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Biophysical Chemistry 123 (2006) 134–140

Biophysical Chemistry

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DNA triple-helix formation at target sites containing duplex mismatches

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Received 28 March 2006; received in revised form 28 April 2006; accepted 29 April 2006
Available online 2 June 2006

Abstract

We have studied the formation of DNA triple helices at target sites that contain mismatches in the duplex target. Fluorescence melting studies were used to examine a series of parallel triple helices that contain all 64 N.XZ triplet combinations at the centre (where N, X and Z are each of the four natural DNA bases in turn). Similar experiments were also performed with N=bis-amino-U (BAU) (for stable recognition of AT base pairs) and N=S (for recognition of TA inversions). We find that the introduction of a duplex mismatch destabilises the C^+.GZ, T.AZ and G.TZ triplets. A similar effect is seen with BAU.AZ triplets. In contrast, other base combinations, based on non-standard triplets such as C.AZ, T.TZ, G.CZ and A.CZ are stabilised by the presence of a duplex mismatch. In each case S binds to sites containing duplex mismatches better than the corresponding Watson–Crick base pairs.

Keywords: Triple helix; Mismatch; Thermal melting

1. Introduction

Triple-helical DNA is formed when a third nucleic acid strand binds within the major groove of a DNA duplex [1–4]. The third strand usually only makes direct contacts with the bases on the purine strand of the duplex, to which it can run either parallel generating C^+.GC and T.AT triplets [5,6], or antiparallel forming A.AT and G.GC triplets [7]. Triplex-forming oligonucleotides have considerable potential as sequence-specific DNA binding agents for the recognition of unique DNA sequences [8–12]. Since the triplex-forming oligonucleotide only recognises the purine strand, its binding within the DNA major groove is asymmetric. Recognition of the duplex purine strand may therefore be insensitive to the identity of the base to which it is paired, i.e. whether it is paired with its Watson–Crick partner or forms a mismatched pair. Previous studies suggested that triplets formed at some duplex mismatches can have increased thermal stability and T.AC and C.GA triplets were shown to be more stable than the standard T.AT and C^+.GC triplets [13]. (The notation N.XZ refers to a triplet in which the third strand base N interacts with the duplex XZ base pair, forming hydrogen bonds to base Z.) This increase in stability was attributed to the greater flexibility of the mismatched base pair, allowing for more favourable interactions with the third strand. In this paper we have examined the thermal stability of all 64 possible triplet combinations (N.XZ, where N, Y and Z are each base in turn), using a fluorescence melting technique [14]. These studies were extended to examine the interaction of nucleoside analogues bis-amino-U (BAU; designed for stable interaction with AT base pairs [15–18]) and S (for recognition of TA interruptions [18–20]) with duplex mismatches (Fig. 1a).

2. Experimental section

2.1. Oligonucleotides

The sequences of the oligonucleotides used in the fluorescence melting experiments are illustrated in Fig. 1b. The purine strand of the duplex (boxed) was labelled at the 5′-end with a fluorophore (F; fluorescein), while the third strand was labelled at the 5′-end with a quencher (Q; methyl red).

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doi:10.1016/j.bpc.2006.04.016

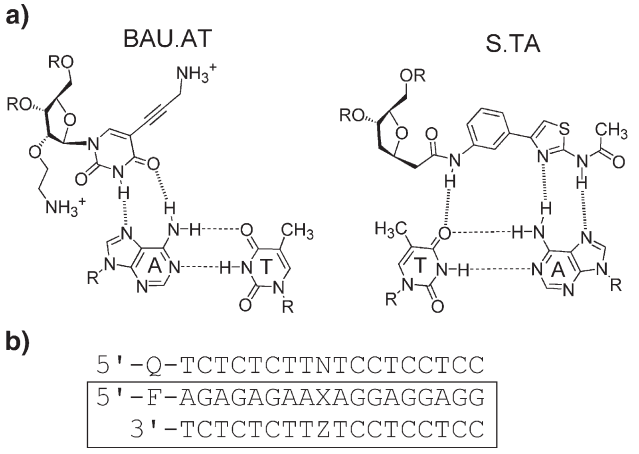

Fig. 1. (a) Chemical structures of the BAU.AT and S.TA triplets. (b) Sequences of the oligonucleotides used in this work. The duplex is boxed and was labelled with fluorescein (F) at the 5′-end of the purine strand, whilst the third strands were labelled with methyl red (Q) at the opposing 3′-end.

