Factors Contributing to Aromatic Stacking in Water: Evaluation in the Context of DNA

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Received September 27, 1999

Abstract: We report the use of thermodynamic measurements in a self-complementary DNA duplex (5′-dXCGCGCG)$_2$, where X is an unpaired natural or nonnatural deoxynucleoside, to study the forces that stabilize aqueous aromatic stacking in the context of DNA. Thermal denaturation experiments show that the core duplex (lacking X) is formed with a free energy (37 °C) of −8.1 kcal·mol^{-1} in a pH 7.0 buffer containing 1 M Na^+. We studied the effects of adding single dangling nucleosides (X) where the aromatic "base" is adenine, guanine, thymine, cytosine, pyrrole, benzene, 4-methylindole, 5-nitroindole, trimethylbenzene, difluorotoluene, naphthalene, phenanthrene, and pyrene. Adding these dangling residues is found to stabilize the duplex by an additional −0.8 to −3.4 kcal·mol^{-1}. At 5 μM DNA concentration, T_m values range from 41.7 °C (core sequence) to 64.1 °C (with dangling pyrene residues). For the four natural bases, the order of stacking ability is A > G ≥ T = C. The nonpolar analogues stack more strongly in general than the more polar natural bases. The stacking geometry was confirmed in two cases (X = adenine and pyrene) by 2-D NOESY experiments. Also studied is the effect of ethanol cosolvent on the stacking of natural bases and pyrene. Stacking abilities were compared to calculated values for hydrophobicity, dipole moment, polarizability, and surface area. In general, hydrophobic effects are found to be larger than other effects stabilizing stacking (electrostatic effects, dispersion forces); however, the natural DNA bases are found to be less dependent on hydrophobic effects than are the more nonpolar compounds. The results also point out strategies for the design nucleoside analogues that stack considerably more strongly than the natural bases; such compounds may be useful in stabilizing designed DNA structures and complexes.

Introduction

The factors contributing to the thermodynamic stability of the DNA double helix have been the focus of intense scrutiny for the past four decades. Both hydrogen bonding and base stacking are important as stabilizing noncovalent interactions in the double helical structure. Of these two, hydrogen bonding is perhaps the simpler and better understood interaction;[1,2] base stacking, although discussed at great length, is more complex and remains considerably less well understood.[3]

Despite this, it is clear that base stacking makes a strong contribution to stabilizing the helical structure of DNA and RNA. A number of studies in short RNA duplexes have made use of the "dangling end" effect, which occurs when a single unpaired base is added to the end of a duplex, stabilizing the helix by stacking on it.[4,5] This method is highly useful since it separates the stacking interaction of a single base from other interactions involved in pairing (namely, stacking of the pairing partner and hydrogen bonding between the bases). Comparable studies of the natural bases have yet to be carried out in DNA, although one preliminary study with two dangling thymidines,[6] and one study with four dangling residues[7] have been reported. In looped RNA or DNA structures, where hydrogen bonding is less extensive, it is likely that base stacking may be relatively even more important as a stabilizing interaction for helical structure.[8−10]

While $\pi-\pi$ stacking is by consensus an important noncovalent interaction in DNA and proteins, the nature of this interaction remains under debate. Theoretical studies have implicated several factors as potentially important in stabilizing the face-to-face base−base interactions.[11−20] Among these are

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10.1021/ja9934854 CCC: $19.00
Published on Web 02/10/2000

electrostatic (dipole−dipole and dipole−induced dipole) interactions, dispersion (momentary dipole−induced dipole) effects, and solvation effects. The electrostatic effects may depend on localized charges that exist at specific parts of a given heterocyclic base, or on electrostatic potentials that may differ between the faces and edges of the bases. Dispersion effects depend on the surface area of contact and on the polarizability of the two species. Finally, solvophobic and other solvent-driven effects will depend on the relative energies of solvation of bases when stacked and unstacked as well as the amount of surface area desolvated on stacking.

While theoretical studies of base stacking exist in relative abundance, there have been fewer experimental studies of stacking in the context of DNA.[21,22] Studies of nucleotide monomers, dimers, and related analogues have demonstrated stacking of unpaired bases in aqueous solution.[23−29] Such studies have indicated that the relative stacking ability of the natural bases qualitatively goes in the order purine−purine > purine−pyrimidine > pyrimidine−purine > pyrimidine−pyrimidine. Since the addition of organic solvents destabilizes this interaction and nucleic acid duplexes in general,[30−32] a solvophobic contribution to stacking has been implicated; however, this is not a classical entropy-driven hydrophobic interaction as seen in protein folding. Since DNA duplex formation is an enthalpy-driven process, researchers have concluded that any entropy-driven hydrophobic effects are hidden by unfavorable entropy of restricted bond rotations, and that electrostatic or van der Waals interactions (enthalpy-driven effects) may be more important in DNA than a solvent-induced interaction. On the whole, there is still not a unified picture as to the relative importance of solvophobic, electrostatic, and dispersive effects on stacking in water.

In addition to studies with nucleic acids, a substantial number of simpler organic structures have been studied as models for aqueous $\pi-\pi$ stacking in the more complex nucleic acid and protein structures. For example, studies by Rebek and co-workers[33] have shown that adenine analogues can be complexed by stacking with simple aromatic hydrocarbon groups; in one study it was found that increasing the size (surface area) of such a group increased binding significantly. Gellman et al. have studied bis-aromatic structures bridged by propylene units,[34] suggesting that classical hydrophobic factors might not be as important in $\pi-\pi$ interactions as was previously thought, although this has been debated.[19,30b] More recently the same group has studied a minimal "molecular balance" system to measure hydrophobic interactions quantitatively.[35] Finally, work by other groups has stressed the importance of electrostatic factors in $\pi-\pi$ interactions. Dougherty and co-workers have pointed out that the negative electrostatic potential in the center of the benzene ring face can be an important factor in its noncovalent interactions.[36] Siegel has shown in elegant experiments that by adjusting this electrostatic potential with substituents one can affect the magnitude of interactions between closely aligned aromatic faces.[37]

Such model systems have led to valuable insights into the stacking question. Yet there is a general need for bridging the gap between these smaller systems and the more complex DNA structure. It is not yet possible to say with confidence what are the most and least important factors in aqueous aromatic stacking, and it is not straightforward to predict how a specific structural change made to an aromatic structure (such as a DNA base) would affect stacking. We have therefore undertaken a study of aromatic stacking in the context of DNA by examining such interactions both with the natural bases as well as with synthetic analogues having altered properties. In this paper we use dangling end studies to evaluate the stacking behavior of the four natural nucleosides as well as that of nine nonnatural analogues in the context of a hexamer DNA duplex and attempt to correlate this behavior with several measured or calculated properties of the individual bases. We find that many of the nonnatural analogues are more proficient at stacking than the natural bases. The results lend insight into the forces involved in aromatic stacking in water in general, as well as their importance in the context of DNA. The results should aid in the design of new synthetically altered DNA bases or analogues which, due to improved stacking properties, can act as helix stabilizers.

