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Base pairing involving deoxyinosine: implications for probe design

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ABSTRACT

The thermal stability of oligodeoxyribonucleotide duplexes containing deoxyinosine (I) residues matched with each of the four normal DNA bases were determined by optical melting techniques. The duplexes containing at least one I were obtained by mixing equimolar amounts of an oligonucleotide of sequence dCA_3XA_3G with one of sequence dCT_3YT_3G where X and Y were A, C, G, T, or I. Comparison of optical melting curves yielded relative stabilities for the I-containing standard base pairs in an otherwise identical base-pair sequence. I:C pairs were found to be less stable than A:T pairs in these duplexes. Large neighboring-base effects upon stability were observed. For example, when (X,Y)=(I,A), the duplex is eight-fold more stable than when (X,Y)=(A,I). Independent of sequence effects the order of stabilities is: I:C > I:A > I:T ≈ I:G. This order differs from that of deoxyguanosine which pairs less strongly with dA; otherwise each deoxyinosine base pair is less stable than its deoxyguanosine counterpart in the same sequence environment. Implications of these results for design of DNA oligonucleotide probes are discussed.

INTRODUCTION

Hypoxanthine, the base found in the nucleosides inosine and deoxyinosine, behaves approximately as a guanine analog in nucleic acids. Poly(rI) and poly(dI) form stable double helices with poly(rC) and poly(dC) (1), and serve as template for the incorporation of cytosine into products of DNA and RNA polymerases (2-4). Inosine occurs naturally in the wobble position of the anticodon of some transfer RNA's, where it appears to pair with adenosine in addition to cytidine and uridine, the nucleosides which pair with guanosine in that position. The conversion of deoxyadenosine to deoxyinosine during nitrous acid mutagenesis (5-7) results in AT → GC transitions, presumably because deoxyinosine tends to pair with deoxycytidine.

There is a large uncertainty in the stability of base pairing of deoxyinosine with the four natural DNA bases. Thermodynamic studies of the sort used to calculate secondary structure stability (8,9) have not been

published for inosine-containing oligonucleotides. Polymer studies provide evidence that dI:dC base pairing is relatively weak and is sequence dependent. The T_m of the homopolymer duplex poly(dI:dC) was reported to be about 8 degrees lower than that of the alternating copolymer poly [d(I-C)] (10,11). Both were significantly less stable than the corresponding dA:dT-containing polymer duplexes.

Knowledge of the base-pairing energies of deoxyinosine with the four normal bases would be of use in the design of oligonucleotide probes. If dI is less specific in its base pairing than the normal four bases, it could be placed at those positions in the probe where the base in the gene being sought is unknown. Such ambiguities arise when the genomic sequence is not known, but is being deduced from a known peptide sequence: the genomic sequence is ambiguous at positions where the genetic code is redundant. The number of different possible sequences of appropriate length for probing (usually a minimum of 17 or 20 bases) corresponding to even the optimal region of the known peptide sequence is usually large. Very many probes have to be used in order to insure that the one which yields exact base pairing has been found. Even a single mismatch can greatly reduce thermal stability of the probe-target duplex (12). Simultaneous screening with mixtures of probes reduces the number of experiments, but interpretation is difficult because the stability of exact base pairing cannot be predicted since the correct base sequence is not known in advance. Tetralkylammonium salts have been used to reduce this unpredictability by selectively stabilizing dA:dT pairs relative to dG:dC pairs (13). Even so, the use of mixed probes results in higher backgrounds and increased radioactivity levels. Ultimately, the length of the probes, (and, as a result, the selectivity and stability of the probe hybrids) is limited by the rapid increase in the number of different possible sequences with probe length.

An alternative approach is to use a longer but unique probing sequence (14,15). The probability of mismatches is reduced by making use of strong codon preferences (16), if any, in the sequence; and the nucleotide used at a position of ambiguity is chosen to minimize the difference in stability of matched and mismatched bases. This approach has been hindered by the limited availability of information on the stability of mismatched base pairs in DNA. Deoxyguanosine has been used opposite dC/dT ambiguities in the belief that dG:dT wobble pairs would be relatively stable (15), but the difference in stability between dG:dC and dG:dT has

been observed to be large (17,18). An examination of the factors involved in the unique probe approach has recently been published (19).