2.2. Fluorescence melting experiments

Fluorescence melting experiments were performed as previously described [14,18]. Triplex formation brings the fluorophore (located at the 5′-end of the duplex) and the quencher (located at the 5′-end of the third strand) in close proximity and the fluorescence is quenched. When the third strand dissociates the fluorophore and quencher are separated and there is a large increase in the fluorescence signal. Melting temperatures (T_m) for these intermolecular triplexes were determined using a Roche LightCycler, increasing the temperature at a rate of 0.2 °C min^{-1}. This slow rate of heating and cooling was achieved by increasing the temperature in 1 °C intervals, leaving the samples to equilibrate for 5 min between each fluorescence reading. Recordings were taken during both the heating and cooling steps and no significant hysteresis was observed. All melting experiments were performed in a total volume of 20 μl in 50 mM sodium acetate buffer, pH 5.5 containing 200 mM sodium chloride. The duplex concentration was 0.25 μM, while the third strand was 3 μM. T_m values were calculated from the first derivatives of the melting and annealing profiles. The plots shown in Figs. 2–4 have each been normalised to the same fluorescence at 70 °C. Each value was recorded in triplicate and these usually differed by less than 0.5 °C.

3. Results

The oligonucleotides illustrated in Fig. 1b were used to generate 64 different triplex combinations, each varying by a single triplet N.XZ in the centre, where N, X and Z are each base in turn. 16 of these combinations contain standard Watson–Crick base pairs, while the remaining 48 contain mismatched base pairs in the duplex. The 5′-end of the duplex purine strand was labelled with fluorescein while the 5′-end of the third strand (which is added in excess) was labelled with methyl red. Triplex formation brings these groups close together and the fluorescence is quenched. When the third strand dissociates these groups are separated and there is a large increase in fluorescence. The fluorescence melting profiles for these complexes are shown in Figs. 2 and 3. In most cases, as we have previously noted [14,18], the temperature-dependent increase in fluorescence, which corresponds to triplex dissociation, is followed by a decrease in fluorescence at higher temperatures. This second transition is caused by melting of the underlying duplex and in all cases it can be seen to occur at

