Nucleic Acids Research, 1992, Vol. 20, No. 24 ***6455–6460***

Solution conformation of an oligonucleotide containing a urea deoxyribose residue in front of a thymine

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Received November 3, 1992; Revised and Accepted November 26, 1992

ABSTRACT

Urea residues are produced by ionizing radiation on thymine residues in DNA. We have studied an oligodeoxynucleotide containing a thymine opposite the urea residue, by one and two dimensional NMR spectroscopy. The urea deoxyribose exists as two isomers with respect to the orientation about the peptide bond. For the *trans* isomer we find that the thymine and urea site are positioned within the helix and are probably hydrogen bonded. The oligonucleotide adopts a globally B form structure although conformational changes are observed around the mismatch site. A minor species is observed, in which the urea deoxyribose and the opposite base adopt an extrahelical position and this corresponds to the isomer *cis* for the peptide bond.

INTRODUCTION

Urea residues are produced by the attack of ionizing radiation on DNA. They are thymine oxydative products generated in minor quantities (1,2). In *E.Coli*, urea residues are recognized by different repair systems (3). The combined N glycosidase/AP (abasic site) endonuclease of the enzyme endonuclease III has been shown to have a specificity in vitro for urea residues in DNA (4–6). Another complex system, exonuclease III, whose major endonuclease activity is for AP sites, recognizes also urea residues, nicking on the 5′ side of the damage and removes the modified base (7,8).

If non repaired, the presence of an urea lesion on a DNA template impedes the DNA polymerase progression so that, in vitro, chain elongation is significantly inhibited. Because these residues constitute replicative blocks for DNA polymerase in vitro, they seem to be potentially lethal in vivo (9,10).

Urea residues could be described as non-instructive lesions and are probably mutagenic like AP sites (11,12). Because part of the coding information has been lost, any base could potentially be incorporated and give rise to a mutation after the first replication. However, if the polymerase inserts preferentially, like for the AP sites (13,14) an adenine in front of the urea lesion, no mutation will occur.

In this study, we have investigated wether it is possible to incorporate a thymine or a guanine opposite the urea lesion.

The urea residue has potential hydrogen bonding sites and these together with base stacking interactions on the opposite strand, could determine the base incorporated opposite a template urea residue.

MATERIALS AND METHODS

The monomer, 5′-O-(4-methoxytrityl)-2′-deoxyribosylurea-3′-O(2-cyanoethyl-N,N′diisopropyl)-phosphoramidite was synthesized as previously reported (15). The oligodeoxynucleotide syntheses were carried out on aminopropylated controlled pore glass support using N-acyl-5′-O-(4,4′dimethoxytrityl)-2′-deoxyribonucleoside-3′-O-(2-cyanoethyl-N,N′diisopropyl)-phosphoramidites with N^6-phenoxyacetyl adenine, N^2-phenoxyacetyl guanine and N^4-acetyl cytosine as base protecting groups (16). Cleavage from the solid support and deprotection of the oligomers was carried out with 28% aqueous ammonia for 6 hours at ambient temperature. The decamers were purified by ion exchange high performance liquid chromatography using a linear gradient of 0.3M potassium phosphate buffer (pH 6.5) with acetonitrile over 40 min. The products were then desalted by dialysis. The duplex studied here has the sequence,

5′ (A1 C2 G3 C4 A5 T6 C7 A8 C9 G10
3′ (T20 G19 C18 G17 T16 ur15 G14 T13 G12 C11

NMR spectra were recorded on BRUKER AMX500 or AMX600 spectrometers in 99,99% D_2O or 90%H_2O/10%D_2O. The duplex was 4mM strand concentration in 10 mM phosphate buffer and 150 mM NaCl. The internal reference for the chemical shifts is tetramethyl ammonium chloride (3.18 ppm).

NOESY spectra were recorded with 400 or 60 ms mixing times, in the phase sensitive mode at 1°C, 12°C, 20°C and 27°C (17).