Experimental Section

Modified Nucleoside Phosphoramidites. The nonnatural benzene, trimethylbenzene, difluorotoluene, 4-methylindole, naphthalene, phenanthrene, and pyrene deoxynucleoside phosphoramidites used in this study were prepared as previously described.[38−40] The pyrrole deoxynucleoside analogue was synthesized as described.[41] The 5-nitroindole deoxynucleoside analogue was purchased from Glen Research.

Oligonucleotide Synthesis. DNA oligonucleotides were synthesized on an Applied Biosystems 392 synthesizer using standard β-cyanoethylphosphoramidite chemistry. Oligomers were purified by preparative 20% denaturing polyacrylamide gel electrophoresis, isolated by the crush and soak method followed by dialysis, and were quantitated by absorbance at 260 nm. Molar extinction coefficients were calculated by the nearest neighbor method. Values for oligonucleotides containing nonnatural residues were calculated as described previously.[42] Oligodeoxynucleotides were obtained after purification as the sodium salt. Intact incorporation of nonnatural nucleoside was previously confirmed by synthesis of short oligomers of sequence T−X−T (where X = nonnatural residue); proton NMR confirmed the presence of the intact structures with the expected integration.

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Thermal Denaturation Studies. Solutions for the thermal denaturation studies were as described.[6] The melt buffer contained 1 M NaCl, 0.1 mM EDTA, and 10 mM Na•phosphate (pH 7.0). After the solutions were prepared, they were heated to 90 °C and allowed to cool slowly to room temperature prior to the melting experiments. The melting studies were carried out in Teflon-stoppered 1 cm path length quartz cells under nitrogen atmosphere on a Varian Cary 1 UV−vis spectophotometer equipped with thermoprogrammer. Absorbance was monitored at 280 nm, while the temperature was raised from 5 to 80 °C at a rate of 0.5 °C/min; a slower heating rate did not affect the results. In all cases the complexes displayed sharp, apparently two-state transitions. Melting temperatures (T_m) were determined by computer-fit of the first derivative of absorbance with respect to $1/T$. Uncertainty in T_m is estimated at ± 0.5 °C based on repetitions of experiments. Free-energy values were derived by two methods: (1) computer-fitting the denaturation data with an algorithm employing linear sloping baselines, using the two-state approximation for melting.[32] Fits were excellent, with χ^2 values of 10^{-6} or better. (2) Van't Hoff thermodynamic parameters were derived from linear plots of $1/T_m$ vs $\ln(C_T)$ by measuring T_m as a function of concentration. Close agreement was seen with the results from curve-fitting, indicating that the two-state approximation may be a reasonable one for this sequence.[43]

Calculated Physical Properties. Geometry-optimized structures calculated using the AM1 Hamiltonian were generated with SPARTAN version 4.0 (Wavefunction, Inc.). The same program was used to calculate Ghose−Crippen log *P* values,[44] polarizabilities, surface area, and dipole moments. Surface areas were calculated with molecular mechanics simulations using the MM2 force field as implemented by MacroModel version 3.4a (W. C. Still, Columbia University).

Water/Octanol Partitioning. The experimental solvent partitioning studies for free nucleosides were carried out as described.[45] Experiments were carried out 4−6 times and the results averaged.

NMR Studies. All 1-D and 2-D NMR data was collected on a Varian Unity 500 MHz spectrometer. The samples were dissolved in buffer (100 mM NaCl, 1 mM EDTA, and 10 mM Na-phosphate, pH 7.4) at duplex concentrations of 3 mM. 1-D data was obtained in H_2O solution using the BINOM pulse sequence with 1:1 water suppression at 0 °C. Proton assignments were made by means of standard two-dimensional techniques, including NOESY, DQF-COSY, and NOESY WATERGATE for both the adenine and pyrene duplexes. Data processing was done using Felix 97 (BIOSYM/Molecular Simulations). All 2-D NOE data for the pyrene- and adenine-containing duplexes in H_2O were collected at 0 °C using the NOESY WATERGATE pulse sequence. In D_2O the pyrene data was collected at 40 °C, while the adenine data was collected at 15 °C using the NOESY pulse sequence.

Results

Structure and Design Aspects of the Nucleosides Studied. The 13 deoxynucleosides studied here are shown in Figure 1; in all cases the deoxyribose is the same, but the "base" moieties are varied in size, shape, and polarity. The varied structures were chosen to examine the importance of these properties in stabilizing aromatic stacking. The four natural deoxynucleosides are included, and for comparison we also studied nonpolar nucleoside shape mimics[46,47] as well as a simple aromatic hydrocarbon series with increasing size (benzene, naphthalene, phenanthrene, and pyrene deoxynucleosides). To test possible