More information on stabilities of mismatches is needed to improve probe design. It would be most useful to find base analogs which would be less discriminatory in base pairing than the normal four bases, so unique sequence probes of greater and more predictable stability could be designed for gene isolation, or at least so that smaller number of mixtures of probes would suffice. Inosine, because it seems to pair less strongly with C and more strongly with A than guanosine does, is a candidate for this purpose. Recently, unique probes containing deoxyinosine at all positions of ambiguity were used in the isolation of the human cholecystokinin gene (4,20). Preliminary data supported the idea that inosine might be an "inert" base, its matched or mismatched base pairs neither stabilizing nor destabilizing a duplex.

We have measured the stabilities of a set of deoxyoligonucleotide duplexes containing each of the four normal DNA bases paired with deoxyinosine. The contributions of matched and mismatched deoxyinosine base pairs to duplex stability have been calculated. Comparison to results obtained with similar duplexes containing only normal bases, described in a previous paper, (18) allows evaluation of the relative base-pairing specificity of deoxyinosine and its possible utility in probes at positions of base ambiguity.

MATERIALS AND METHODS

Deoxyoligonucleotides were synthesized by the phosphoramidite method (21). Dimethoxytrityl deoxyinosine was synthesized from deoxyinosine and dimethoxytrityl chloride by standard methods, but dimethylsulfoxide was used as solvent instead of methylene chloride due to the insolubility of deoxyinosine in the latter solvent. Oligomers were purified by RPC-5 chromatography after deblocking.

Melting curves were obtained and analyzed as described previously (18).

RESULTS

Each oligodeoxyribonucleotide exhibited gradual absorbance increases upon heating, as expected for single strand melting. Melting of 1:1 mixtures of appropriate pairs of oligomers exhibited the sigmoidal

Table I. Van't Hoff Thermodynamic Values for Double Helix Formation of Deoxyinosine Containing $dCA_3XA_3G+dCT_3YT_3G$ in 1 M NaCl, pH7.

X•Y	ΔG°, 25° C (kcal mol^{-1})[a]	ΔH° (kcal mol^{-1})[b]	ΔS° (cal $deg^{-1} mol^{-1}$)[c]	T_m(°C)[d] C_T=400μM
I•C	-8.8	-66	-191	41°
C•I	-8.1	-58	-168	39°
-•-[e]	-7.8	-59	-172	37°
I•A	-7.5	-63	-186	35°
I•G	-6.3	-57	-168	30°
A•I	-6.3	-48	-141	30°
I•T	-5.9	-58	-176	27°
T•I	-5.8	-50	-147	27°
G•I	-5.7	-52	-154	26°
I•I	-5.7	-47	-140	27°

[a]Estimated precision in ΔG° is ± 0.1 kcal mol^{-1}
[b]Estimated precision in ΔH° is ± 0.3 kcal mol^{-1}
[c]Estimated precision in ΔS° is ± 9 cal deg^{-1} mol^{-1}
[d]Estimated precision in T_m is ± 1°
[e]dCA_6G •dCT_6G. Data from Morden et al., (1983) Biochemistry 21, 428-436.

absorbance-temperature profiles usually observed for double helix-single strand transitions.

