Effect of terminal amino acids on the stability and specificity of PNA–DNA hybridisation

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The effect of various charged or hydrophobic amino acids on the hybridisation of fully complementary and mismatch PNA–DNA duplexes was investigated *via* UV melting curve analysis. The results described here show that the thermal stability and binding specificity of PNA probes can be modified by conjugation to amino acids and these effects should be considered in experimental design when conjugating PNA sequences to solubility enhancing groups or cell transport peptides. Where stabilisation of a duplex is important, without there being a corresponding need for specific binding to fully complementary targets, the conjugation of multiple lysine residues to the C-terminus of PNA may be the best probe design. If, however, the key is to obtain maximum discrimination between fully complementary and mismatch targets, a replacement of glutamic acid for lysine as the routine solubility enhancing group is recommended.

Introduction

Peptide nucleic acids (PNA) are analogues of DNA with an *N*-(2-aminoethyl)glycine backbone,[1] that bind to DNA and RNA *via* Watson–Crick base-pairing rules.[2] Due to the lack of a negatively-charged backbone, hybridisation of PNA to DNA or RNA occurs without electrostatic repulsion thus binding is typically stronger and more rapid than when traditional DNA probes are used[3] and this is reflected in the increased melting temperature (T_m) of the conjugates. These properties, as well as the chemical and biological stability of PNA, make these molecules attractive for use in diagnostic and therapeutic applications, such as PCR blocker probes, PNA FISH, affinity capture and antisense technologies (described by Nielsen[4] and Stender *et al.*[3]).

One of the advantages of PNA is that in addition to hybridising single-stranded oligonucleotides, PNA can bind to double-stranded DNA by triplex formation, strand invasion or tail-clamping (Fig. 1).[6] Despite the advantages PNA offers over more traditional DNA technologies, there are certain features that still need to be improved before their applications become more widespread. One such aspect is that while PNA–DNA hybrids generally have a greater thermal and thermodynamic stability than the corresponding DNA–DNA hybrids, this difference can be small and ΔT_m's as low as 4 °C have been reported.[7] Covalent attachment of amino acids or peptides to PNA probes is one way in which the stability of PNA–DNA duplexes can be increased.

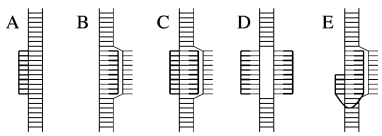

Fig. 1 Binding modes of PNA to double stranded DNA. (A) triplex, (B) duplex invasion, (C) triplex invasion, (D) double duplex invasion and (E) tail clamp by bis-PNA; adapted from Nielsen.[5]

Several groups[8–12] have investigated the effect of covalently attached peptides on the stability of DNA–DNA hybridisation. Specifically, peptides containing positively charged or neutral, hydrophobic residues have been investigated. While there has been widespread agreement that the presence and number of cationic residues influence the degree to which stability of binding is enhanced, there is some controversy over whether the position of residues alters the outcome to any extent. Similarly, while some authors have reported a stabilisation effect arising from the use of neutral hydrophobic residues,[9,11] others have reported no significant increase in binding stability by tryptophan.[8,10] Harrison and Balasrubramanian[8] found that the identity of the cationic residue was important, with increased binding stability in the order arginine > ornithine > lysine > histidine. They also noted that the stabilisation effect was greater when there was a single-stranded oligonucleotide overhang from the DNA-peptide–DNA duplex.

PNA probes are commonly synthesised with one or more C-terminal solubility enhancing groups (most often lysine).[13,14] Polycationic peptides are also frequently conjugated to PNA for use in cellular uptake.[15–17] Despite this frequent attachment of amino acids and peptides to PNA probes, there appears to have been few quantitative studies into the effect that these residues have on PNA–DNA duplex stability and sequence specificity. To date, the only studies we are aware of in this field have been with respect to bis-PNA probes for strand invasion and the attachment of short lysine chains to PNA probes for enhancement of cellular uptake. Kaihatsu *et al.*[18] investigated the attachment of 3-lysines and polycationic peptides (containing 2, 4, 6 and 8 lysine residues as AAKK, $(AAKK)_2$, $(AAKK)_3$ and $(AAKK)_4$) to bis-PNAs. They reported that the more lysine residues present

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in the peptide, the higher the efficiency of binding, with a further increase when using D-rather than natural L-amino acids. Like the unmodified bis-PNA, the bis-PNA—$(AAKK)_4$ conjugates were found to bind specifically to complementary DNA duplexes. However the authors note that the specificity of the binding of modified PNA should be examined.