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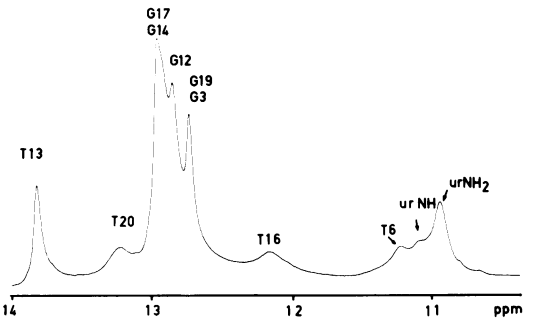

Figure1. One dimensional spectrum (low field region) of the duplex in H_2O at 1°C, pH 5.

For the H_2O spectra a jump and return sequence was used for the observation pulse (18).

TOCSY spectra have been recorded, with mixing times of 60 and 121 ms, in the phase sensitive mode (19).

RESULTS

In order to mix the two strands in equimolar quantities, the T strand was progressively added to a solution of the Ur strand and NMR spectra have been recorded at 50°C and integrated. We observed for the Ur strand alone, two resonances for a TH6, presumably T16, in the ratio of ca. 2:3. This corresponds to the two isomers of the urea deoxyribose analysed during the synthesis of the oligonucleotide, as previously reported (15).

Spectra in H_2O

The downfield region of the 1D spectrum recorded at pH 5, 1°C, is shown in Figure 1. Only one resonance is observed below 13.5 ppm where we would expect to find the T imino resonances. Three relatively broad resonances are observed around 11 ppm and another at 12.1 ppm. All four resonances get narrower as the pH is lowered from 7 to 5 but no changes in the chemical shifts of the non-exchangeable protons are observed.

The assignment of the resonances has been obtained from analysis of a NOESY spectrum recorded in H_2O with a mixing time of 250 ms. Two regions are shown in Figure 2 : imino/imino (Figure 2A) and imino/amino, H2 (Figure 2B). In Figure 2A, the T imino proton shows interactions with 2 G imino protons at 12.9 and 12.82 ppm and from the sequence this corresponds to G^{12}-G^{14}. We also observe a cross peak between 2 G imino protons. The assignment, based partly upon NOEs observed with the CH5 protons (assigned below), is shown in Figure 2B. The imino resonance at 12.1 ppm shows an NOE to the A^5H2 proton as does the imino proton of G^{17} although in this region there are NOEs to CH6 protons via spin diffusion. This former imino resonance is very broad and shows no other NOEs. This NOE suggests that the imino resonance is that of T^{16}. The three resonances at ca. 11 ppm show no cross peaks in the NOESY spectrum.

To probe further the environment of the protons giving rise to these broad resonances we have recorded 1D difference spectra. Presaturation of the resonance at 12.1 ppm, Figure 3A, gives NOEs to G^{17} and A^5H2 confirming that it is the T^{16} imino proton. Presaturation of the 10.9 ppm resonance, Figure 3B, gives NOEs to the T^{16} and G^{14} imino protons, to A^5H2 and