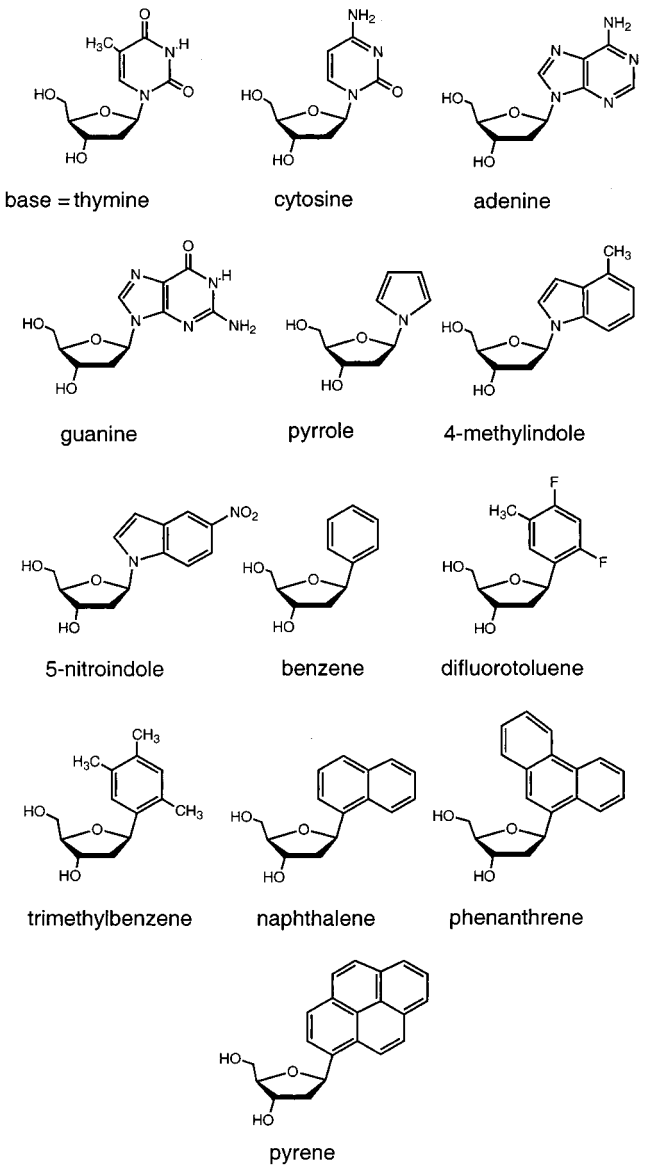

Figure 1. The structures of natural and nonnatural deoxynucleosides in this study.

effects of electron-withdrawing substituents we included a known 5-nitroindole analogue[48,49] and a difluorotoluene analogue.[50] Finally, the smallest member of the series, a deoxynucleoside of pyrrole,[41] was included as a truncated analogue of the indole deoxynucleosides. A previous preliminary study reported dangling end measurements for a few of these analogues but did not include all four natural bases, did not include structural data, and did not correlate the results with physical properties or solvent effects.[50]

Physical Properties of the Aromatic Stacking Species. To aid in relating physical properties of these compounds to their stacking ability, we carried out calculations on the aromatic "bases" in the nucleoside series. Calculated values are given in Table 1. Hydrophobicity was evaluated for the bases methylated at the same site normally attached to deoxyribose. This was done by calculating log *P* values for water/octanol partitioning

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Table 1. Calculated Physical Properties of DNA Bases and Related Structures in This Study[a]

aromatic structure	log *P* (octanol−water)[b,d]	polarizability (Å^3)[b]	dipole moment (debye)[b]	surface area (Å^2)[c,e]	stacking area (Å^2)[c,f]
thymine	−0.36	12.3	4.51	142	95
adenine	−1.07	13.7	2.27	142	128
cytosine	−0.76	11.3	6.02	127	102
guanine	−1.36	15.0	6.55	154	139
pyrrole	+0.82	7.8	2.25	96	77
4-methylindole	+2.91	15.5	1.94	165	157
5-nitroindole	+2.46	16.9	8.19	173	165
benzene	+2.52	9.1	0.26	110	88
difluorotoluene	+3.32	12.5	1.84	144	96
trimethylbenzene	+3.98	14.0	0.00	173	114
naphthalene	+3.52	15.7	0.27	158	134
phenanthrene	+4.52	19.8	0.29	203	122
pyrene	+4.67	23.9	0.35	217	184

[a] log *P*, polarizability, and dipole moment are for the methylated compounds,[b] and surface and stacking areas are for the unsubstituted compounds. [b] Values for bases having methyl group at position where it is attached to a nucleoside. [c] Values for the unsubstituted bases alone. [d] Values are for the Ghose−Crippen method. Negative values indicate water preference, while positive values indicate octanol preference.[e] Half of the calculated surface area of base. [f] Estimated by multiplying surface area number by fraction of surface covering adjacent base pair.

using the Ghose−Crippen method.[44] Polarizability and dipole moment were also calculated for the methylated bases. Surface area was calculated for the free (unsubstituted) bases. We also generated estimates of stacking surface area by examining models of possible B-form stacking geometries (see below) and multiplying the estimated fraction of a given aromatic base overlapping with the neighboring base pair times half the total surface area of the aromatic base.

It was not practical to obtain experimental log *P* (water/octanol) values for the majority of the bases alone in the series because extreme polarity or hydrophobicity of several cases made it difficult to measure very low solute concentrations in the less-populated fraction. However, to test the calculation method we did carry out water/octanol partitioning experiments with a number of whole nucleosides, whose polarity properties are leveled substantially by the presence of the sugar. We studied deoxyadenosine (dA), thymidine (dT), difluorotoluene deoxynucleoside (dF). The corresponding experimental log *P* values were −0.89, −1.27, +1.39, respectively. The calculated values for these same compounds were −2.00, −2.12, +1.57, respectively, which, although not quantitatively accurate, agree qualitatively in rank order and sign. Thus, the experiments tend to lend qualitative confidence in the calculated values for the methylated bases.

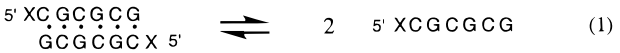
The calculated log *P* values for the series (Table 1) range quite widely from −1.36 (guanine, the most hydrophilic) to +4.67 (for pyrene, the most nonpolar). On this scale, thymine and pyrrole are closest to neutral polarity. Of the natural bases, guanine is predicted to be the most water soluble and thymine the most octanol soluble.

The calculated polarizability values also vary widely, and not surprisingly, they correlate quite well with size (surface area). The least polarizable and smallest of the group is pyrrole (7.8 Å^3), and the most polarizable and largest is pyrene (23.9 Å^3). The values for the natural bases are all quite similar, ranging from 11.3 to 15.0 Å^3; thus, the nonnatural analogues are useful in expanding the overall range quite considerably.

As for dipole moments, of the natural bases guanine has the largest dipole moment (6.55 D) and adenine the smallest (2.27 D). The nonnatural analogues in the series generally have very low dipole moments, and the benzene through pyrene series all have values of 0.35 D or lower. The two nonnatural purine mimics, 4-methylindole and pyrrole (a truncated purine), have dipole moments very similar to their natural counterpart, adenine, while 5-nitroindole has the largest value of the series (8.19 D). The two thymine mimics (difluorotoluene and trimethylbenzene) have considerably lower dipole moments than those of their natural counterpart (Table 1). We chose not to evaluate the overall direction of the dipoles (see below).

A final potentially important parameter is stacking surface area, which depends not only on the size of the molecule in question but also on its shape and geometry of overlap with the neighboring base pair (see below). Estimated values range from 77 Å^2 for the smallest base analogue (pyrrole) to 184 Å^2 for the largest analogue (pyrene). Among the natural bases, the estimated stacking area ranges from 95 Å^2 for thymine to 139 Å^2 for guanine.

