways. In the *imino*-OMe^{6}A:C base pair, proton exchange occures between a cytosine *amino* proton and the N^6-H of the tautomer *amino*-OMe^{6}A, which does not associate with cytidine, and is coupled with proton exchange between the *imino*- and *amino*-tautomers of OMe^{6}A. In the hetero-associate of *amino*-OMe^{6}A with uridine, exchange proceeds directly between the two associated bases, i.e. between the N^6-H of *amino*-OMe^{6}A and the N(3)-H of the uracil ring. Hence, tautomeric exchange, while accentuated by base pairing, apparently does not dictate the pathway for proton exchange.

The process of exchange must necessarily proceed *via* some intermediary state. In a study of proton exchange in the base pairs G:C and A:T in non-aqueous medium, Iwahashi and Kyogoku [34] considered two possible intermediates: (a) the rare enol and imino forms, resulting from tunneling transfer of two protons in the complementary base pair, and (b) protonated and ionized forms as a result of the transfer of a single proton in two steps. Their observed rates of proton exchange are comparable to those found in the present investigation. It should, however, be noted that the exchange process in our system is the first to demonstrate directly coupling between intermolecular proton exchange with tautomerism, as well as the possibility of different mechanisms of exchange (and different intermediate forms) with different base pairs.

Proton exchange in base pairs at the level of oligo- and polynucleotides has received little attention, in part because of difficulties associated with parallel exchange of such protons with water, resulting from the known "breathing" of a duplex. Ruterjans et al. [35] followed the imino proton resonances of ^{15}N-labeled tRNA, which showed up as asymmetric doublets, and initially interpreted this as due to exchange by tunneling, of the type N–H $\cdots$ N $\rightleftharpoons$ N $\cdots$ H–N, i.e. via formation of intermediate rare enol and imino tautomers in the base pairs. This led to the conclusion that 10% of the bases were present as rare tautomers, a highly improbable result. An alternative, and more probable interpretation, is anisotropy of the proton chemical shifts [35,36], which should be more pronounced at higher field strengths, as actually observed by the authors.

The existence of rare tautomeric forms of the bases found in nucleic acids, since they were advanced by Watson and Crick as a possible source of spontaneous point mutations, has remained controversial. Although there have been recent reports on the existence of the enol form of 9-methylguanine, and the imino form of 1-methylcytosine, in low-temperature matrices [37,38], there is very extensive evidence that the natural bases, in solution and in the solid state, and in synthetic and natural oligo- and polynucleotides, exist as the keto and amino species (for recent review, see ref. [39]). OMe^{6}A, which exists in solution as an equilibrium mixture of amino and imino tautomers with comparable populations, and each of which base pairs with a different complementary base, provides for the first time a suitable model system for more detailed investigations along these lines.

Acknowledgments

This work was supported by grants from the KBN through the Polish Ministry of National Education (Projects GR-144 and BST-61).

References

1 E.I. Budowsky, E.D. Sverdlov, T.N. Spasokukotskaya and J. Kudelka, Biochim. Biophys. Acta 390 (1981) 1–12.
2 B. Singer and S. Spengler, FEBS Lett. 139 (1982) 69–75.
3 M. Thabit Abdul-Masih and M.J. Bessman, J. Biol. Chem. 261 (1986) 2020–2026.
4 B. Kierdaszuk, R. Stolarski and D. Shugar, Acta Biochim. Polon. 27 (1984) 57–65.