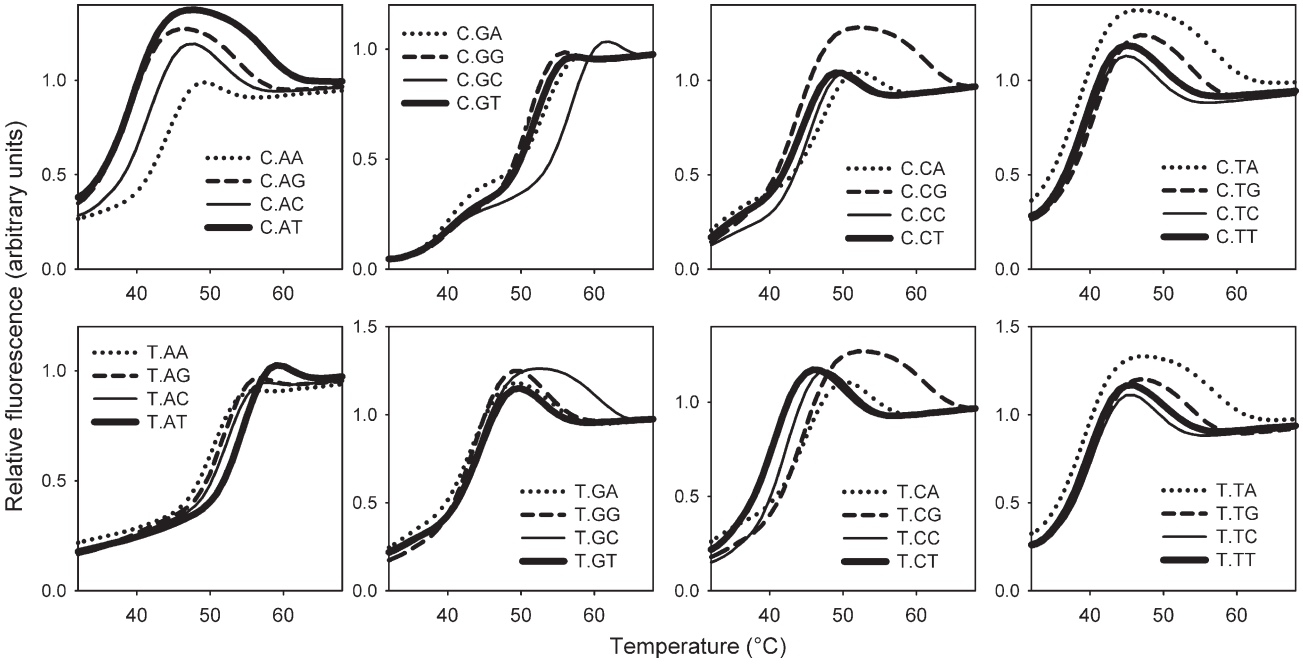

Fig. 2. Representative fluorescence melting curves for different triplexes containing C.XZ (top row) and T.XZ triplets (bottom row). N.XA, dotted line; N.XG, dashed line; N.XC thin line; N.XT, thick line. The experiments were performed in 50 mM sodium acetate, pH 5.5 containing 200 mM NaCl. The *y*-axis show the normalised fluorescence (arbitrary units), while the *x*-axis shows the temperature (°C). The samples were heated at a rate of 0.2 °C min^{-1}.

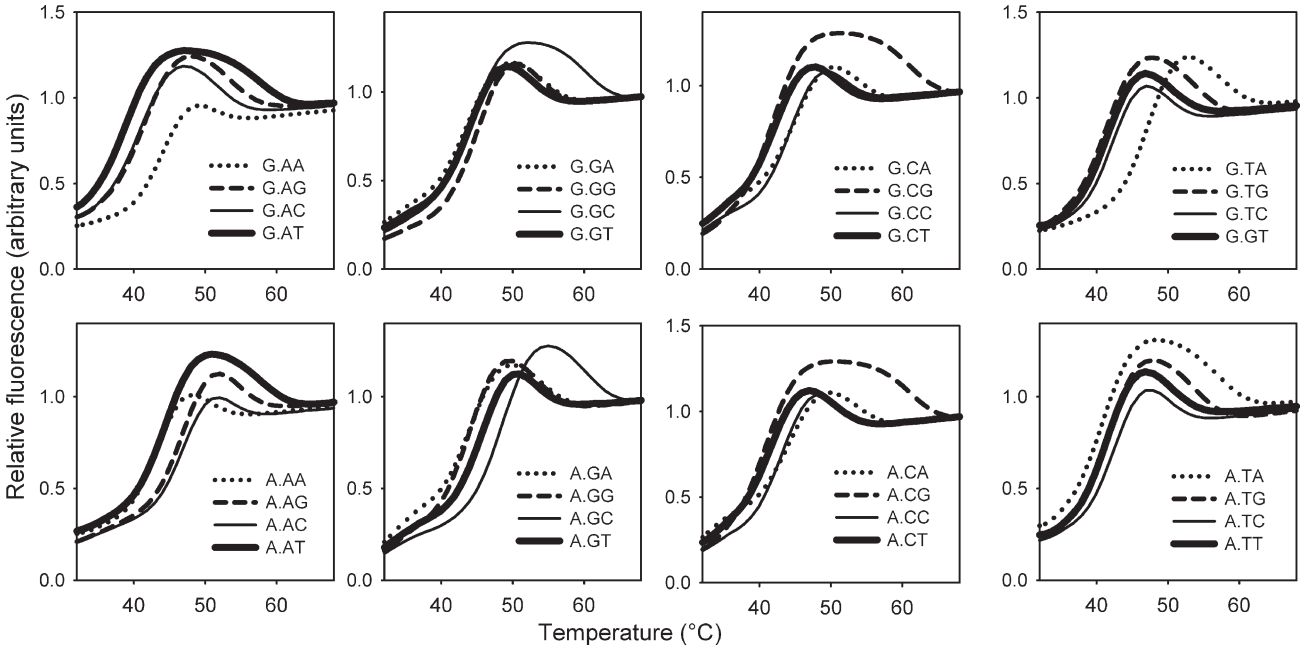

Fig. 3. Representative fluorescence melting curves for different triplexes containing G.XZ (top row) and A.XZ triplets (bottom row). N.XA, dotted line; N.XG, dashed line; N.XC thin line; N.XT, thick line. The experiments were performed in 50 mM sodium acetate, pH 5.5 containing 200 mM NaCl. The *y*-axis show the normalised fluorescence (arbitrary units), while the *x*-axis shows the temperature (°C). The samples were heated at a rate of 0.2 °C min^{-1}.

higher temperatures for the duplexes that contain only Watson–Crick base pairs. Only one transition is observed for the most stable triplexes (C.GZ and T.AZ).