Dangling End Experiments. To evaluate experimentally the ability of the aromatic "base" analogues to stack on the end of a DNA duplex we carried out a series of UV-monitored thermal denaturation experiments in which the nucleoside being tested is placed at the 5′-end of a short self-complementary duplex. Since the aromatic species in question is not in a position to base pair with another base in the complementary strand, the most likely remaining interaction is stacking on the neighboring C−G pair, which can be evaluated by its effect on the helix−coil equilibrium (eq 1).[4−7]



(1)

It should be noted that a possible alternative stabilizing interaction might be minor groove binding of the dangling residue; however, this is quite unlikely in the present case because the core duplex sequence consists only of G and C, which generally prevents groove binding.

The results are presented in Table 2. The duplexes all appear to behave in a two-state fashion, with all-or-none melting behavior indicating cooperative interactions by the dangling residues. Examples of melting curves and van't Hoff plots are given in Figure 2. All of the test bases stabilize the duplex significantly relative to the core sequence. The least stabilization is seen for a dangling pyrrole, which increases the T_m by 4.9 °C and contributes −0.8 kcal·mol^{-1} of stability ("$\Delta\Delta G°$ stacking" in Table 2) with two symmetrical substitutions. The most stabilizing interactions are those with pyrene and 5-nitroindole, which increase the T_m of the duplex by 19−23 °C and add a large −3.4 kcal·mol^{-1} of stabilization to the core structure.

Results show that the entropy and enthalpy terms for the various dangling end sequences vary considerably and do not correlate with stacking ability in any obvious way. The core sequence forms a duplex stabilized by a large enthalpy term (−45.9 kcal·mol^{-1}) and nearly counterbalanced by an entropy term (−122 eu) which is somewhat smaller at 37 °C. Examination of the additional changes in enthalpy and entropy on adding a dangling base shows a general trend similar to this finding. For 11 of 13 cases, enthalpy is made even more favorable (by ∼2−21 kcal·mol^{-1}) by addition of a dangling residue, and opposing this is a less favorable entropy term (by ∼1−18 kcal·mol^{-1} at 37 °C). Two cases are found to be somewhat different: first, addition of a dangling deoxyguanosine results in a small unfavorable change in enthalpy (∼2.6 kcal·mol^{-1}, relative to the core sequence) and a slightly larger favorable change in entropy (∼4.0 kcal·mol^{-1}). The dG stacking data must be regarded with caution due to the possibility of an equilibrium

Table 2. Free Energy of Stacking for Natural Nucleosides and Related Analogs, as Measured by Dangling End Thermal Denaturation Studies with Self-Complementary Strands (dXCGCGCG)[a]

dangling residue	T_m (°C)[b]	ΔT_m (°C)	$-\Delta H°$ (kcal) (van't Hoff)	$-\Delta S°$ (eu) (van't Hoff)	$-\Delta G°_{37}$ (kcal) (van't Hoff)	$-\Delta G°_{37}$ (kcal) (fits)[c]	$\Delta\Delta G°$ stacking
none (core duplex)	41.7	--	45.9	122	8.1 ± 0.2	8.1 ± 0.1	
thymine	48.1	6.4	47.9	125	9.2 ± 0.2	9.2 ± 0.9	1.1 ± 0.2
adenine	51.6	9.9	54.7	144	10.1 ± 0.2	10.0 ± 0.4	2.0 ± 0.2
cytosine	46.2	4.5	50.4	133	9.1 ± 0.2	8.9 ± 0.1	1.0 ± 0.2
guanine	51.5	9.8	43.3	109	9.4 ± 0.2	9.9 ± 0.3	1.3 ± 0.2
pyrrole	46.6	4.9	50.8	135	8.9 ± 0.2	9.5 ± 0.2	0.8 ± 0.2
4-methylindole	54.6	13.6	66.7	179	11.2 ± 0.2	10.5 ± 0.1	3.1 ± 0.3
5-nitroindole	60.6	18.9	53.9	137	11.4 ± 0.2	11.6 ± 0.3	3.4 ± 0.3
benzene	48.3	7.3	51.4	135	9.4 ± 0.2	9.4 ± 0.7	1.4 ± 0.2
difluorotoluene	54.4	13.4	60.5	161	10.7 ± 0.2	10.3 ± 0.2	2.6 ± 0.3
trimethylbenzene	51.4[d]	9.7	51.6	135	9.7 ± 0.2	9.8 ± 0.4	1.6 ± 0.3
naphthalene	56.2	15.2	58.7	154	10.9 ± 0.2	10.9 ± 0.1	2.9 ± 0.3
phenanthrene	57.3	16.3	51.3	131	10.7 ± 0.2	10.6 ± 0.1	2.6 ± 0.3
pyrene	64.1	23.1	48.6	120	11.4 ± 0.2	11.3 ± 0.3	3.4 ± 0.3

[a]Free energy of stacking ($\Delta\Delta G°$) is obtained by subtracting the free energies of the duplexes with dangling residues from the energy of the core hexamer duplex.[b] [b] Conditions: 1 M NaCl, 10 mM Na·phosphate pH 7.0; 5.0 μM DNA strand concentration for T_m value shown. [c] Average free energies from fits to individual melting curves. [d] Concentration 6 μM.

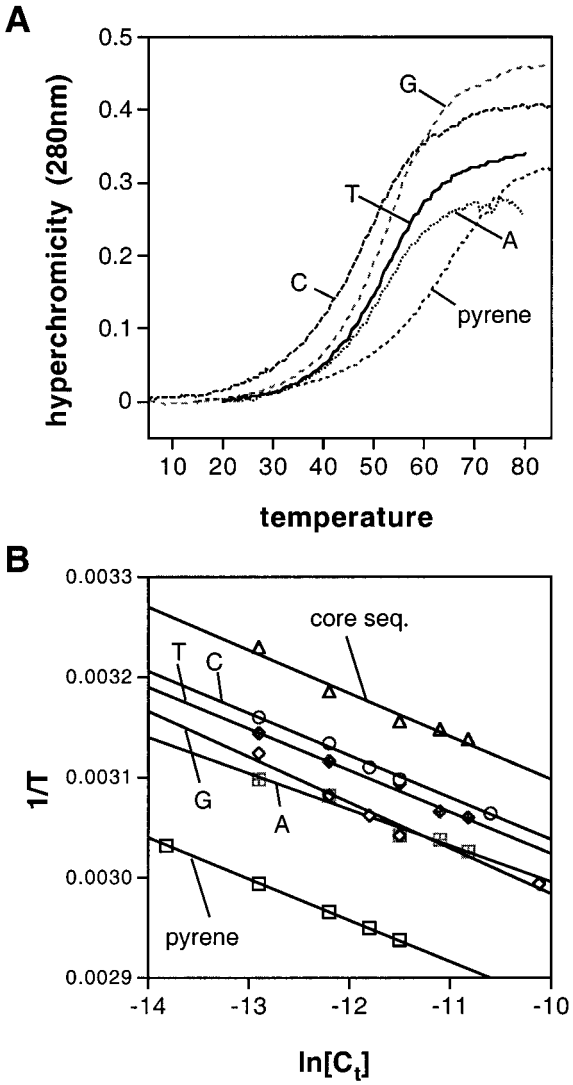


Figure 2. (A) Examples of thermal denaturation data for several of the dangling end sequences in this study. The dangling residues for each melting curve are given on the plot. (B) Van't Hoff plots for the same sequences as in A.

between the structure illustrated in Figure 4 and an alternative slipped structure in which guanine becomes a 3′-dangling end on the same core duplex. Finally, addition of pyrene results in a small favorable change in enthalpy (by 2.7 kcal·mol^{-1}), and a near-zero change in entropy (favorable by 0.7 kcal·mol^{-1}).