In this paper we report the extent to which the incorporation of representative positively charged, negatively charged and neutral hydrophobic amino acids affects duplex stability. The impact of the resultant change in stability of single mismatched duplexes on the specificity of PNA–DNA hybridisation is also discussed.

Results and discussion

To investigate the effects of terminal amino acid residues on PNA–DNA hybridisation, we constructed a nine-residue PNA sequence (P1, H-TGTTTCTAC-NH_2) and used 15-mer oligonucleotide targets, thereby providing a 3-nucleobase overhang at both ends of the duplex with which the terminal amino acids may interact. In this work, one aim was to extend our investigations into the effects of sequence mismatches in PNA–DNA duplexes. We have previously found, not unexpectedly, that while all single-base mismatches had a destabilising effect on PNA–DNA duplexes those mismatches that were located near either terminus had a much lower thermodynamic impact on duplex stability than those located in the middle of the sequence. Under these conditions discrimination between sequences possessing a terminal mismatch and fully complementary sequences were the most difficult to distinguish (results not shown). For this study, we chose two mismatches to investigate, one being a C–G mismatch proximal to the 3′-terminus (T2) and the other a T–A mismatch proximal to the 5′-terminus (T3). These sequences were tested alongside a fully complementary target (T1), to compare the effect that terminal amino acids have on the stability and specificity of PNA–DNA hybridisation. Full details of the probes and targets used are given in Table 1. All melting curve experiments were carried out in 5 mM sodium phosphate buffer without the addition of salts so that ionic effects on charged amino acids were not introduced into the system.

As lysine is frequently used as a solubility-enhancing group for PNA probes, we chose the PNA–lysine system as our benchmark sequence for investigation into the impact of positive charges on the stabilisation and specificity of PNA–DNA hybridisation. We expected that positive charges conjugated to PNA would increase the stability of PNA–DNA duplexes due to electrostatic interactions with the negatively charged oligonucleotide backbone. Similarly, it was expected that the conjugation of negatively charged amino acids would decrease the stability of the PNA–DNA duplex. Glutamic acid was chosen as the representative negatively charged amino acid as aspartic acid can undergo cyclisation to form aspartamide during solid phase synthesis.[19] The hydrophobic amino acids phenylalanine and tryptophan have been studied previously[8–11] in relation to DNA–DNA-peptide duplex stability, with contradictory results, and were therefore also included in this analysis.

Standard melting temperature (T_m) analysis was carried out on all of the duplexes by calculating the fraction of strands existing as the duplex (a), as described by Marky and Breslauer.[20] The T_m of a duplex is the point at which $a = 0.5$ and is calculated *via* eqn (1), where A_s and A_D are the absorbance contributions from single strands and the duplex

$$a = \frac{(A_s - A)}{(A_s - A) + (A - A_D)} \quad (1)$$

respectively, calculated by linear fitting of the upper and lower baselines of the melting curve.

A typical melting curve of a duplex with a mid-range T_m and its analysis is given in Fig. 2 and all T_m values obtained in this study are listed in Table 2. The value of T_m indicates the thermal stability of the duplex and, as discussed below, for suitable systems it indirectly reflects the relative thermodynamic stability.

Thermodynamic analysis of PNA–DNA duplexes

To investigate the full thermodynamic impact of conjugated amino acids on the formation of PNA–DNA duplexes the dependence of the equilibrium constant, K, was determined from the fraction of strands in the double-stranded state (a) by eqn (2),

$$K = \frac{2a}{(1 - a)^2 C_T} \quad (2)$$

where C_T is the total concentration of strands and in this work $C_T = 2\ \mu M$.

The standard change in Gibbs free energy (ΔG°) on association is then $\Delta G^\circ = -RT \ln K$ where $R = 8.314\ J\ K^{-1}\ mol^{-1}$ (the ideal gas constant) and T is the temperature (in Kelvin).