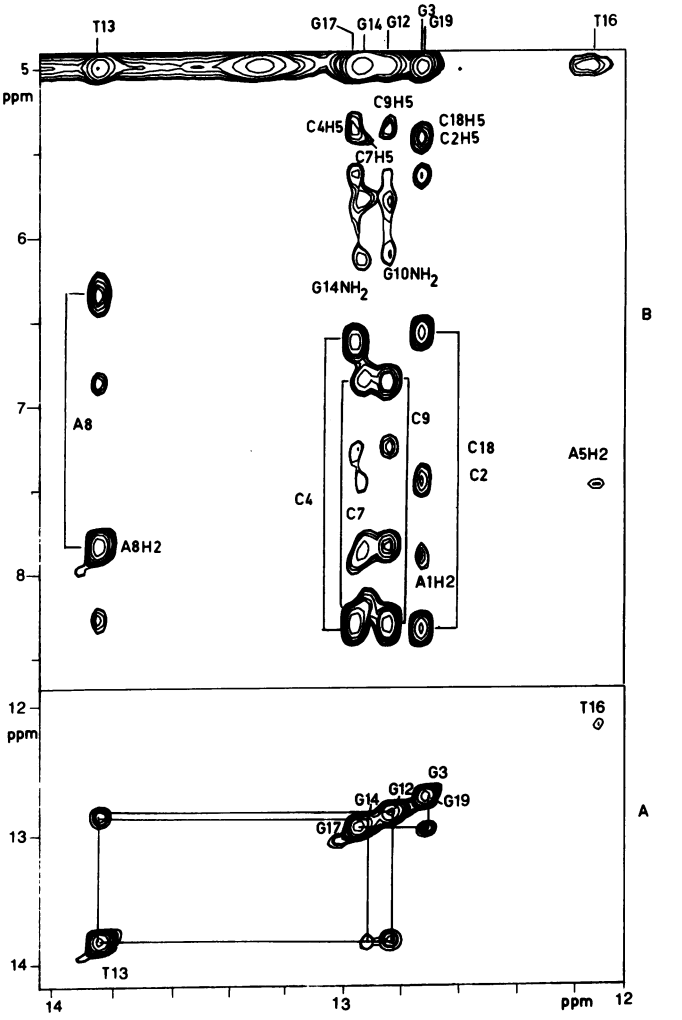

Figure 2. Expanded contour plots of a NOESY spectrum with a 250 ms mixing time in H_2O at 1°C , pH 5. **A.** region of the interactions between the imino protons. **B.** region showing the interactions beween the imino protons and the amino/H2 protons.

weakly to the hydrogen bonded C^7 amino proton. Difference spectra after presaturation of the other two neighbouring resonances have been recorded (not shown). Weighted difference spectra were calculated to remove the effects of spill over. They give the same interactions as for the peak at 10.9 ppm although the relative effects are different. The peak at 11.2 ppm shows the strongest NOE to A^5H2. Further, presaturation at 11.05 ppm results in NOEs to the G^{14} H2'/H2'' protons (identified below) and weakly to a resonance at 2.34 ppm.

Given that all the other exchangeable proton resonances have been assigned, these three peaks must arise from the urea NH, NH_2 and the T^6 imino protons and all must lie inside the helix. The peak integrals are in the ratio ca. 1:1:2 which suggests that the high field one can be assigned to the NH_2 protons. We can tentatively assign the resonance at 11.2 ppm to the T^6 imino proton on the grounds of its proximity to A^5H2. Further the 11.05 ppm resonance gives NOEs to the G^{14}H2'/H2'' protons which would be possible from the urea NH proton but not from

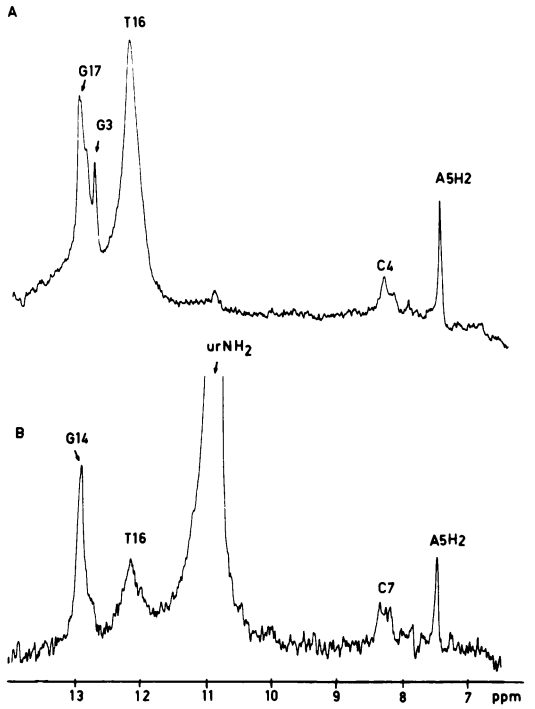

Figure 3. Difference spectra after presaturation for 0.25 s of the resonances T^{16} imino **(A)** and Ur^{15} amino **(B)**.