Although RNA nucleosides and bases have been studied in RNA duplexes by the dangling end method,[4,5] few previous studies have used this method for DNA. Thus, we report here the first single-base dangling end thermodynamic data for the four natural DNA bases. We find that in this context the relative apparent stacking ability is A > G ≥ T = C.

Relationships between Physical Properties and Stacking Propensity. To test for general correlations between these relative apparent stacking free-energy values and calculated physical properties of the aromatic bases, we plotted the various properties as a function of $\Delta\Delta G°$ (stacking). The results are shown in Figure 3. Perhaps not surprisingly, there appears to be no quantitatively close linear correlation between any single property and stacking ability, indicating that more than one property is important in the energetics of stacking. However, examination of the plots does reveal some potentially useful qualitative relationships and also allows us to rule out some properties as not predictive at all.

As a whole, the plotted data show clearly that log *P* and dipole moment are very poorly correlated (if at all) with stacking energy. This is especially true for dipole moment, which shows no suggestive trends at all. If one focuses only on the nonnatural DNA base analogues in Figure 3A, there appears to be a weak qualitative correlation between increasingly positive log *P* and stacking ability. However, there are distinct cases where pairs of structures have similar log *P* and quite different stacking ability. For example, benzene and methylindole have similar log *P* values, while the difference in stacking ability is large. The same is true for trimethylbenzene and pyrene. Thus, log *P* alone is a very poor predictor of stacking ability, and this is especially evident when the natural bases are included in the analysis.

The plot of polarizability vs stacking energy appears to show a rough qualitative correlation between increasing polarizability and increasing stacking ability. The most polarizable "base", pyrene, is the strongest stacker, and the least polarizable, pyrrole, is apparently the weakest stacker. In general this trend holds true for the others as well, including the natural bases. Clearly, this is not completely predictive, however. For example, difluorotoluene and phenanthrene differ quite strongly in their polarizability, and yet they stack almost equally well. In another example, difluorotoluene and trimethylbenzene have similar

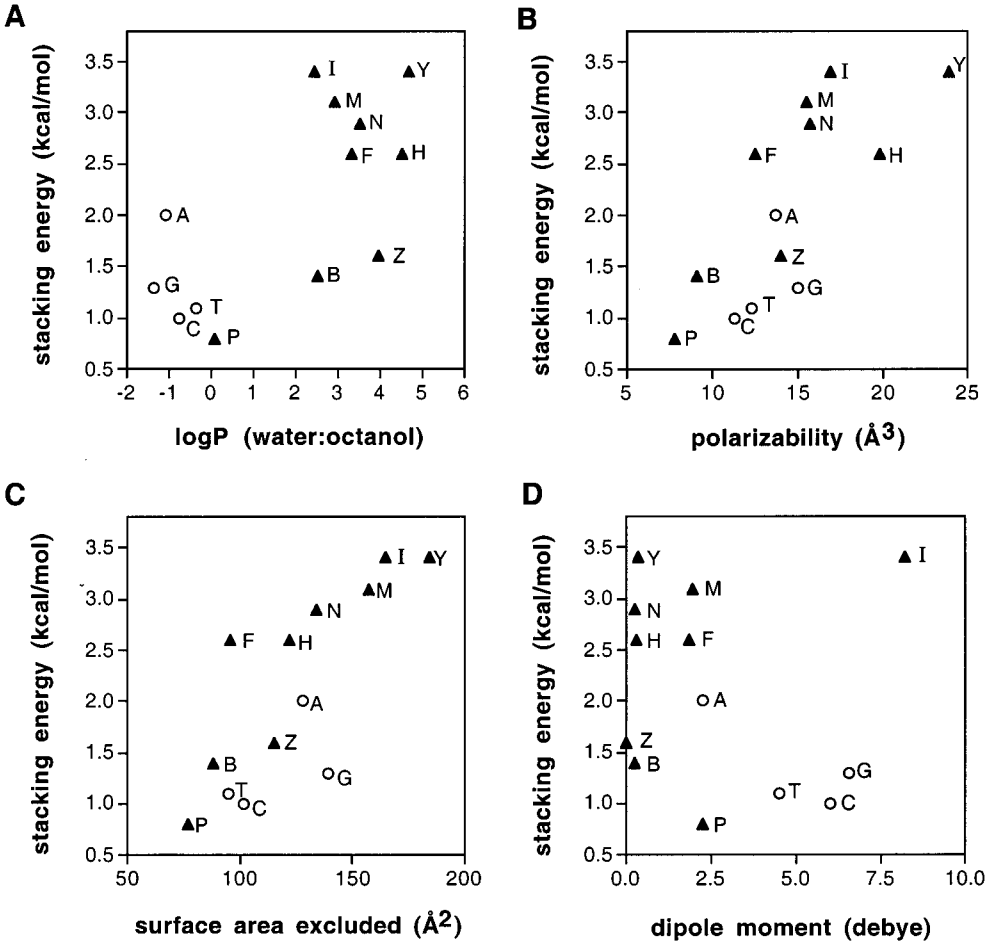


Figure 3. Relationships between stacking free energies (Table 2) and calculated physical properties of the DNA bases and aromatic analogues in this study (Table 1). (A) Hydrophobicity, as measured by log *P* for the methylated bases; (B) calculated polarizability; (C) estimated surface area of dangling residue excluded from solvent on stacking; (D) dipole moment of the methylated base. Data for natural DNA bases are given as circles, and other analogues, triangles. Abbreviations for the bases are as follows: A (adenine), B (benzene), C (cytosine), F (difluorotoluene), G (guanine), H (phenanthrene), I (5-nitroindole), M (4-methylindole), N (naphthalene), P (pyrrole), T (thymine), Y (pyrene), Z (trimethylbenzene).

polarizability, and yet difluorotoluene stacks much more strongly. It is also worth noting that polarizability correlates very well with the total surface area of the bases, which may also be an important factor in stacking (see discussion below). We did not analyze possible effects of base orientation on polarizability, since not enough information is available on the specific geometries of the bases in question in DNA.