Table 1 PNA probe and oligonucleotide target sequences

	Sequence
PNA probes	
P1	H-TGTTTCTAC-NH_2
P2	H-TGTTTCTAC-Lys-NH_2
P3	H-Lys-TGTTTCTAC-NH_2
P4	H-Lys-TGTTTCTAC-Lys-NH_2
P5	H-TGTTTCTAC-Lys-Lys-NH_2
P6	H-TGTTTCTAC-Lys-Lys-Lys-NH_2
P7	H-TGTTTCTAC-Glu-NH_2
P8	H-TGTTTCTAC-Phe-NH_2
P9	H-TGTTTCTAC-Trp-NH_2
Oligonucleotide targets	
T1	5′-GAA**GTAGAAACA**GCC-3′
T2	5′-GAA**GTAGAAATA**GCC-3′
T3	5′-GAA**G**C**AGAAACA**GCC-3′

Table 2 $T_m(a)$ for PNA–DNA thermal melting curves

	T1		T2		T3	
	T_m/°C	ΔT_m/°C[a]	T_m/°C	ΔT_m/°C[a]	T_m/°C	ΔT_m (°C)[a]
P1	38.7		26.1		26.7	
P2	44.8	+6.1	32.6	+6.5	32.6	+5.9
P3	41.1	+2.4	30.5	+4.4	28.3	+1.6
P4	44.8	+6.1	31.6	+5.5	31.5	+4.8
P5	47.2	+8.5	35.7	+9.6	37.3	+10.6
P6	54.3	+15.6	41.1	+15.0	43.6	+16.9
P7	31.1	−7.6	22.8	−3.3	23.4	−3.3
P8	37.6	−1.1	27.5	+1.4	26.4	−0.3
P9	39.8	+1.1	28.8	+2.7	27.2	+0.9

[a] Change in T_m compared to duplex with P1.

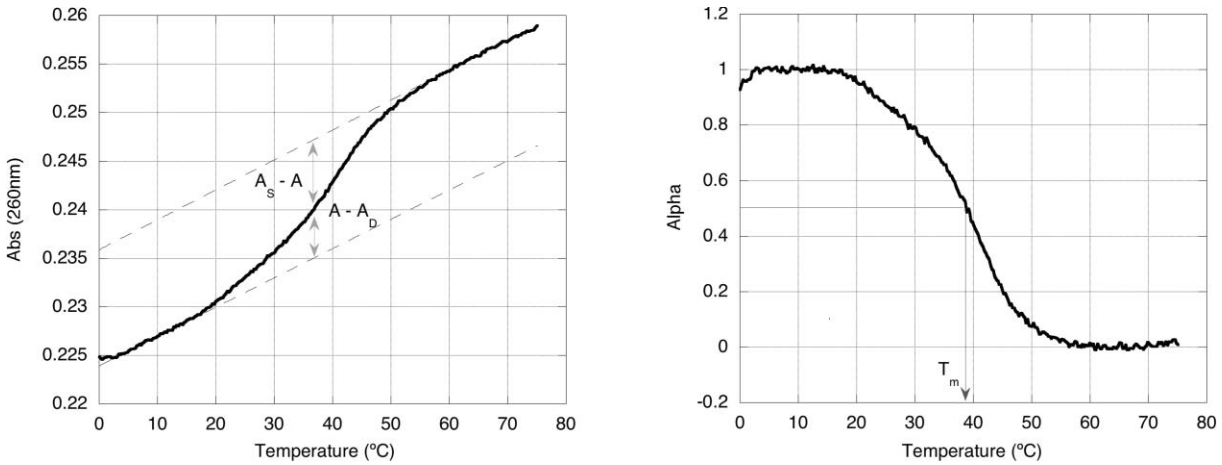

Fig. 2 A representative absorbance melting curve of 1 μM P1–T1 in 5 mM sodium phosphate buffer, pH 7.5, with fitted upper and lower baselines (left) and the resultant α curve (right).

In Fig. 3 we show the dependence of ΔG° on T for duplexes of all nine PNA probes investigated with T1, T2 and T3. For each system, the results were repeated 3 to 10 times in order to obtain an indication of the precision (the standard error for the thermodynamic data was within ±5%) and the figure shows the results of a single melting curve experiment for each case. The dependence of ΔG° on T is quite linear over the accessible temperature ranges, as would be expected. On the graph, we also show ΔG° for the case where $\alpha = 0.5$ and $C_T = 2\,\mu M$, and therefore the intercept of this line with the ΔG° lines for each system gives the T_m. Since the lines generally do not cross in this region, an increase in stability due to a more negative ΔG° will result in an increase in the T_m. This means that in these cases the T_m reflects increases in the duplex stability. T_m values are often used in the literature in discussion of stability, however caution should be used in comparing changes in T_m for different systems under different conditions.