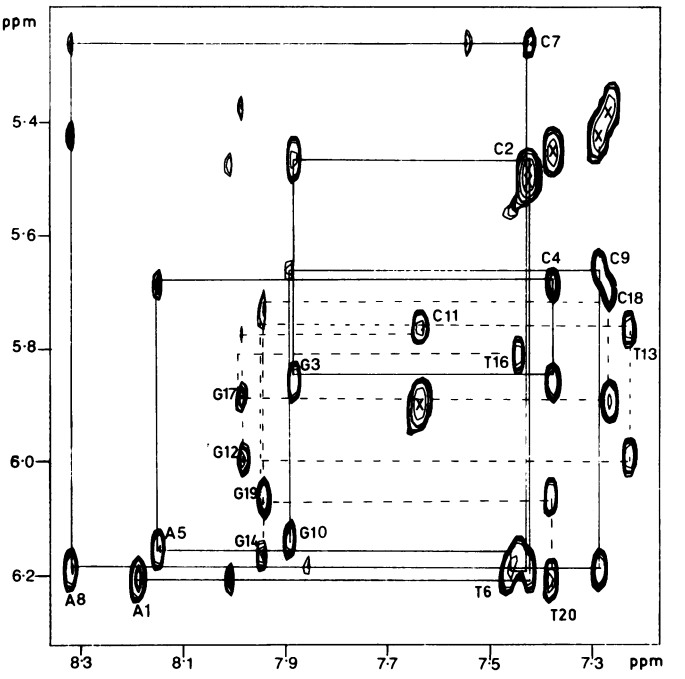

Figure 4. Expanded contour plot of the H6H8/H1'H5 region of the NOESY spectrum recorded with a 400 ms mixing time at 27°C, pH 7.3 in D_2O. Cross-peaks marked X correspond to the cytidine H5/H6 interactions.

Table 1. Observed chemical shifts for the non-exchangeable protons at 27°C and exchangeable protons at 1°C.

	H8/H6	CH_3/ H2/H5	H1'	H2'	H2''	H3'	H4'	NH	NH2
A^1	8.19	8.01	6.21	2.77	2.62	4.81	4.06		
C^2	7.43	5.49	5.47	2.1	2.36	4.83	4.15		8.40/6.60
G^3	7.88		5.85	2.59	2.65	4.98	4.15	12.70	
C^4	7.38	5.45	5.69	2.01	2.35	4.81	4.17		8.35/6.65
A^5	8.15	7.47	6.17	2.72	2.60	4.98	4.17		
T^6	7.47	1.65	6.19	2.30	2.47	4.84	4.26	11.2	
$T^{6'}$	7.63	1.87							
C^7	7.42	5.48	5.26	1.61	2.19	4.71	4.24		8.30/6.80
A^8	8.32	7.86	6.19	2.75	2.85	5.01	4.38		
C^9	7.29	5.42	5.65	1.83	2.29	4.76	4.14		8.40/6.75
G^{10}	7.89		6.17	2.60	2.38	4.66	4.16		
C^{11}	7.64	5.89	5.76	1.98	2.40	4.69	4.07		
G^{12}	7.98		6.0	2.70	2.73	4.97	4.36	12.82	
T^{13}	7.23	1.49	5.77	2.01	2.36	4.86	4.21	13.80	
G^{14}	7.94		6.15	2.73	2.78	5.05	4.35	12.90	
Ur^{15}			5.48	2.34	2.34	4.83	4.15	11.05	10.90
ext.base			6.25	2.35	2.35	4.45	3.79		
T^{16}	7.45	1.45	5.81	1.74	2.16	4.84	4.35	12.10	
G^{17}	7.99		5.88	2.66	2.75	4.97	4.11	12.95	
C^{18}	7.27	5.36	5.72	1.81	2.26	4.76	4.13		8.35/6.60
G^{19}	7.94		6.06	2.63	2.70	4.97	4.37	12.70	
T^{20}	7.38	1.64	6.21	2.25	2.25	4.53		13.30	

the others. We have carefully integrated this region relative to the T^{13} imino proton in an experiment in which the pulse maximum was placed at 12.5 ppm. The integration corresponds to 2.2–2.7 protons rather than 4 as expected. Similar integration relative to the broader signal of T^{16} confirms this result.