3.1. Triplexes containing C.XZ triplets

Representative melting profiles for triplexes containing cytosine in the third strand are shown in the top row of Fig. 2. For each panel the N.X bases of the N.XZ triplet are constant, while Z is substituted with each base in turn. The T_m values determined for these profiles, which were performed at pH 5.5, are shown in Table 1. The profiles obtained for the triplexes containing central C.AZ triplets are shown in the first panel. It can be seen that, when cytosine is place opposite a mismatched AZ base pair, the triplexes are more stable than with the standard AT base pair (thick line). This is most notable

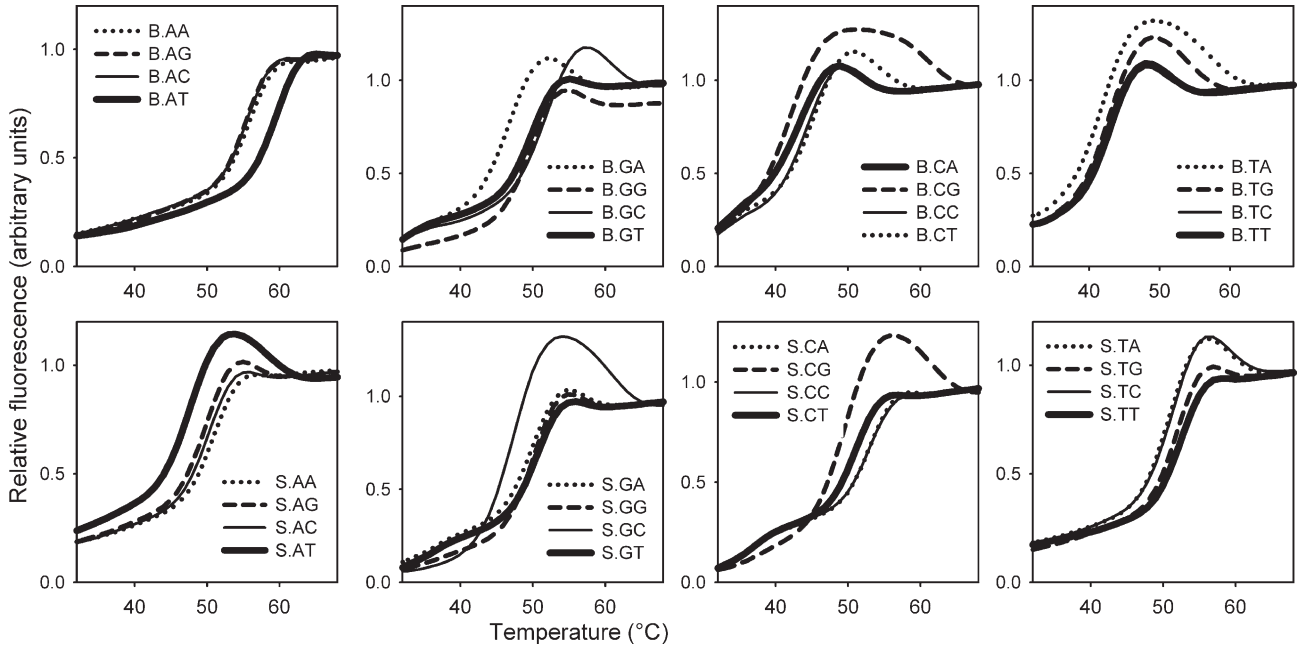

Fig. 4. Representative fluorescence melting curves for different triplexes containing BAU.XZ (top row) and S.XZ triplets (bottom row). N.XA, dotted line; N.XG, dashed line; N.XC thin line; N.XT, thick line. The experiments were performed in 50 mM sodium acetate, pH 5.5 containing 200 mM NaCl. The *y*-axis show the normalised fluorescence (arbitrary units), while the *x*-axis shows the temperature (°C). The samples were heated at a rate of 0.2 °C min^{-1}.

Table 1
T_m values for different triplet combinations determined by fluorescence melting