ΔG° at 298 K is routinely used for the study of duplex stability, however we were also interested in the stability of duplexes at physiological temperature, therefore ΔG° was also obtained for 310 K. Experimental detection limitations restrict the range of T for which these values can be determined reliably, and for the most stable duplexes values of 298 K and 310 K were not accessible, as seen in Fig. 3. Linearity of the temperature dependence of ΔG° over the accessible values enabled a van't Hoff analysis (ln K *vs.* $1/T$) to interpolate or extrapolate the ΔG°_{vH} values of interest. It also allowed estimation of the van't Hoff enthalpy (ΔH°_{vH}) and entropy (ΔS°_{vH}) values, as shown in Table 3. Correlation coefficients were typically $R = 0.98$–0.998. We note that since the temperature range in the van't Hoff plot was small, the errors in ΔH° and ΔS°, determined from the standard error of several independent calculations, are quite large. However, since 298 K and 310 K are either within or close to the accessible region, errors in ΔG° from the van't Hoff analysis at these temperatures are relatively small. The ΔG° values for all duplexes at 298 K and 310 K are listed in Table 4.

In order to determine the impact on duplex stability arising from the modification of the PNA probes, the change in ΔG°_{vH} was determined ($\Delta\Delta G_{vH}$). Fig. 4 shows the results at 298 K, where a negative value indicates a more favourable free energy and an increased stability is observed for most modified PNA probes (except P7 and P8). Not surprisingly, these trends reflect the T_m results.

Fully complementary duplexes. Of all the C-terminal conjugated amino acids investigated lysine and glutamic acid resulted in the most significant change in duplex stability. As predicted, the incorporation of a single lysine residue at the C-terminus of PNA (P2) resulted in a significant increase in duplex stability, demonstrated by a decrease in $\Delta G^\circ_{298\,K}$ of 4.5 kJ mol^{-1} (from −46.0 to −50.5 kJ mol^{-1}) and an increase in the T_m of approximately 6 °C (from 38.7 to 44.8 °C) with respect to the unmodified PNA (P1). Conversely, the incorporation of a glutamic acid at the C-terminus (P7) resulted in an increase in $\Delta G^\circ_{298\,K}$ of 5.2 kJ mol^{-1}

Table 3 Thermodynamic data (van't Hoff analysis) for fully complementary PNA–DNA duplexes. Standard errors are shown in brackets

	ΔH°_{vH}/kJ mol^{-1}	ΔS°_{vH}/kJ mol^{-1}	$\Delta G^\circ_{298\,K}$/kJ mol^{-1}	$\Delta G^\circ_{310\,K}$/kJ mol^{-1}
P1	−228 (3)	−612 (9)	−46.0 (0.3)	−38.3 (0.3)
P2	−232 (10)	−610 (33)	−50.5 (0.8)	−42.9 (0.4)
P3	−263 (16)	−715 (49)	−49.7 (1.0)	−41.4 (0.4)
P4	−238 (18)	−628 (57)	−51.0 (1.5)	−43.3 (0.9)
P5	−238 (19)	−621 (58)	−52.8 (1.5)	−45.5 (0.8)
P6	−274 (12)	−716 (36)	−60.8 (1.7)	−52.0 (1.3)
P7	−232 (12)	−642 (40)	−40.8 (0.9)	−33.0 (0.6)
P8	−232 (13)	−627 (40)	−45.6 (0.8)	−37.6 (0.3)
P9	−271 (16)	−746 (51)	−49.0 (1.0)	−39.4 (1.0)