NOESY spectra in D_2O: assignment of non exchangeable protons

In order to assign the base and sugar proton resonances of the duplex, we have recorded a NOESY spectrum at 27°C with a 400 ms mixing time. The region of the spectrum, that corresponds to interactions between the aromatic protons H6/H8 and H1' sugar protons is shown Figure 4. The assignment starts from the 5' terminal bases, A^1 and C^{11}. In this region, we can follow the classical connectivities for a right-handed B-DNA helix, from A^1 to G^{10}. Unfortunately, the interaction between C^7H6 and T^6H1' overlaps with the cross peak T^6H6/A^5H1'.

The region H6H8/H2'2'', Figure 5 confirms these assignments. However, in Figure 5 we do not observe a cross peak between the aromatic proton of T^6 and H2'H2'' protons of A^5. By contrast, the interaction between these two bases is emphasized by the cross peak T^6CH3/A^5H8, peak A. Also, the aromatic proton C^7H8 gives rise to a single weak cross peak at 2.47 ppm, peak B, which corresponds to T^6H2''. These 2 cross peaks A and B indicate that the thymine T^6 is inside the helix.

For the second strand, the assignment begins from the terminal 5' residue C^{11}. In the region H6H8/H1', Figure 4, one cross peak corresponds to the interaction between C^{11}H6 at 7.64 ppm and its own H1' sugar proton at 5.89 ppm. Then, the chain can be followed, in a classical manner for a right handed B-DNA from C^{11}H1' to G^{14}H1'. However, the urea site has no aromatic proton, so that the chain is broken. We continue the assignment from the terminal T^{20} base up to G^{17}. In Figure 4, no cross peak between G^{17}H8 and T^{16}H1' is observed. We have also run NOESY spectra at 1°C, 12°C and 20°C (not shown), these spectra confirm the assignment and do not show the cross peak G^{17}H8/T^{16}H1'. However, the column obtained from the aromatic proton of G^{17} presents a weak signal corresponding to

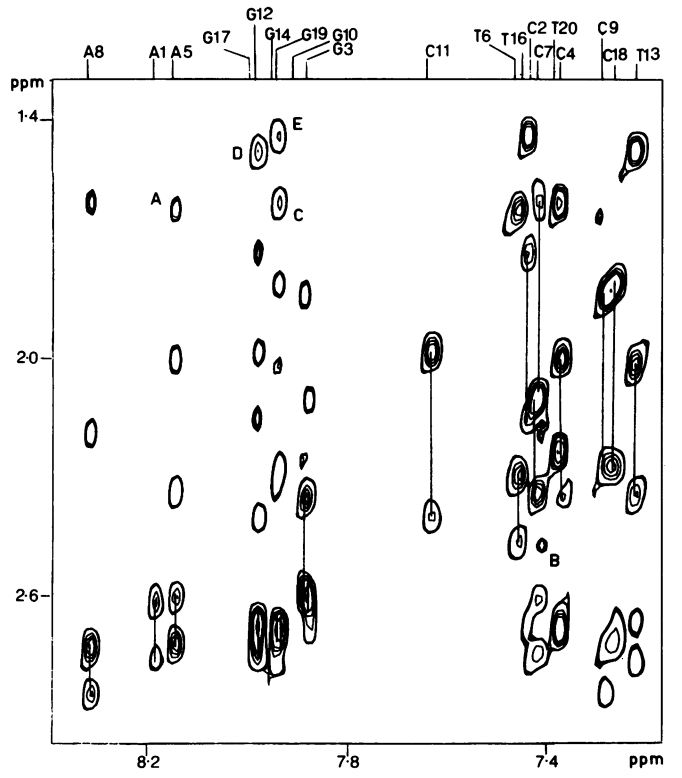

Figure 5. Expanded contour plot of the H6H8/H2'H2'' region of the NOESY spectrum (400 ms mixing time) of the duplex at 27°C in D_2O. Vertical lines connect intraresidues cross peaks. Peaks A-E are described in the text.