XZ	N			
	A	G	C	T
AA	43.5 (−0.9)	44.3 (+5.3)	44.2 (+4.4)	50.4 (−4.1)
AG	46.6 (+2.2)	41.3 (+2.3)	39.2 (−0.8)	51.6 (−2.9)
AC	47.0 (+2.6)	40.7 (+1.7)	41.4 (+1.6)	52.1 (−2.4)
AT	44.4	39.0	39.8	**54.5**
GA	44.1 (−3.9)	43.6 (−0.4)	53.4 (−3.2)	43.2 (−0.5)
GG	43.7 (−4.7)	45.2 (+1.1)	51.1 (−5.5)	43.6 (−0.1)
GC	48.4	**44.1**	**56.6**	43.7
GT	45.7 (−2.7)	43.8 (−0.4)	51.9 (−4.7)	44.4 (+0.8)
CA	43.9 (+2.2)	44.8 (+2.9)	47.0 (+3.3)	44.8 (+0.3)
CG	40.7	41.9	**43.7**	**44.5**
CC	43.1 (+2.4)	44.3 (2.4)	45.5 (+1.8)	42.3 (−2.2)
CT	41.3 (+0.6)	41.9 (0.0)	44.2 (+0.5)	40.5 (−4.0)
TA	40.7	**47.3**	38.7	37.8
TG	41.5 (+0.8)	41.3 (−6.0)	40.3 (+1.6)	40.3 (+2.5)
TC	42.3 (+1.6)	42.1 (−5.2)	39.4 (+0.7)	40.2 (+2.4)
TT	41.3 (+0.6)	41.3 (−6.0)	39.0 (+0.3)	39.8 (+2.0)

Experiments were performed in 50 mM sodium acetate pH 5.5 containing 200 mM NaCl. The purine strand of the duplex was labelled with fluorescein while the third strands were labelled with methyl red. The values in parentheses show the differences in T_m between the triplet formed at a Watson–Crick base pair and the triplet containing a duplex mismatch. The values in bold correspond to the well known C^+.GC, T.AT, G.TA, G.GC, T.CG and C.CG triplets.

for the C.AA triplet (dotted line) for which the T_m is 4.4 °C higher than for C.AT. The second panel shows the profiles for the triplexes containing C.GZ triplets. In this instance the triplex containing the C^+.GC triplet (thin line) is more stable than any of the triplets at C.GZ mismatches, with a T_m of 3.2 °C higher than the next most stable. As expected, these C.GZ triplets are more stable than all the other C.XZ triplets. Further examination of the melting profiles reveals another transition at lower temperatures. This transition is peculiar to the C.GZ triplets and is not observed with any of the other triplet combinations. The T_m for this transition (about 40 °C) is the same for all four bases (Z) on the complementary strand, suggesting that it represents the formation of a Hoogsteen duplex between the third strand and the purine-containing strand of the duplex.

The third and fourth panels show the profiles for the triplexes containing C.CZ and C.TZ triplets. In all these cases the triplexes generated with C against a mismatched base pair are more stable than with a Watson–Crick base pair (CG or TA). It is interesting to note the increased affinity of C for CA relative to CG, as C has been proposed for recognition of CG base pairs [21–23].

3.2. Triplexes containing T.XZ triplets

Representative melting profiles for the triplexes containing thymine in the third strand are shown in the bottom row of Fig. 2. The first panel shows the profiles for the triplexes containing T.AZ triplets. The most stable triplex is generated with T opposite an AT base pair, forming the usual T.AT triplet (thick line) rather than with an AZ mismatch. The T.AA triplet (dotted line) is the least stable, with a T_m of 4.1 °C lower than T.AT. The melting curves obtained for the triplexes containing T.GZ triplets are shown in the second panel. All these triplexes exhibit similar T_m values (~44 °C), irrespective of the base that is paired with G.

The third panel shows the profiles for triplexes containing T.CZ triplets. In this instance the triplexes containing T.CT and T.CC triplets are between 2 and 4 °C less stable than T.CG and T.CA. The most stable triplets produced similar T_m values to the T.GZ triplets. The melting profiles for the triplexes containing T.TY triplets are shown in the fourth panel. The triplets containing mismatched base pairs are more stable than the T.TA triplet displaying T_m values between 2 and 5 °C higher, although these triplets are less stable than T.AT.

3.3. Triplexes containing G.XZ triplets

Representative melting profiles for the triplexes containing guanine in the third strand are shown in the top row of Fig. 3. The first panel shows the profiles for the triplexes containing G.AZ triplets. It is evident that the triplexes generated opposite a mismatched base pair are more stable than against AT. The G.AA triplet (dotted line) is the most stable with a T_m that is 5.3 °C higher than G.AT (thick line). The second panel shows the profiles obtained for the G.GZ triplets. These all have similar stabilities with T_m values of about 44 °C.

The third panel illustrates the profiles for the triplexes containing G.CZ triplets. The triplex containing a G.CG triplet (dashed line) exhibits a T_m of 41.9 °C, while the triplexes with duplex mismatches are more stable; G.CA (dotted line) and G.CC (thin line) triplets produce T_m values of about 44.5 °C. The fourth panel shows the profiles for the G.TZ-containing triplexes. In this instance the most stable triplex is generated with G opposite a TA base pair (generating the well-known G.TA triplet [24–27]) with a T_m of 47.3 °C. This triplet is at least 6 °C more stable than with any G.TZ mismatch.