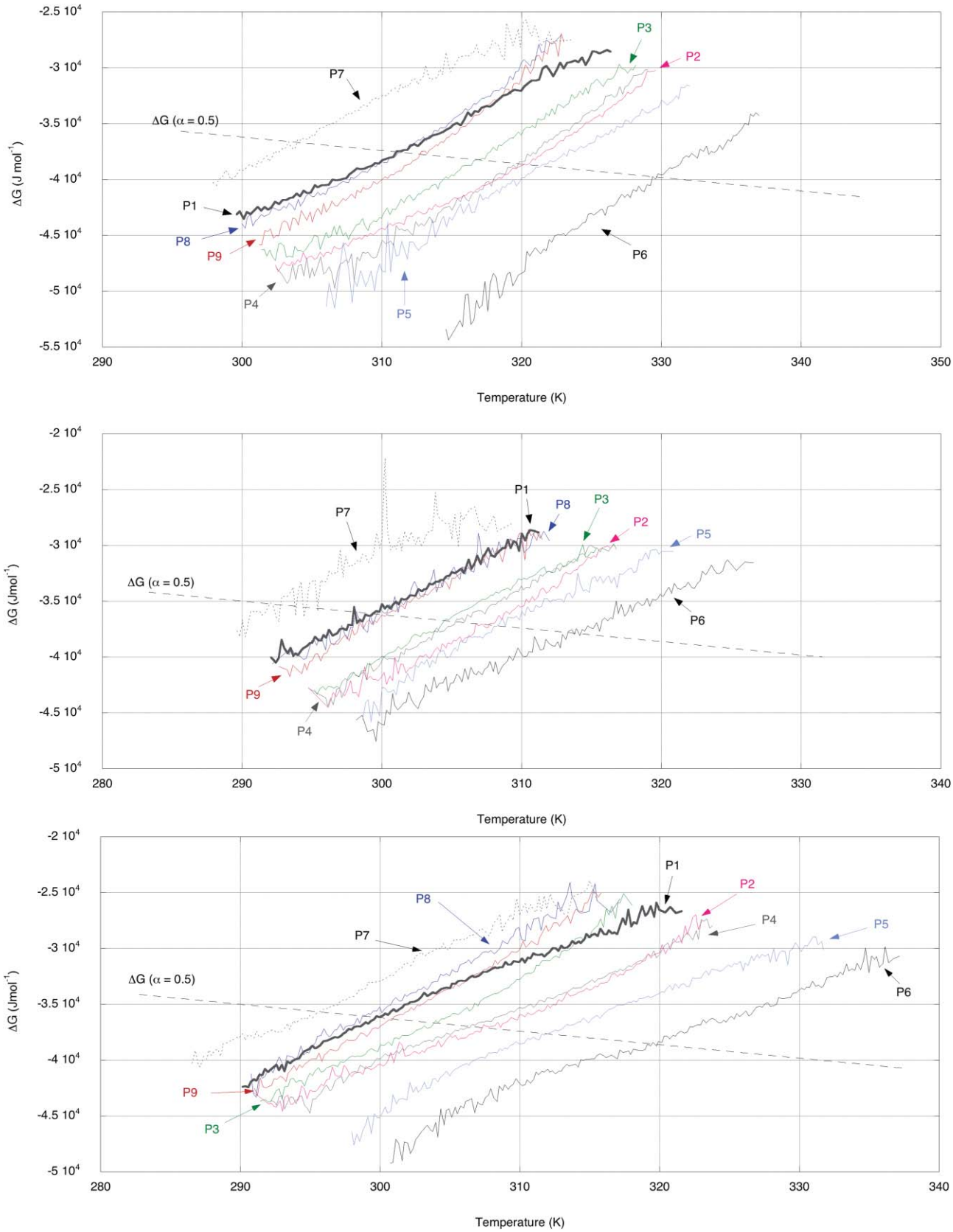

Fig. 3 ΔG° as a function of temperature for duplexes of all nine PNA probes with (top) T1, (middle) T2 and (bottom) T3. Also marked is the ΔG° line for $\alpha = 0.5$ and $C_T = 2\ \mu M$.

Table 4 ΔG° values for 298 K and 310 K for all single complementary and mismatch duplexes

	T1		T2		T3	
	$\Delta G^\circ_{298\,K}$/kJ mol^{-1}	$\Delta G^\circ_{310\,K}$/kJ mol^{-1}	$\Delta G^\circ_{298\,K}$/kJ mol^{-1}	$\Delta G^\circ_{310\,K}$/kJ mol^{-1}	$\Delta G^\circ_{298\,K}$/kJ mol^{-1}	$\Delta G^\circ_{310\,K}$/kJ mol^{-1}
P1	−46.0	−38.3	−37.0	−30.5	−37.4	−30.3
P2	−50.5	−42.9	−41.4	−34.6	−41.4	−35.2
P3	−49.7	−41.4	−40.7	−32.0	−37.9	−32.1
P4	−51.0	−43.3	−40.9	−33.9	−39.4	−34.7
P5	−52.8	−45.5	−45.2	−36.9	−43.2	−38.3
P6	−60.8	−52.0	−47.0	−40.3	−46.0	−40.9
P7	−40.8	−33.0	−34.5	−29.2	−34.8	−28.0
P8	−45.6	−37.6	−38.0	−30.1	−37.4	−30.1
P9	−49.0	−39.4	−38.9	−31.2	−37.6	−30.7