Thermodynamic parameters for the helix-coil transition of all nine deoxyinosine-containing duplexes were calculated from the absorbance curves as in our earlier work (18) and are shown in Table I. In Table II, nearest-neighbor contributions to double strand formation are listed for dI:dC pairs, whereas in Table III, nearest-neighbor contributions have been calculated for the mismatched duplexes, treating the mismatched base pair as a two base internal loop, in accordance with convention (18). In terms of nearest neighbor interactions, the duplexes may be considered as equivalent to dCA_5G:dCT_5G plus either two additional base pair stacking interactions, $\left(\begin{smallmatrix}AX\\TY\end{smallmatrix}\right)$ + $\left(\begin{smallmatrix}XA\\YT\end{smallmatrix}\right)$, or a two base internal loop, $\left(\begin{smallmatrix}A\text{-}X\text{-}A\\T\text{-}Y\text{-}T\end{smallmatrix}\right)$. We have chosen the former treatment for dI:dC pairs (matched base pairs) and have treated the other dI oppositions as internal loops (mismatched bases),

Table II. Neareast-neighbor Contributions of Deoxyinosine to Double Strand Formation in 1 M NaCl, pH 7.[a]

Nearest Neighbor	ΔG°, 25 °C (kcal mol^{-1})	ΔH° (kcal mol^{-1})	ΔS° (cal $deg^{-1} mol^{-1}$)
$\frac{1}{2}\left(\begin{smallmatrix}-A-I-\\ -T-C-\end{smallmatrix} + \begin{smallmatrix}-I-A-\\ -C-T-\end{smallmatrix}\right)$	-1.1 ± 0.2	-9.3	-27
$\frac{1}{2}\left(\begin{smallmatrix}-A-C-\\ -T-I-\end{smallmatrix} + \begin{smallmatrix}-C-A-\\ -I-T-\end{smallmatrix}\right)$	-0.8 ± 0.2	-5.3	-15

[a]The values given are for the reaction $\begin{smallmatrix}-W_1 & & W_2-\\ & \diagdown\diagup & \\ -C_1 & & C_2-\end{smallmatrix}$ → $-W_1-W_2-$ + $-C_1-C_2-$

in order to maintain consistency with earlier treatments. The distinction between matched pairs and mismatched bases is merely formal, and is summarized by the mathematical relations below:

$$\Delta G^0(dCA_3XA_3G{:}dCT_3YT_3G) - \Delta G^0(dCA_5G{:}dCT_5G) =$$
$$= \Delta G^0\left(\begin{smallmatrix}AX\\ TY\end{smallmatrix}\right) + \Delta G^0\left(\begin{smallmatrix}XA\\ YT\end{smallmatrix}\right) \quad \text{(matched pairs)}$$
$$= \Delta G^0\left(\begin{smallmatrix}A-X-A\\ T-Y-T\end{smallmatrix}\right) \quad \text{(mismatched bases)}$$

It should be noted that while $dCA_5G{:}dCT_5G$ is the appropriate reference compound for computation of nearest-neighbor stacking interactions (Tables II and III), we have chosen $dCA_6G{:}dCT_6G$, which lacks only the X:Y pair, as

Table III. Destabilization of Double Helices by Deoxyinosine in Base-Base Mismatches or Wobble Base Pairs.[a]

Mismatch/ Wobble	ΔG°, 25°C (kcal mol^{-1})	ΔH° (kcal mol^{-1})	ΔS° (cal deg^{-1} mol^{-1})
$\begin{smallmatrix}-A-I-A-\\ -T-A-T-\end{smallmatrix}$	-1.0	-15.5	-48
$\begin{smallmatrix}-A-A-A-\\ -T-I-T-\end{smallmatrix}$	+0.2	-0.5	-3
$\begin{smallmatrix}-A-I-A-\\ -T-G-T-\end{smallmatrix}$	+0.2	-9.5	-30
$\begin{smallmatrix}-A-I-A-\\ -T-T-T-\end{smallmatrix}$	+0.6	-10.5	-38
$\begin{smallmatrix}-A-T-A-\\ -T-I-T-\end{smallmatrix}$	+0.7	-2.5	-9
$\begin{smallmatrix}-A-G-A-\\ -T-I-T-\end{smallmatrix}$	+0.8	-4.5	-16
$\begin{smallmatrix}-A-I-A-\\ -T-I-T-\end{smallmatrix}$	+0.8	0.5	-2

[a]The values are obtained by subtracting nearest-neighbor contributions present in $dCA_5G\cdot dCT_5G$ from the data given in Table I. See reference 18.