3.4. Triplexes containing A.XZ triplets

Representative melting profiles for the triplexes containing adenine in the third strand are shown in the bottom row of Fig. 3. The first panel shows the results for the A.AZ triplets. It can be seen that the most stable triplexes are generated when adenine is placed opposite an AC (thin line) and AG (dashed line) duplex mismatch. These complexes exhibit T_m values of about 47 °C, compared with 44 °C for the A.AT-containing triplex (thick line). In contrast, the triplex containing the A.AA (dotted line) triplet is no more stable than A.AT. The second panel shows the profiles for the A.GZ triplets. The most stable triplex is generated with adenine opposite a GC base pair; the other bases at position Z produced triplexes that are at least 2 °C less stable. The third and fourth panels show the profiles obtained for the A.CZ or A.TZ triplets. In both cases positioning A opposite a duplex mismatch generated a complex that is more stable than opposite a Watson–Crick base pair; this is most evident for the A.CA and A.CC triplets (thin and dashed lines). The triplexes formed with these base

Table 2
T_m values of different triplet combinations formed with BAU or S in the third strand, determined by fluorescence melting

XZ	N	
	BAU	S
AA	55.7 (−3.8)	50.9 (+3.4)
AG	55.2 (−4.3)	49.9 (+2.4)
AC	55.4 (−4.1)	50.2 (+2.7)
AT	**59.5**	47.5
GA	46.8 (−5.0)	50.0 (+3.2)
GG	49.8 (−1.9)	50.1 (+3.3)
GC	51.8	46.8
GT	49.9 (−1.6)	50.7 (+4.0)
CA	45.2 (+3.2)	53.1 (+3.6)
CG	42.0	49.5
CC	44.3 (+2.4)	53.0 (+3.5)
CT	43.4 (+1.4)	51.1 (+1.6)
TA	41.8	**51.0**
TG	42.9 (+1.1)	51.9 (+0.9)
TC	43.3 (+1.5)	51.2 (+0.2)
TT	42.8 (+1.0)	52.5 (+1.5)

Experiments were performed in 50 mM sodium acetate pH 5.5 containing 200 mM NaCl. The purine strand of the duplex was labelled with fluorescein while the third strands were labelled with methyl red. The values in parentheses show the differences in T_m between the triplet formed at a Watson–Crick base pair and the triplet containing a duplex mismatch. The values in bold correspond to the BAU.AT and S.TA triplets.

pairs are less stable than those generated with AZ and GZ base pairs.

3.5. Triplexes containing BAU.XZ triplets

We have developed bis-amino-U (BAU, Fig. 1a) as a nucleoside analogue for generating stable triplets at AT base pairs [15,16]. It is has the same selectivity as T, but shows some interaction with GC base pairs, which is greater than at CG or TA [16]. We have examined the thermal stability of triplexes containing this modified nucleoside opposite each of the possible mismatched base pairs. Representative melting profiles for the triplexes containing this analogue are shown in the top row of Fig. 4. The T_m values determined from these profiles are summarised in Table 2. The first panel shows the profiles obtained for triplexes containing BAU.AZ triplets. It can be seen that the most stable triplex is formed with BAU opposite the Watson–Crick AT base pair (thick line) with a T_m of 59.5 °C. Substituting T of the BAU.AT triplet with A, G and C results in complexes with T_m values that are all about 4 °C lower. The second panel shows the melting curves for the BAU.GY triplets. It can be seen that BAU displays a lower affinity for GZ mismatches than the GC base pair (thin line), generating triplexes with T_m values that are 1.6–5 °C lower. This destabilisation is greatest for the BAU.GA triplet.

The melting profiles for the BAU.CZ and BAU.TZ triplets are illustrated in the third and fourth panels. All CZ mismatches generates triplexes with T_m values that are 1.4–3.2 °C higher than at CG; the greatest stabilisation is with CA, with a T_m of 45.2 °C. All TY mismatches are also 1–2 °C more stable than TA.