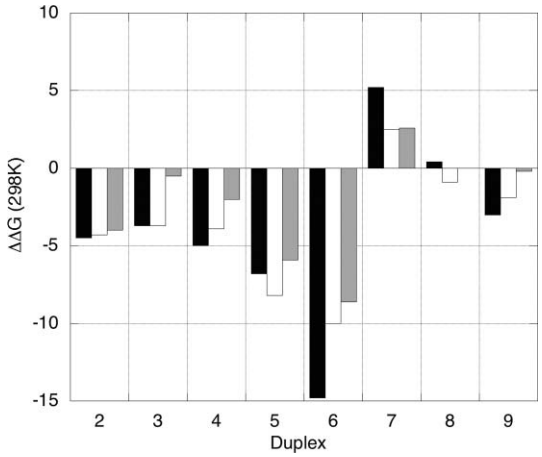

Fig. 4 Change in the free energy for duplexes of P2, P3, P4, P5, P6, P7, P8 and P9 compared to P1. Duplexes with the fully complementary target T1 are shown in black, and with the mismatched targets T2 and T3 in white and grey respectively.

(from −46.0 to −40.8 kJ mol^{-1}) and a decrease in the T_m of the duplex of over 7 °C (from 38.7 to 31.1 °C). The increased stability of P2 duplexes and the decreased stability of P7 duplexes at physiological temperature, compared to unmodified PNA, are suggested by the change in the T_m for the respective duplexes. This is supported by the change in $\Delta G^\circ_{310\,K}$, of −4.6 and +5.3 kJ mol^{-1} for P2 and P7 respectively, which is comparable to $\Delta\Delta G^\circ_{298\,K}$. These changes in the thermodynamic stability of the PNA–DNA duplexes with respect to physiological temperature are important when determining the suitability of PNA probes for use in cellular systems. Whilst the conjugation of either a positive or negatively charged moiety at the C-terminus of PNA probes alters duplex stability as expected, what was not expected was the magnitude of the destabilising effect of glutamic acid. The two hydrophobic amino acids investigated, phenylalanine and tryptophan, caused no significant change in the duplex thermal stability with only a 1.1 °C decrease and increase in the T_m respectively (a change essentially within the standard error range of ±1 °C). As the T_m for both of these duplexes were close to physiological temperature, the change in ΔG° at 310 K was also very small (+0.7 kJ mol^{-1} for P8 and −1.1 kJ mol^{-1} for P9). However, the shape of the melting transition resulted in an increase in stability of P9 at 298 K with a $\Delta\Delta G^\circ_{298\,K}$ of −3.0 kJ mol^{-1}. The small size of the effects shown for P8 and P9 may explain the controversy in past literature on the stabilisation contribution by tryptophan and phenylalanine.

Single base mismatches. The introduction of terminal single mismatches into the PNA–DNA duplexes universally caused a decrease in the stability of the duplex with an increase in $\Delta G^\circ_{298\,K}$ of approximately 9 kJ mol^{-1}, and a decrease in T_m of the unmodified PNA mismatch duplexes of approximately 12 °C, compared with the fully complementary duplex. Conjugation of a lysine to the C-terminus of PNA resulted in a decrease in $\Delta G^\circ_{298\,K}$ of approximately 4 kJ mol^{-1} and an increase of about 6 °C for single mismatch duplexes compared to duplexes of the same target with unmodified PNA. As was the case with the fully complementary duplexes, the incorporation of glutamic acid into the PNA sequence resulted in a decrease in the duplex stability with an increase in $\Delta G^\circ_{298\,K}$ of about 2.5 kJ mol^{-1} and a decrease in the T_m of 3.3 °C compared to unmodified PNA, for duplexes with those single mismatched bases.