Table IV. Average Net Stability and Base Discrimination Free Energies of Probe Bases at Two-Fold Codon Redundancies at 25°C and 50°C[a,b]

Base Ambiguity in Target DNA	Probing Base (X)	Average Net Free Energy of Stabilization (kcal mol^{-1})[c] 25°C	50°C	Discrimination Free Energy (kcal mol^{-1})[d] 25°C	50°C
A/G	T	+0.4	+0.3	2.9	2.1
	C	+0.8	+0.3	5.3	3.6
	I	+1.5	+1.4	0.9	0.9
T/C	G	0	0	3.6	2.9
	I	+0.8	+0.9	2.6	2.2
	A	+1.2	+0.7	4.6	2.8
A/C	G	+0.2	0	4.0	2.9
	I	+0.3	+0.4	1.6	1.2
	T	+0.9	+0.9	3.9	3.3
	A	+3.2	+1.8	0.5	0.6
	C	+3.5	+2.8	0	1.5
G/T	C	+0.5	+0.5	4.6	4.0
	A	+0.6	+0.4	3.3	2.1
	G	+1.8	+1.4	0.1	0
	I	+2.1	+1.9	0.2	0.2
	T	+2.4	+2.2	1.2	1.6

[a]Data for natural base pairs is from Table I, reference 18.

[b]$\Delta G°(50°C) = \Delta G°(25°C) - 25\Delta S°$.

[c]Average Net Free Energy of Stabilization = Difference between the average free energy of the four duplexes which contain the probe base paired with the two possible target bases and the free energy of the reference duplex $dCA_6G{+}dCT_6G$. The reference values for $dCA_6G{\cdot}dCT_6G$ of $\Delta G°(25°C)=-8.0$ kcal mol^{-1} and $\Delta G°(50°C)=-3.8$ kcal mol^{-1} are obtained by a least squares fit to data for $dCA_nG{\cdot}dCT_nG$, n=5,6,7 (Ref. 18). For example for T probing A/G, the net free energy is $\frac{1}{4}[\Delta G^0(G{\cdot}T) + \Delta G^0(T{\cdot}G) + \Delta G^0(A{\cdot}T) + \Delta G^0(T{\cdot}A)] - \Delta G^0$ (reference).

[d]For example the discrimination free energy for T probing A/G is

$$\frac{1}{2}[\Delta G^0(G{\cdot}T) + \Delta G^0(T{\cdot}G)] - \frac{1}{2}[\Delta G^0(A{\cdot}T) + \Delta G^0(T{\cdot}A)].$$

the reference duplex for Tables IV and V to allow a more direct intuitive determination of the stabilizing effect of each X:Y pair.

DISCUSSION

The results reported in Table I give information on the stability of base pairs containing deoxyinosine matched with each of the four normal bases, each in two orientations in the duplexes $dCA_3XA_3G{:}dCT_3YT_3G$. The oligonucleotide duplex lacking the central X:Y pair, $dCA_6G{:}dCT_6G$, is included for comparison; an "inert" X:Y base pair would give a duplex with the same stability as $dCA_6G{:}dCT_6G$. As expected, I:C pairs are more stable

Table V. Stability and Base Discrimination Free Energies by Probes at Four-fold Codon Redundancies.[a,b]

Stable Probing Base	Average Net Free Energy of stabilization (kcal mol^{-1})[c] 25°C	50°C	Average Base Discrimination Free Energy (kcal mol^{-1})[d] 25°C	50°C	Free Energy Difference Between Least and Most Stable (kcal mol^{-1})[e] 25°C	50°C
G	+1.0	+0.7	3.7	2.9	4.0	2.9
I	+1.2	+1.1	2.2	1.8	2.6	2.0
T	+1.7	+1.6	3.7	3.0	4.1	3.7
A	+1.9	+1.1	4.0	2.4	4.6	2.8
C	+2.0	+1.7	5.1	4.2	5.3	5.1

[a]Data for natural base pairs is from Table I, reference 18.