3.6. Triplexes containing S.XZ triplets

Representative melting profiles for the triplexes containing the nucleoside analogue S in the third strand are shown in the bottom row of Fig. 4 and the T_m values determined from these are shown in Table 2. This nucleoside (Fig. 1a) has been designed for triplex formation at TA base pair inversions [19,20], although it shows little selectivity and also binds well to CG [18]. The first panel shows the profiles obtained for triplexes containing S.AZ triplets. It can be seen that most stable triplexes are formed opposite the three mismatches with T_m values that are between 2.4 and 3.4 °C higher than for the triplet containing an AT base pair. The second panel shows the profiles for the S.GZ triplets. Again, triplexes generated with duplex mismatches are more stable than with a GC base pair, with a difference of between 3.2–4.0 °C, which is greatest for the S.GC triplet. The melting temperatures of these triplexes are similar to those obtained for S.AZ. The third panel shows the profiles for triplexes containing S.CZ triplets. The triplexes generated at the duplex mismatches are between 1.6 and 3.6 °C more stable than with S.CG. The fourth panel shows the melting curves for triplexes containing S.TZ triplets. Again, the triplexes generated with the mismatched base pairs are more stable than with TA, although the difference is less pronounced than with CZ mismatches. Overall, it can be seen that the T_m values of triplexes containing vary between 46.8 and 53.1 °C and the most stable complexes are generated with S.CA, S.CC and S.TT triplexes.

4. Discussion

The results presented in this paper demonstrate that triplex thermal stability is affected by the identity of the base that is paired with the recognised duplex base; in some instances a duplex mismatch increase triplex affinity, while others show a decrease in stability.

4.1. Natural bases

With cytosine in the third strand the most stable triplet is generated opposite a GC base pair, generating the familiar C^+.GC triplet. Substituting the duplex C with A, G or T destabilises the triplet by at least 4 °C and this effect is greatest with GG. This contrasts with a previous study [13] which suggested that C.GA is more stable than C.GC. This difference is presumably due to the different sequence contexts in which these triplets have been assessed. In the present study the N.XZ triplets are flanked by T.AT triplets, whereas the previous study had a C^+.GC triplet on one side and T.AT on the other. The mismatches in the present study are sandwiched between two T.AT triplets and it is possible that other base pair stack environments will yield different thermal stabilities. The C.AZ, C.CZ and C.TZ triplexes are all more stable when Z is a mismatch instead of a Watson–Crick base pair. In particular C.CA and C.AA display T_m values which are 3.3 and 5 °C higher than C.CG and C.AT, respectively.

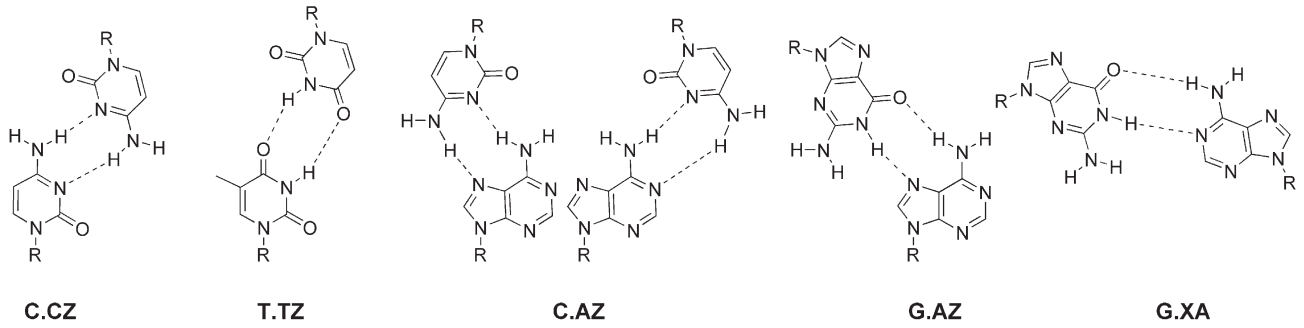

Fig. 5. Examples of the types of interaction that may be formed between a third strand base (N) and one of the base pairs (X) in an N.XZ triplet. Examples are shown for C.CZ, T.CZ, C.AZ, and G.AZ. In each case the widowed base (Z), which is not involved in base pairing, is not shown. The final example corresponds to a possible interaction of G with an A in the opposite strand, forming a G.XA triplet.

With thymine in the third strand the most stable triplex is generated with the AT-containing target, generating the T.AT triplet. Substituting the duplex T leads to a decrease in T_m of about 3–4 °C. Again this contrasts the previous results which suggested that T.AC is more stable than T.AT [13]. T also recognises GC and CG base pairs with moderate affinity. Replacing the G of T.CG decreases the stability of this triplet, while replacing the C of T.GC has little effect. This suggests that the interaction of T with GC is flexible and can tolerate some structural distortion. The weaker binding of T to CA than to CG contrasts with C, for which C.CA is more stable than C.CG. This difference is surprising since both T and C are thought to interact with CG, via the 2-carbonyl groups.