Whilst it would be expected that a C–G mismatch would cause a greater destabilising effect than an A–T mismatch, such was not seen to be the case in these experiments. The melting temperatures for duplexes of PNA with T2 (3′ C–G mismatch) and T3 (5′ A–T mismatch) were mostly within 1.1 °C of each other (for each individual PNA, both modified and unmodified) and where the $\Delta\Delta G^\circ_{298\,K}$ was more significant in some cases, the T2 duplexes generally had a more favourable $\Delta G^\circ_{298\,K}$. However, the other important difference in the duplexes of PNA with T2 and T3 targets is the terminal location of the mismatches (the mismatch occurring near the N-terminus of PNA for PNA–T2 duplexes and near the C-terminus for PNA–T3 duplexes) and this may have some impact on the extent of duplex destabilisation, although we have not investigated this further.

Importance of the position and number of positively charged residues

Amino acids are typically attached to the C-terminus of a PNA sequence so that in addition to the solubility-enhancing effects of the free sequence, PNA–bead aggregation and steric hindrance are reduced during synthesis.[21] However, there are several reports of amino acids or peptides being conjugated to the N-terminus of PNA sequences.[16,22] To investigate whether the terminus to which the amino acids are attached impacts on the stabilisation effect, PNA with an N-terminal lysine was synthesised (P3). We found that while an N-terminal lysine increases the stability of the duplex ($\Delta\Delta G^\circ_{298\,K}$ of −3.7 kJ mol^{-1} and ΔT_m of +2.4 °C for the T1 duplex), it does so to a lesser degree than a C-terminal lysine ($\Delta\Delta G^\circ_{298\,K}$ of −4.5 kJ mol^{-1} and ΔT_m of +6.1 °C), as shown in Tables 2 and 4 and

by comparing duplexes 2 and 3 in Fig. 4. One possible explanation for this effect might be interactions between the terminal amino acid residue and the PNA dipole.

As the stabilisation effect of a single lysine residue is dependent on the position of its attachment to the PNA, we were interested in the outcome of a PNA probe with lysine conjugated to both the N- and C-termini (P4). The T_m results for these duplexes were surprising. Whilst the P3 sequence causes an increase in duplex stability, the $\Delta G^\circ_{298\,K}$ of P4 is approximately the same as that of P2, and therefore the effect from each terminal lysine is not additive. Multiple lysine residues incorporated onto PNA at the same terminus however, does result in a step-wise increase in duplex stability, with the greatest increase occurring with addition of the first and third lysine. This difference is more exaggerated with fully complementary duplexes (a decrease in $\Delta G^\circ_{298\,K}$ of 4.5, 2.3 and 8 kJ mol^{-1} and an increase of 6.1, 2.4 and 9.5 °C with the addition of the first, second and third lysine respectively) but is also evident for all mismatch duplexes (Tables 2 and 4 and Fig. 4).

Stability, specificity and discrimination

The ΔG° results obtained using PNA probes with conjugated lysine residues suggest that lysine is the best solubility enhancing group to use when the aim is to improve the discrimination between fully complementary and mismatch duplexes. The difference in ΔG° between the mismatched duplexes with T2 and T3, compared to that of the fully complementary with T1, for P1 was 9 and 8.6 kJ mol^{-1} respectively. These increased to 9.1 kJ mol^{-1} for P2 and 13.8 and 14.8 kJ mol^{-1} for P6, whilst decreasing to 6.3 and 6.0 kJ mol^{-1} for P7. These results suggest that probes with three lysine residues conjugated to the C-terminus provide maximum thermodynamic stability and discriminatory power. The difference in ΔG° between fully complementary and mismatch duplexes however is not the only consideration, it is also important to examine the absolute values of the ΔG° for each duplex. The increased stability obtained with P6 duplexes results in a more favourable $\Delta G^\circ_{298\,K}$ of P6–T2 and P6–T3 duplexes to −47 and −46 kJ mol^{-1} respectively, which are comparable to the $\Delta G^\circ_{298\,K}$ of P1–T1 at −46 kJ mol^{-1}. This indicates that at room temperature, at which many biological screening applications are performed, PNA probes such as P6 will bind stably with mismatch duplexes as well as complementary duplexes. Conversely, the addition of a glutamic acid to the C-terminus of PNA probes decreases the stability of mismatched duplexes sufficiently to suggest that applications at room temperature will not result in enhanced binding of the mismatched duplexes.