Finally, examination of the plot of surface area excluded (i.e., stacking surface area, Figure 3C) versus stacking energy shows perhaps the best correlation of the four. Stacking areas were estimated from the calculated total surface areas of the base analogues (Table 1) and from model building of the hexamer duplexes with a 5′-dangling residue in B-form geometry (Figure 4). This core sequence was previously shown to adopt a B-like conformation.[6] Results show that the compound that is likely to have the smallest overlap (pyrrole) stacks the least strongly, while pyrene, which likely has the largest area of overlap, stacks the most strongly. Ignoring (for the moment) the natural bases, there is good qualitative ordering of stacking area and stacking free energy. The exceptions to this are difluorotoluene, 5-niroindole, and trimethylbenzene; here we find that the first two stack more strongly than predicted by surface area, whereas the third stacks less strongly than expected. Turning to the natural bases, one finds that A, T, and C also fall reasonably well into the correlation. G shows the weakest correlation with surface area, since it stacks more than 1 kcal/mol less well than predicted from its estimated stacking area; again, in this case we cannot rule out an equilibrium between structures having 3′- and 5′-dangling ends.

Structural Confirmation of Stacking. Although stacking in DNA is a favorable interaction, the thermodynamic data alone do not guarantee a face-to-face stacked geometry for the dangling bases, as modeled in Figure 4. It is difficult to see how these residues could stabilize the helix by an interaction other than stacking, since it is unlikely (as mentioned above) that groove binding would occur with this sequence. In addition, an NMR study of dangling TT residues in this same sequence showed clear evidence of stacking by the two thymines.[6] Nevertheless, we tested this question directly for two quite different cases, with adenine and pyrene in the dangling positions, and for comparison we studied the core duplex. We measured 1-D spectra at 0 °C in H_2O for all three duplexes, and 2-D NOE data was obtained in both H_2O and D_2O for both the dangling pyrene- and adenine-containing duplexes. All three imino proton spectra are shown in Figure 5. The water data clearly show a strong upfield shift of one of the imino resonances (the terminal C−G pairs) consistent with ring current effects in a stacked geometry. Thus, we rule out groove binding or edge-to-face interactions by the dangling residues, at least in these two cases. Also consistent with face-to-face stacking in the pyrene case are two NOEs observed in D_2O between two pyrene protons, distal to the sugar attachment, and the H1′ of the 3′-G of the partner strand (data not shown). In addition, NOEs were seen from the terminal imino proton to pyrene.

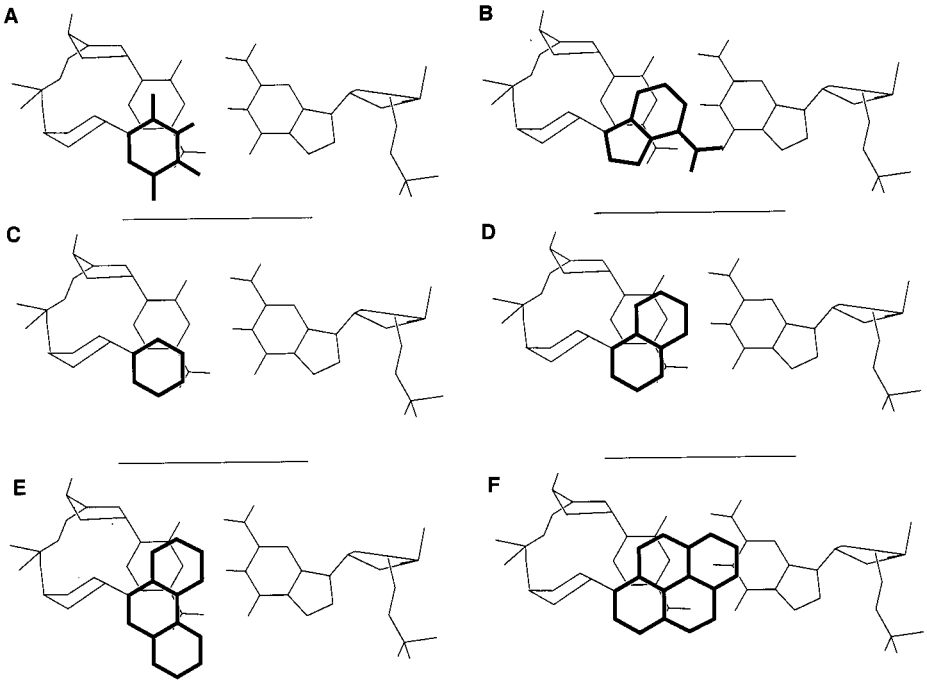


Figure 4. Illustrations of possible 5′-end stacking geometries for the aromatic rings in this study. Models were built using canonical B-form geometry and placing the dangling residue at the 5′-end adjacent to a C−G pair. (A) Dangling pyrimidine (specifically thymine or difluorotoluene), (B) purine (specifically adenine or 4-methylindole), (C) benzene, (D) naphthalene, (E) phenanthrene, (F) pyrene. Stacked geometries were confirmed for adenine and pyrene by NMR experiments.