With G in the third strand the most stable triplex is formed opposite a TA base pair, generating the G.TA triplet. Positioning this base opposite a TZ mismatch decreases the T_m by about 6 °C. The stability of the G.TA triplet is known to be dependent on the formation of an additional hydrogen bond to the T of an adjacent T.AT triplet [25], and it is possible that this contact is not made when G is positioned opposite a mismatched T. G is also known to form a parallel triplet with GC base pairs [27], though this isolated triplet is less stable than G.TA. Substituting the C of the G.GC triplet for the three other bases leads a decrease in triplex thermal stability, though the destabilisation is less than that with G.TZ mismatches. This suggests that the affinity of G for GC is less dependent on the position of G. The triplets generated with G opposite AZ and CZ are more stable when Z is a mismatch and is greatest for the G.AA, G.CA and G.CC triplets.

With adenine in the third strand the most stable triplexes are generated with the GC base pair. Replacing the C of the A.GC triplet with A, G or T decreases the thermal stability. This is greatest for the purines, presumably due to their larger size, distorting the triplex structure. The triplexes generated with A opposite the AZ, CZ and TZ base pairs are more stable when Z is a mismatch and this is greatest for the A.AC and A.AG triplets.

Overall, it can be seen that the most stable triplexes are generated when T is positioned opposite AT and C opposite GC base pairs. In this sequence context no triplet combinations produced triplexes of greater thermal stability than those containing C^+.GC and T.AT triplets. In some instances the triplet is more stable when the third strand is placed opposite a duplex mismatch than at a Watson–Crick base pair. These are always found with non-canonical triplet combinations, such as C.AA and C.CA. We suggest that this is facilitated when the duplex mismatch is especially weak, and the third strand may therefore be able to form hydrogen bonds to base substituents that are normally involved in Watson–Crick base pairing. A few speculative examples are shown in Fig. 5. It should be noted that the widowed base (i.e. Z in the N.XZ triplet) which is not shown in these illustrations, can also partake in base stacking interactions which may be optimised due to the extra flexibility incurred by the lack of a base pairing partner. It should also be noted that these fluorescence melting curves were carried out at pH 5.5, at which the cytosines will be partially protonated, which could result in the formation of other competing structures (such as the i-motif). These structures, if formed, may affect the shapes of the melting curves, and the relative stability of the different triplet combinations.

4.2. Modified nucleosides

We have previously shown that BAU forms more stable triplets with AT than does T [15,16], while S recognises TA base pairs with a higher affinity than G [19,20]. Replacing the T of the BAU.AT triplet with A, G or C leads to a decrease in triplex melting temperature, as seen with T.AT. Each of these AZ duplex mismatches decreases the T_m by 4 °C, which is slightly more than most of the T.AZ mismatches. Replacing the C of the BAU.GC triplet is also destabilising, whereas the transition temperature of T.GZ is very similar for all values of Z. In contrast, positioning BAU opposite CZ or TZ increases triplex melting temperature relative to CG and TA. This is greatest with the CZ mismatches and is most notable for the BAU.CA triplet. Again this contrasts to the T.CZ triplets, where the mismatches are destabilising. Previous studies have shown that the high affinity of BAU for AT and GC base pairs is due to the interaction the 5-propargylamino and 2′-aminoethoxy groups with the phosphodiester backbone. The destabilisation exhibited by BAU against AY and GY mismatches is likely to be a consequence of altering the positioning of the backbone, disrupting these interactions. Previous studies also demonstrated that BAU exhibits

enhanced discrimination against CG base pairs. The increase in affinity of BAU compared to T for CY mismatches is likely to result from the increased flexibility of the duplex at the mismatch.

Positioning S opposite any duplex mismatch increases the triplex transition temperature, in contrast to any of the other bases studied. This is greatest for S.CA and S.CC triplets, which are 4 °C more stable than S.CG. The S.GZ and S.AZ triplex mismatches are the next most stabilising, and increase the melting temperature by 2–3 °C, depending on the mismatch. The least stabilising mismatches are formed at S.TZ, where a difference in T_m of only 1 °C is observed relative to interaction with the Watson–Crick pairs. The low selectivity of this base has previously been noted and is attributed to its conformational flexibility, an intercalative mode of binding or the presence of hydrogen bond contacts that span the entire major groove [19,20].

Acknowledgements

DAR was supported by a research studentship from EPSRC. This work was supported by grants from BBSRC and the European Union.

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