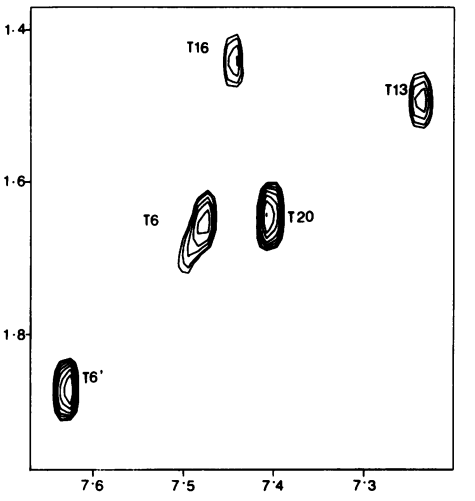

Figure 6. Expanded contour plot of a TOCSY spectrum in D_2O at 27°C with a 121 ms mixing time.

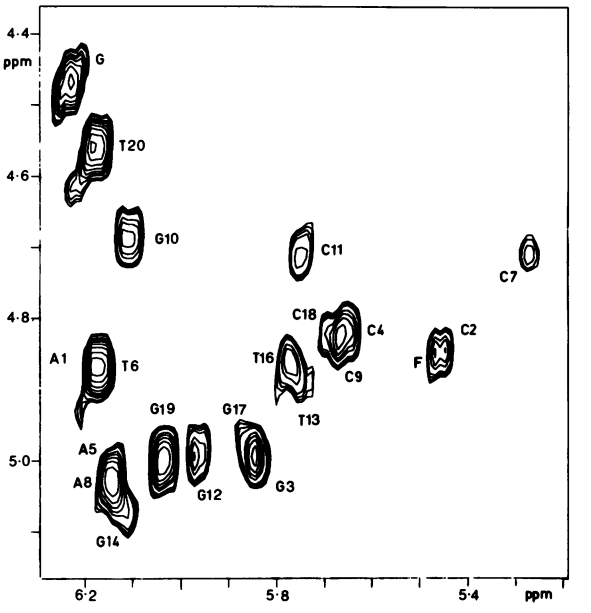

Figure 7. Expanded contour plot of a TOCSY spectrum in D_2O at 27°C with a 70 ms mixing time.

this interaction so that we can say that the interaction exists although weakly. On the other hand, if we consider the H6H8/H2'2'' region, Figure 5, for $G^{17}H8$, two cross peaks at 1.74 and 2.16 ppm are observed, corresponding to the protons H2'H2'' of T^{16} showing that T^{16} is inside the helix.

This region shows two normal interresidue cross peaks, C and D but also another between $G^{14}H8$ and $T^{16}CH_3$, peak E. This observation indicates the presence of a species in which the urido is extrahelical, in which G^{14} and T^{16} are stacked on each other.

In Figures 4 and 5, we can not check that C^7 interacts with A^5 because of the overlapping cross peaks C^2/A^1. The spectra recorded at lower temperatures, 1°C, 12°C and 20°C, do not improve the resolution. However, in the case of an interaction C^7/A^5, we could show that T^6 can also adopt an extrahelical position. To search for an extrahelical thymine, we have recorded a TOCSY spectrum at 27°C with a mixing time of 121 ms. Figure 6 shows TH6/CH_3 interactions for the 3 intrahelical thymine residues plus a fourth one at 7.63 ppm. We observe different intensities for the peaks CH_3-H6 of thymines since the line widths are not the same for all the base protons. Besides, we note that we observe no cross peak in the NOESY spectrum at the downfield chemical shift. The integration of the low field methyl resonance in the 1D spectrum corresponds to ca. 35% of that of the A.T methyl resonances.

In order to identify the resonances of the deoxyribose of ur[15], we have searched for any non-assigned cross peaks in the different regions of a TOCSY spectrum recorded at 27°C with a mixing time of 70 ms. In the region H1'/H3', Figure 7, we observe a cross peak at 4.83 ppm and 5.48 ppm, peak F. These both show a cross peak with a resonance at 2.34 ppm. In Figure 4, the aromatic proton of T^{16} could interact with a H1' proton at 5.48 ppm and similarly, in Figure 5 an interaction is possible between $T^{16}H6$ and H2'H2'' situated at 2.34 ppm. It is probable that these resonances belong to the urea sugar protons of the intrahelical species. However, in both cases there is resonance overlap with protons of C^2.