Conclusions

Given that there has been avid interest in the use of PNA as biological reagents in the medicinal chemistry field, it is necessary that the stability and specificity of these molecules are investigated. Amino acids and peptides are often conjugated to PNA molecules to assist in their delivery or synthesis[23] therefore it is important to understand the effects these conjugated amino acids may have on the function of the PNA molecules. The routine use of amino acids as a solubility enhancing group for PNA probes also invites the question of what effect this modification will have on the ability to detect a target sequence with high levels of discrimination.

We found that, as expected, the conjugation of lysine to the C-terminus of PNA probes increases the stability of fully complementary and mismatched duplexes, while a glutamic acid decreases duplex stability. With the increase in the thermodynamic stability of duplexes however, a corresponding increase in experimental temperature is required to maintain the ability to differentiate between complementary and mismatched duplexes. For the PNA sequence studied, three C-terminal lysine residues increased the T_m of all duplexes above physiological temperature (37 °C) and decreased the $\Delta G^\circ_{310\,K}$ to less than −40 kJ mol^{-1} for the mismatched duplexes, which could increase the occurrence of non-specific effects in some experiments. One such consideration is that the use of conjugated cationic peptides is commonplace for cellular delivery of PNA,[15–17] therefore there may be problems with specificity of action within the cell if the peptide is attached *via* a permanent link rather than a cleavable or temporary linker, creating a probe able to bind stably to many mismatched sequences. This added stability of the single mismatch duplexes suggests that screening experiments, usually performed at or around 298 K, using these probes will result in unsatisfactory discrimination between fully complementary and mismatched target molecules.

These results show that consideration must be given to the design of experiments using PNA for detection and antisense applications, to ensure that addition of solubility enhancing groups or transport peptides do not adversely affect the efficacy of the probe. In the majority of experiments where maintaining sequence specificity (*i.e.* discriminatory power) of PNA–DNA hybridisation is important, the routine use of lysine as a solubility enhancing group for PNA should be replaced by the use of glutamic acid.

Experimental

Materials

All oligonucleotides were obtained from Sigma-Genosys (Sigma-Aldrich, Australia). Fmoc-protected PNA monomers were purchased from Applied Biosystems (Australia) and Fmoc-protected amino acid monomers from Auspep and Novabiochem (Australia). All PNA synthesis solvents were peptide synthesis or biotech grade and supplied by Sigma-Aldrich (Australia) and Auspep (Australia). HATU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) was purchased from GT Biochem (China) and PAL resin from Advanced ChemTech (Kentucky, USA). HPLC grade acetonitrile was purchased from LabScan (Thailand). Biotech grade buffer components were supplied by Sigma-Aldrich (Australia).

PNA Synthesis

PNA was synthesised on an Advanced ChemTech Omega 396 peptide synthesiser (Kentucky, USA). Each cycle consisted of 2 min deprotection (20% piperidine in dry DMF), 30 min coupling (125 μL 0.2 M PNA monomer in dry NMP or 125 μL 0.5 M amino acid monomer in dry DMF; 125 μL 0.3 M DIPEA, 0.2 M lutidine in dry DMF; 125 μL 0.19 M HATU in dry DMF) and 2 min capping (6% acetic anhydride, 5% lutidine in dry DMF) with DMF washes in between each. The first monomer was double-coupled onto the resin after swelling of the resin with dry DMF

(30 min). PNA was purified by reverse-phase HPLC using an Agilent Zorbax 300SB C18 5 μm, 9.4 × 250 mm column at 55 °C with a flow rate of 1 mL min^{-1}, monitoring at 260 nm using a 0.5% TFA water (A)–acetonitrile (B) gradient.

Thermal melting curves

DNA melting curves were carried out in 1 cm path length quartz cells on a Varian Cary 300 spectrophotometer with a peltier temperature controller. Samples consisted of 1 μM of each appropriate PNA and oligonucleotide, in 5 mM sodium phosphate buffer (pH 7.5). Samples were annealed by heating at 90 °C for 5 min before cooling at 20 °C for 20 min and for a further 10 min at 0 °C. The thermal melting curves were measured with a temperature ramp of 0 °C to 75 °C at a rate of 0.5 °C min^{-1}, with data points collected every 0.2 °C, monitoring at 260 nm. Melting temperature and thermodynamic analysis was performed using calculations for α (fraction of duplex molecules) and van't Hoff analysis as described previously.[20,24] Each duplex melting curve was performed in triplicate, as were the calculations for each individual curve. Error in T_m calculation is approximately ±1 °C and for $\Delta G°$ thermodynamic parameters approximately ±2%.

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