[b]$\Delta G°(50°C) = \Delta G°(25°) - 25\Delta S°$

[c]Difference between average free energy of the eight duplexes which contain the probing base matched with each of the four natural bases in both orientations and the free energy of the reference duplex $dCA_6G \cdot dCT_6G$.

[d]Difference between free energy of Watson-Crick duplex and the average free energy of the three non Watson-Crick duplexes containing the probing base, averaged over both orientations, e.g. for A,

$$\tfrac{1}{2}\{\tfrac{1}{3}[\Delta G^0(A\cdot C) + \Delta G^0(A\cdot G) + \Delta G^0(A\cdot A)] - \Delta G^0(A\cdot T) + \tfrac{1}{3}[\Delta G^0(C\cdot A) + \Delta G^0(G\cdot A) + \Delta G^0(A\cdot A)] - \Delta G^0(T\cdot A)\}$$

[e]Averaged over both orientations for example, in case of

$$A = \tfrac{1}{2}\{\Delta G^0(A\cdot C) + \Delta G^0(C\cdot A) - \Delta G^0(A\cdot T) - \Delta G^0(T\cdot A)\}$$

than the other I-containing pairs, but they contribute less stability than standard Watson-Crick pairs. Replacing I:C in the duplex by G:C increases the stability by an average of -1.4 kcal mol^{-1} in standard free energy at 25°C; replacing I:C by A:T the average increase in stability is -0.6 kcal mol^{-1} (18). Insertion of an I:A pair into the middle of a dCA_6G:dCT_6G duplex is only slightly destabilizing in one orientation, but the other mismatches are more strongly destabilizing. The decrease in stability from dCA_6G:dCT_6G ranges from +1.5 kcal mol^{-1} (X:Y = I:G, A:I) to +2.1 kcal mol^{-1} (X:Y = G:I, I:I) in standard free energy at 25°C. Most mismatches in the same duplex not involving I are more destabilizing (18); for example, a C:C or A:C mismatch reduces the stability by +3.3 kcal mol^{-1} in free energy at 25°C.

The relative stabilities of the various base oppositions depend on the number of hydrogen bonds that can be formed within the constraints of the glycosidic bonds, and on the stacking interactions with the neighboring bases. Possible hydrogen bonding schemes are shown in Figure 1. We note