In Figure 7, we can observe an additional peak, G that shows a H1' proton shifted downfield, 6.25 ppm, that does not appear in NOESY spectra. This may arise from an extrahelical species but we can not assign it with certainty.

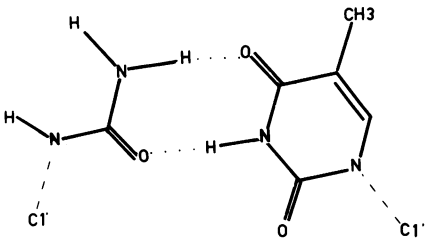

Figure 8. Possible pairing structure for Ur-T.

We have measured in a NOESY spectrum recorded with a 60 ms mixing time the ratio of the cross peak volumes for the intraresidue H6H8/H2′ and H6H8/H3′ interactions to determine the major sugar conformation (20). We have found for all non-terminal sugars a predominant C2′ *endo* conformation. This measure cannot be done for the ureadeoxyribose group. This was confirmed from analysis of the phase sensitive COSY spectrum. Similarly, we have determined the ratio of H6H8/H2′ to H6H8/H1′ NOEs; the results show that all the bases adopt an *anti* configuration.

We have followed the chemical shift of the different thymine methyl resonances as a function of temperature. The melting temperature obtained for the intrahelical T^6 is 48°C, the same as for all the central bases. So at 27°C the mismatched T, for which the TCH_3 is observed at 1.65 ppm is predominantly intrahelical. We do not observe a clear melting curve for the low field TCH_3 consistent with it being extrahelical even at low temperature.

We have previously reported studies on oligonucleotides with a central abasic site and with each of the four natural bases in front (21−22). For a T residue opposite the abasic site we observed both an intrahelical and an extrahelical structure. Equal populations were found at ca. 30°C. We have now measured the Tm of the surrounding bases of this abasic site-T sequence and found values of 41−43°C. In this oligonucleotide only stacking interactions keep the T residue inside the helix as no hydrogen bonds can be formed and the T residue melts ca. 12°C below that of the surrounding bases. This is in contrast to what we observe for the urea system and strongly indicates that at least one hydrogen bond is formed.

Guanosine can potentially form the same pattern of hydrogen bonding with urea as thymine. NMR spectra on the same sequence with a G at position 6 show, in the range 0°C-35°C, certain aromatic resonances which are sharp and others very broad. NOESY spectra give an almost complete assignment, however resonances corresponding to the central part of the oligonucleotide cannot be assigned with certainty. Under NMR conditions, the Tm of the central part of the oligonucleotide is ca. 25°C lower than that of the T analogue.

DISCUSSION

With the oligonucleotide containing a thymine opposite the urea lesion, our results show that for the major species the Watson Crick base pairs are integrated in a right-handed B-DNA helix. In the NOESY spectrum in D_2O recorded at 27°C, we can observe that T^6 and the urea are stacked into the helix. This is confirmed in the spectra in H_2O, where the imino proton of T^6, the imino and amino protons of urea are observed; this means that they are protected from the exchange with water. Further, the observed NOEs from presaturation of the resonances of these protons confirm that they are intrahelical.

We do not observe interresidue NOEs betwen T^6H6 and A^5H2′/H2″. Also, we observe the cross peak C^7H6/T^6H2′ whereas C^7H6/T^6H2′ is not detected and these results suggest that T^6 is shifted towards the major groove. We also note that the cross peaks G^{17}H8/T^{16}H1′, H2′, H2″ are weak. The insertion of the urea group leads to a local perturbation of the duplex geometry and disturbs the adjacent base pairing.