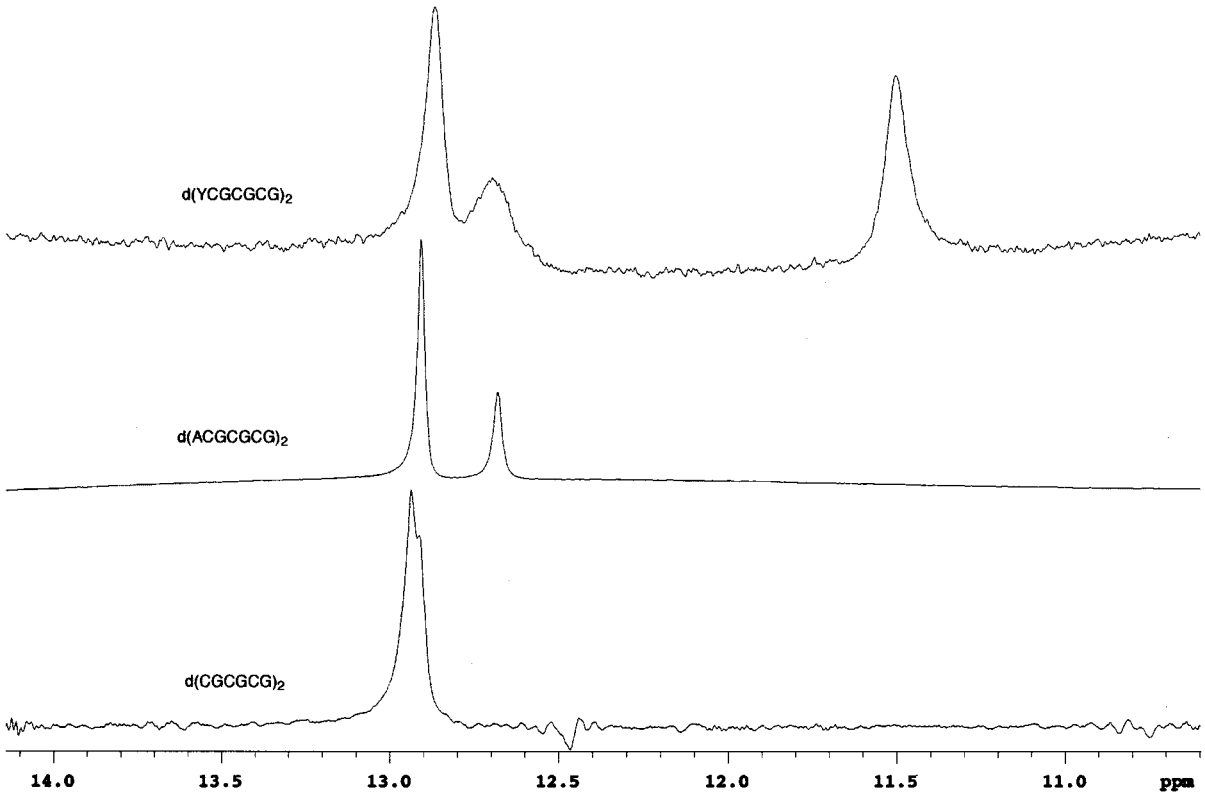


Figure 5. Imino proton spectra at 0 °C for (5′-dCGCGCG)$_2$ (bottom), (5′-dACGCGCG)$_2$ (center), and (5′-dYCGCGCG)$_2$ (top). Note the strong upfield shift of the imino proton in the terminal C−G pair caused by the adjacent stacking of the 5′-dangling bases A and Y.

Modeling studies indicate that the only conformation that can satisfy both sets of NOEs is one in which the pyrene is stacked in a dangling position as illustrated in Figure 4. NOE studies similar to these were also done with the dangling adenine case; however, adenine has a paucity of ring protons, and no NOE was seen from the adenine H2 to the terminal imino proton. We believe this may be due to the inability to see long NOEs, such as this one, from terminal imino protons, due to their facile exchange with H_2O. Because adenine has no other ring protons that would be useful as NOE probes, in this case the stacked conformation was confirmed by the chemical shift of the imino protons rather than by NOE data.

Effect of Ethanol Cosolvent. The results above indicated that surface area excluded might be a useful predictor of stacking

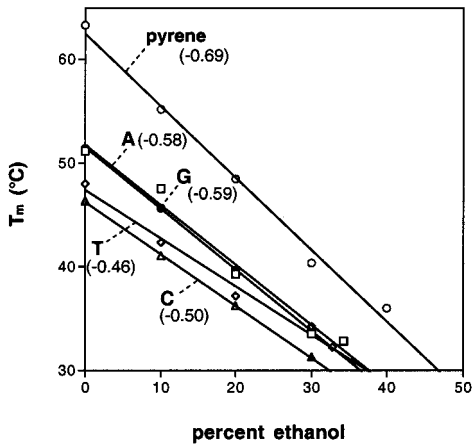


Figure 6. The effect of added ethanol (as volume percent) on the T_m for five different dangling residues. The slopes of the lines are given in parentheses. Correlation coefficients for the lines range from 0.98 to >0.999.

ability. Since solvophobic effects are also correlated with the extent of surface area excluded from solvent,[51] we examined the role of such effects in DNA base stacking using an organic cosolvent. Cosolvents such as ethanol or ethylene glycol have been useful previously as probes of solvophobic effects.[30−32] It is expected that the greater the extent of solvophobic contributions to molecular interactions, the more that such a cosolvent will weaken the interaction. Significantly, it has been noted that the thermal melting temperature (T_m) of short RNA duplexes is linearly dependent on ethanol concentration.[32] Thus, we examined five different short DNA duplexes for their sensitivity to added ethanol, as measured by the slope of the line of vol % ethanol versus T_m (Figure 6). The five duplexes contain the same core sequence $(dCGCGCG)_2$, and differ only by a varied 5′ dangling residue.

Interestingly, the results show very good linear correlations of thermal stability with ethanol content, and also clearly show differences for the five duplexes, despite the fact that they vary only by one residue. The largest slope is for the pyrene-containing sequence (slope = −0.69, corresponding to a 6.9 °C drop in T_m per each 10 vol % ethanol added), and the smallest slope is for the thymine-containing sequence (−0.46). Thus, the pyrene−DNA interaction is significantly more sensitive to added ethanol than are the interactions of the natural bases. Among the natural bases in dangling positions, the relative sensitivity to ethanol decreases in the order G = A > C ≥ T, which is similar in ordering to the relative surface areas of overlap predicted for the four bases.

Discussion

Factors Affecting Aromatic Stacking in DNA. First, it should be noted that these experiments evaluate stacking by measuring thermodynamics of ssDNA−dsDNA equilibria. Although stacking is likely to be stronger in the duplex state, possible stacking effects in the single-stranded state may be significant. For example, the thermodynamic data with the pyrene and guanine cases shows no added entropic cost for adding these residues to the core, which might be explained by strong preorganization of the single-stranded state. However, we are hesitant to over-interpret these data because of the known effects of entropy−enthalpy compensation.[52]

Our results indicate that in the present sequence context the relative stacking ability of the natural bases is A > G > T = C. This is in reasonable agreement with previous studies of dinucleotides and related analogues, which also find the purines to be more effective than pyrimidines.[23−29] It also agrees reasonably well with a study of dangling tetranucleotides, which found A,G > T > C,[53] and a preliminary study which found decreasing thermal stabilities in the order A > T > G > C.[54] In addition, previous studies of dangling bases at the 3′-end of RNA duplexes has given a relative order of stacking affinity of A = G > U ≥ C with a neighboring C−G pair,[5] which is also in reasonable agreement with our findings.