5 B. Kierdaszuk, R. Stolarski and D. Shugar, FEBS Lett. 158 (1983) 128–130.
6 R. Stolarski, B. Kierdaszuk, C.-E. Hagberg and D. Shugar, Biochemistry 23 (1984) 2906–2913.
7 R. Stolarski, B. Kierdaszuk, C.-E. Hagberg and D. Shugar, Biochemistry 26 (1987) 4332–4337.
8 T. Fujii, T. Saito, T. Date, T. Itaya, T. K. Kizu, Y. Kumazawa and S. Kakajima, Chem. Pharm. Bull. 35 (1987) 4482–4493.
9 J.T. Kuśmierek, J. Giziewicz, J. and D. Shugar, Biochemistry 12 (1973) 194–199.
10 J.T. Kuśmierek and D. Shugar, Acta Biochim. Polon. 17 (1970) 259–266.
11 H. Iwahashi, H. and Y. Kyogoku, J. Am. Chem. Soc. 99 (1977) 7761–7765.
12 C.L. Perrin and E.R. Johnston, J. Magn. Reson., 33 (1979) 619–626.
13 G.I. Birnbaum, B. Kierdaszuk and D. Shugar, Nucleic Acids Res. 12 (1984) 2447–2460.
14 T. Fujii, T. Saito, T. Date and Y. Nishibata, Chem. Pharm. Bull. 38 (1990) 912–916.
15 S.B. Petersen and J.J. Led, J. Am. Chem. Soc. 103 (1981) 5308–5313.
16 N.G. Williams, L.D. Williams and B.R. Shaw, J. Am. Chem. Soc. 111 (1989) 7205–7209.
17 J. Wierzchowski and D. Shugar, Photochem. Photobiol. 35 (1982) 445.
18 J. Sepiol, Z. Kazimierczuk and D. Shugar, Z. Naturforsch. 31c (1974) 361–370.
19 F.A. Fuhrman, G.J. Fuhrman, Y.H. Kim, L.A. Pavelka and H.S. Mosher, Science, 207 (1980) 193–195.
20 F.A. Fuhrman, G.J. Fuhrman, R.J. Nachman and H.S. Mosher, Science, 212 (1981) 557–558.
21 D. Shugar and B. Kierdaszuk, Proc. Int. Symp. Biomol. Struct. Interact. Suppl. J. Biosci. 8 (1985) 657–668.
22 E.I. Budowsky, Prog. Nucleic Acids Res. Mol. Biol. 16 (1976) 125–188.
23 F. Marfey and E. Robinson, Mutat. Res. 86 (1981) 155–191.
24 H. Nishio, A. Ono, A. Matsuda, A. and T. Ueda, Nucleic Acids Res. 20 (1992) 777–782.
25 H. Nishio, A. Ono, A. Matsuda and T. Ueda, Chem. Pharm. Bull. in press.
26 R.A. Flavell, D.L. Sabo, E.F. Bandle, E.F. and C. Weissmann, J. Mol. Biol. 89 (1974) 255–262.
27 D.M. Brown, M.J.E. Hewlins and P. Schell J. Chem. Soc. C (1968) 1925–1929.
28 D. Shugar, C.P. Huber and G.l. Birnbaum, Biochim. Biophys. Acta 447 (1976) 274–284.
29 G.I. Birnbaum, T. Kulikowski and D. Shugar, D. Can. J. Biochem. 57 (1979) 308.
30 B. Kierdaszuk, R. Stolarski and D. Shugar, Eur. J. Biochem. 130 (1983) 559–464.
31 L. Van Meervelt, M.H. Moore, P. Kong Thoo Lin, D.M. Brown and O. Kenard, J. Mol. Biol. 216 (1990) 773–781.
32 Y.V. Morozov, F.A. Savin, V.O. Chekhov, E.I. Budowsky and D.Y. Yakovlev, J. Photochem. 20 (1982) 229–252.
33 M.J. Stone, A.N.R. Nedderman, D.M. Williams, P. Kong Thoo Lin and D.M. Brown, J. Mol. Biol. 222 (1991) 711–723.
34 H. Iwahashi and Y. Kyogoku, J. Am. Chem. Soc. 102 (1980) 2913–2917.
35 H. Ruterjans, E. Kaun, W.E. Hull and H.H. Limbach, Nucleic Acids Res. 10 (1982) 7027–7039.
36 C. Dalvit, J. Magn. Reson. 97 (1992) 645–650.
37 K. Szczepaniak, M. Szczęśniak and W.B. Person, Chem. Phys. Lett. 153 (1988) 39–43.
38 M. Szczęśniak, J. Leszczynski and W.B. Person, J. Am. Chem. Soc. 114 (1992) 2731–2733.
39 D. Shugar and A. Psoda, in: Landolt–Bornstein New Series—Biophysics, ed. W. Saenger, Nucleic Acids, vol. 1 (Springer-Verlag, Berlin, 1990) pp. 308–348.