Imino resonances in the region 10−12 ppm have been observed for NH--O=C pairing in wobble structures (23,24) or for imino protons non-hydrogen bonded in loop and other structures (25,26). The chemical shift of 11.2 ppm of the T^6 imino proton is the same as that observed for the monomeric T residue (27). Base stacking will induce an upfield chemical shift displacement as has been observed in either T.T mismatches (27,28) or in a T frameshift (29). Hydrogen bonding would induce a downfield shift which may explain the observed chemical shift. Comparison of the melting behaviour of this system with that of an abasic site-T mismatch strongly suggests hydrogen bond formation. In this case the T residue melts with the oligonucleotide whereas when there are no hydrogen bonds, the T residue melts ca. 12°C lower.

We observe for the T^{16} imino proton a very large linewidth and a unusual chemical shift of 12.1 ppm: this value is intermediate between that of a base paired and unpaired T imino resonance. The chemical shift suggests a weak hydrogen bond for this imino proton. This is, however, not reflected by the Tm of the base pair which is the same as others further away from the mismatch site. The enhanced proton exchange with solvent may arise from a mechanism which does not involve base pair opening but through solvent access into the interior of the helix. The same does not appear to be so for the G.C pair next to the mismatch site. This result confirms the idea of a perturbation of the adjacent base pairing.

At all temperatures we observe the presence of a species in which the thymine and the urea group are extrahelical as shown by additional peaks in the TOCSY spectra.

We propose a scheme for the pairing between the urea group and the thymine, Figure 8. The synthesis gave rise to 2 isomers *trans* and *cis* for the peptide bond, in a ratio of ca. 3:2. From preliminary model building studies the *trans* isomer can pair with T with two hydrogen bonds as shown in Figure 8. The T residue has to move towards the minor groove relative to a Watson Crick conformation. On the other hand, the *cis* isomer is highly unfavourable for the formation of hydrogen bonds. Direct evidence for this is lacking, though a number of observations support this interpretation. Integration of the resonances around 11 ppm gives 2.2−2.7 protons in agreement with the population of the *trans* isomer. This species is shown to be intrahelical. It is difficult to explain the thermal stability of the mismatch other than by the presence of one or two hydrogen bonds. The formation of such a structure requires significant helix distortion in agreement with the observed NOEs for the central part of the helix. The extrahelical T species corresponds to ca. 35% of the total population judging from integration of its methyl resonance in agreement with the population of the *cis* isomer.

The relative population of the two isomers is known and even at high temperature and for the single strand, we do not observe interconversion of them. This ratio appears fixed. We observe

the same ratio for this intrahelical versus extrahelical species as measured from the different integrations. The population of the intrahelical species corresponds well with that of the *trans* isomer and that of the extrahelical species with that of the *cis* isomer. Although there is no direct proof of this correspondence, the relationship appears very probable. This could only be confirmed by studying the isomers separately.

Studies, in vitro, show that the DNA polymerase is frequently stopped at one nucleotide before the urea site (9). This observation indicates that urea sites act like AP sites and we have previously proposed a model to explain why the polymerase progression is stopped (21). When a thymine is incorporated in a duplex structure opposite an AP site, intrahelical and extrahelical forms have been observed for both the thymine and the deoxyribose (21). It has been assumed that in the case of a pyrimidine, the stacking energy is more important when the structure is collapsed, so that the pyrimidine is shifted out of the helix.

The situation that we observe here for a T opposite the urea residue is quantitatively different. For the species that we have assigned to the *trans* urea isomer, the mispair is thermally stable and we do not observe an equilibrium of intra and extrahelical species different from global melting of the duplex. On the other hand, the *cis* isomer is entirely extrahelical even at low temperature. This difference would appear to arise from the different hydrogen bonding potential of the two isomers.

For a related lesion, β-ureidoisobutyric acid (30), which has the same hydrogen bonding potential as the urea residue and with a polymerase lacking $3' \rightarrow 5'$ exonuclease activity, purine nucleotides were preferentially incorporated opposite the lesion. We have also recorded spectra on our reference sequence, but containing a G residue instead of a thymine opposite the urea site; in this case, the duplex gave rise to very broad resonances which precludes a detailed analysis.

Due to resonance overlap and the presence of the two urea isomers, a detailed structural analysis has not been possible. As yet, we have not been able to separate oligonucleotide strands of the two isomers.

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