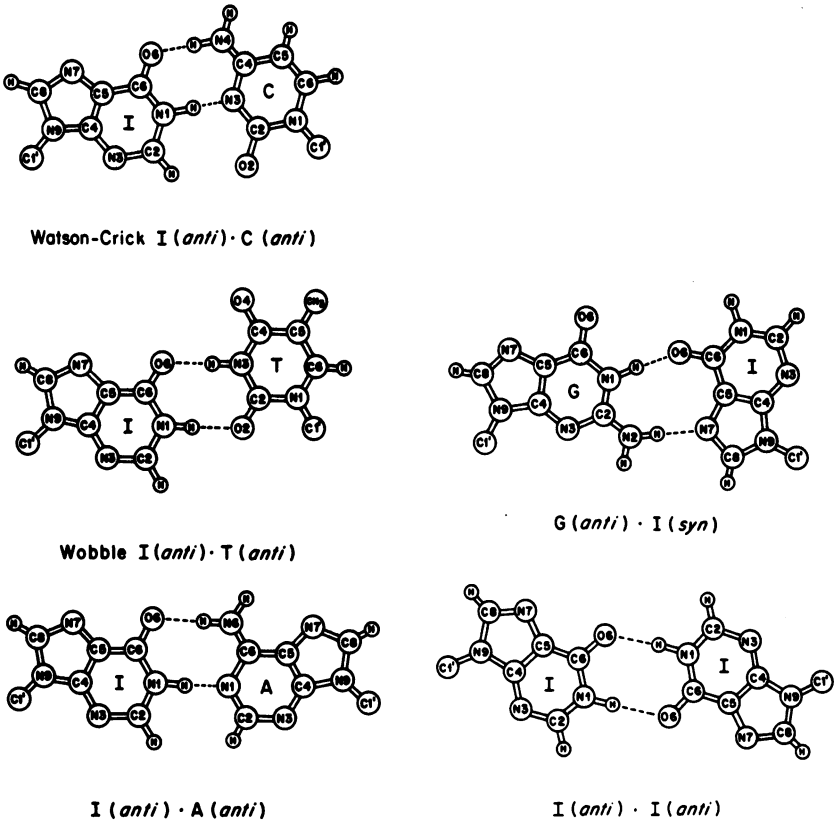

Fig. 1. Postulated base-pairing schemes for pairs containing deoxyinosine. The pairs I:C, I:T and I:A are exact analogs of G:C, G:T and G:A.

that two hydrogen bonds per base opposition can be drawn for all pairs. This is in contrast to A:C and C:C for which at most one hydrogen bond is reasonable. The first three base pairs in Figure 1 are precise analogs of established G-containing pairs (Watson-Crick G:C, wobble G:T and G:A) (22,23). The G:I pair is drawn by analogy with the G:G pairing proposed for a quadruple-stranded poly rG structure (24). The I:I pair is the most distorted from DNA geometry, and is the least stable thermodynamically.

Stability of base pairing is not determined solely by the number of hydrogen bonds. The pair I:T and I:A form the same hydrogen bonds as G:T and G:A, respectively, yet $(\frac{A-T-A}{T-G-T})$ is more stable than the other G:T or I:T containing sequences, whereas $(\frac{A-I-A}{T-A-T})$ is the most stable of the G:A or I:A containing sequences. The magnitudes of these differences are evidence

of the importance of sequence-dependent factors such as stacking interactions and local structural variations.

The environment of the X:Y pair in the duplexes studied here is very asymmetric; the X base is in the middle of an oligopurine sequence, the Y base is in an oligopyrimidine region. We observed large differences in the stabilities of the two opposite orientations of some of the base pairs. For example, the two duplexes containing I:A pairs differ in stability by a factor of 8 at 25°C (a difference in ΔG° of 1.2 kcal). Large sequence-dependent variations in the stability parameters of each base pair may be expected in duplexes different from those studied here. Although sequence dependence limits the universality of the parameters derived in studies of small groups of oligonucleotide duplexes, these variations are of interest in themselves in gaining an understanding of the factors involved in the stability of DNA duplexes. For example, some of the thermodynamic effects seen here may result from interrupting the A tract, as we have discussed elsewhere (18). Ability to predict DNA duplex stabilities will improve as the oligonucleotide data base expands.

DNA hybridization probing experiments involve kinetic and solid-phase heterogeneity complications not present in our solution equilibrium measurements. Nevertheless, some parameters relevant to unique sequence DNA probe design can be derived from the duplex stabilities reported here and in reference 18. At positions of ambiguity in the gene sequence, the ideal probe base would pair with equal stability to all bases which might occur at that position. Further, the average stability of pairing should be great enough that probe target duplex stability would accumulate with increasing probe length. The criteria for judging the utility of a base analog are that it be (1) non-destabilizing and (2) non-discriminating when paired with each of the possible bases. The extent to which inosine and the four normal DNA nucleotides meet these criteria can be judged from the data in Table IV (two-base ambiguities) and Table V (four base ambiguity). The overall base pairing by each probe base can be judged by comparing the stability of all dCA_3XA_3G:dCT_3YT_3G duplexes containing that base to the stability of dCA_6G:dCT_6G. The degree of base discrimination by a probe base at a two-fold ambiguity (Table IV) is measured by the difference in free energy of the two corresponding duplexes. At a position of four-fold ambiguity (Table V) an average free energy of discrimination by each probe base is calculated: the average difference between the three non-Watson-Crick base pairs and the Watson-Crick pair. The energy

difference between the most and least stable pairs formed by each probe base is also shown. In the case of inosine, the I:C pair is considered to be Watson-Crick. A small free energy of discrimination means the probe will be non-selective at that position, as desired. It is not clear to what extent the results can be applied to DNA sequences different from these AT rich duplexes. For simplicity of presentation, data in Tables IV and V are average values for the two opposite orientations of each base pair. While there was some resulting loss of detailed information on orientation-dependent effects at 25°C, the energies are almost orientation-independent at 50°C.

In our study (Table IV), the least discriminatory pyrimidine for probing an A/G ambiguity is T, and I is least discriminatory purine at a T/C ambiguity. As expected, purines other than I are not useful for probing A/G ambiguities and pyrimidines are not useful for probing T/C ambiguities because all the base pairs are too destabilizing, both in absolute terms and in comparison to the Watson-Crick pairs, whose relative stability could lead to background problems. The superior non-selectivity of T over C at A/G ambiguities arises equally from the relative instability of A:T compared to G:C and to the relative stability of G:T compared to A:C. The two pyrimidines are comparable by the second criterion--the average stability of pairing with A/G. The superior non-selectivity of I at T/C ambiguities as compared to the other purines A and G appears to decrease with increasing temperature, although the increased uncertainties of extrapolation to high temperature must be kept in mind. The average net instability of I:(C/T) pairs (0.8 kcal/mol) is not likely to be large enough to cause problems, being smaller than the 1-2 kcal/mol of stability contributed by each normal base pair; probe-target duplex stability will increase with probe length as required.

A special case of two-fold ambiguity occurs at the first base of the arginine codon, where either A or C can occur in the sense strand, and either G or T occurs in the anti-sense DNA strand. Inosine is clearly superior to the four normal bases opposite A/C ambiguities, with a small average destabilization and only about a ten-fold (>1.3 kcal) preference for C over A. There is no single base which is ideal for probing G/T ambiguities but both G and I are almost totally non-selective against G/T while yielding a destabilization of about 2 kcal/mol at 25°C. I would be preferable to G here because G would have a greater tendency to increase background by binding of the probe to incorrect, related sequences

containing C at the corresponding position. At four-fold ambiguities (Table V), I is clearly the best probe base to use; it is least selective. Surprisingly, at 50°C or higher, A appears to be next best; and C is clearly the most selective (worst) base. At three base ambiguities (not shown), results are similar to the four base case: inosine is somewhat preferable.

It should be noted that the "best" probe base in each of the cases studied is still significantly selective; for example, the difference in stability between the strongest and weakest base pair of I is between 2 and 3 Kcal of free energy or about a factor of 100 in stability constant. In solution, this difference in stability constant would mean that the less stable duplex would dissociate at 100 times the rate of the more stable duplex, since the association rates would be approximately the same. Deoxyinosine is not an inert base in the sequences we studied, although it is less selective than the other bases. In a study of dissociation temperatures (T_d) of a deoxinosine-containing 26-mer probe bound to target DNA on nitrocellulose filters (4), no change in T_d was reported when two I:C pairs were replaced by I:T pairs. From our data we would have predicted a change in T_d of about 8°C; the difference between I:C and I:T pairs is more than twice the difference between G:C and A:T pairs. Possible sources of this discrepancy include secondary structure effects in the two target DNA's studied in reference 4, large sequence dependence of the stabilities of I:C and I:T pairs, and differences between T_d and T_m measurements. Other sequences need to be studied to determine the generality of our results both in solution and on solid supports.

The current results indicate a definite advantage in the use of deoxyinosine to reduce specificity of DNA probes, especially at A/C or G/T ambiguities and at three- and four-fold ambiguities. The use of I instead of G at T/C ambiguities is less clear-cut, since I is less discriminatory against mismatches, but also less stabilizing on the average. Deoxyinosine is not nearly inert as proposed, except at A/C ambiguities, so the use of natural bases against T, A or G where those bases are indicated by strong codon preferences or other evidence may be advantageous. Other base analogs should be investigated for more favorable combinations of high base-pairing stability and lack of A/G or T/C discrimination. Finally, a base analog which is more discriminatory against mismatches than the normal bases could be used to increase the selectivity of the probe at positions of unambiguous sequence.

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