Examination both of the data as a whole and of pairwise comparisons between related molecules allows us to draw some conclusions regarding the different forces that may contribute to aromatic stacking in aqueous solution in the context of DNA structure:

(1) *Dispersion forces.* It is no doubt true that dispersion (momentary dipole−induced dipole) forces do in fact contribute to the stacking of aromatic systems in aqueous solution. Such attractive forces depend on the surface area of overlap, on the goodness of fit (or closeness of contact), and on the polarizability of the two molecules in question. Since two flat aromatic systems are more polarizable than water and fit together more efficiently than with water, the dispersion forces should virtually always be stabilizing in DNA helix formation. The more relevant question to be addressed experimentally is what is the magnitude of this attraction relative to the other forces making contributions. We do note an apparent rough correlation between polarizability and stacking free energy; however, polarizability also depends on size, which also strongly influences hydrophobic effects as well as dispersion effects (see discussion below).

Thus, we can examine pairwise comparisons to attempt to separate dispersion effects from other effects. One particularly pertinent comparison here is that of naphthalene and phenanthrene, which stack equally strongly even though polarizability of the latter is considerably greater than that of the former. Models suggest that the surface area of overlap on stacking is virtually the same in the two cases. If dispersive forces were significant or dominant, then one would expect phenanthrene to stack more strongly. Since it does not, one is led to conclude that dispersive forces are weak contributors relative to other forces. Also consistent with this idea is the comparison of thymine and difluorotoluene, which have similar polarizability but which stack considerably differently. The same is true for 4-methylindole or naphthalene as compared to guanine. Thus, it appears that factors other than dispersive forces are exerting more significant effects in the series as a whole.

(2) *Permanent polar/electrostatic effects.* Three other electrostatic effects are worth examining for their influence on stacking. First there is the dipole (or multipole)-induced−dipole dispersive force, which would depend in part on the magnitude of the dipole (multipole) in one aromatic system and on the polarizability of its stacking partner, and on the relative orientations of the bases. Since cytosine has one of the strongest dipole moments of the analogues in this study, one might expect that a dipole-induced−dipole interaction involving the neighboring cytosine in the core duplex would be dependent primarily on the polarizability of the dangling aromatic structure in

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question. Yet, as we pointed out above, polarizability, and dispersive forces depending on it, do not appear to be a dominant factor here.

A second electrostatic effect to be examined is the permanent interaction between partial charges (or multipoles) in the two stacking participants. These can be stabilizing or destabilizing, depending on the orientation and magnitude of local bond polarization for each stacking participant. While the current results cannot rule out a contribution by this effect for the natural bases and 5-nitroindole, which do have significant overall dipoles as well as local charge differences, we note that most of the strongly stacking species have virtually no total dipole or local bond polarizations. Thus, the majority of the stabilizations seen in this study very likely arise from factors other than these electrostatic effects. As for the natural bases, it is likely that permanent electrostatic interactions play some part in the total stacking interaction, since there is significant charge localization for all four bases (which differs among them). We do note that guanine in this context stacks significantly less strongly (by ~1 kcal/mol) than predicted from its expected surface area of overlap (Figure 3C), while adenine, cytosine, and thymine fall more in line with the other analogues. It is possible that this difference arises from less stabilizing electrostatic contributions to its interaction of G with the neighboring base pair, although the GpC base step is known to be reasonably stable in DNA duplexes,[55,56] or from an alternative duplex conformation in the G case (see above).

A third electrostatic effect worth noting is the quadrupolar effect, which has been suggested to be important when aromatic species are adjacent in a face-to-face orientation.[17,37,57] Although we have no cases here with strongly electron donating groups, the trimethylbenzene case has the most donating substituents of those we did examine. Interestingly, we find it to be a poorer stacker than predicted by surface area. On the other end of the spectrum, the current data include two cases with strongly electron-withdrawing groups: difluorotoluene and 5-nitroindole. Significantly, we find that they both stack more strongly than expected from surface area alone. Thus, the current data suggest that a quadrupolar effect may be a significant factor in stacking, particularly with nonnatural bases having strong electron-donating or -withdrawing groups. More study is clearly warranted on this effect in the DNA context.

(3) *Solvent-driven effects.* A number of lines of evidence in this study point to solvent-driven effects as perhaps the dominant factor or factors in stacking of most of the compounds examined. We consider here two possible solvent effects: first there is the hydrophobic effect, which arises from energetically unfavorable solvation of the flat aromatic surface or surfaces undergoing stacking during helix formation. This tends to cause the system to minimize its contact with water, and thus, given two compounds with similarly poor solvation, the major determining factor should be surface area of overlap on stacking. This is for the most part what we observe experimentally, and surface area is found to be the best single predictor of stacking among the factors examined (Figure 3C).

The four compounds least well correlated with this factor are difluorotoluene, trimethylbenzene, 5-nitroindole, and guanine. It is possible that in the first three of these cases, quadrupolar effects may also play a role (see above). In general, the four natural bases stack somewhat less strongly than the correlated effects for the other analogues; we surmise that this is due to the lower hydrophobicity (higher polarity) of the stacking surfaces of the natural compounds.

Similarly, comparison of difluorotoluene with thymine and of methylindole with adenine indicates strongly that, if surface area of overlap is constant, decreasing polarity leads to considerably stronger stacking. These effects cannot be attributed either to polarizability (dispersive effects) since the structures being compared have similar polarizability, or to electrostatic effects, since the dipoles decrease greatly while the stacking strength increases. Thus we are led to conclude that solvation-driven hydrophobic effects are the largest single factor in the stacking of most of the structures studied here.

Although hydrophobic effects may be dominant for many of the structures studied here, it is less clear whether this is the case for the natural bases, which stack less strongly. If hydrophobic forces are dominant, then why does not log *P* (water−octanol partitioning), a widely used measure of hydrophobicity, correlate well with stacking? For example, the natural bases stack significantly better than log *P* values would seem to indicate (Figure 4A). The answer may well lie in the fact that the polar functional groups of the natural bases lie along the edges, rather than on the aromatic faces, of the bases. Thus, they are likely to be influential in stacking only indirectly, since they can probably remain in contact with water whether or not the base is stacked.[44,45] Even a nitrogen incorporated into a ring (such as N-7 of A and G) is probably solvated much more strongly from its edge (using the free lone pair) rather than from the π-system. Thus, we surmise that polarity for these bases is much higher along the edges than on the π-surfaces, and it is possible that some degree of hydrophobic effect can occur even though the bases are quite polar as a whole. This is consistent with our observation that, while log *P* is a poor predictor of stacking, surface area of overlap is a much better predictor (with the exception of G, as noted above).

A related solvent effect seen here is that observed with added ethanol, which destabilizes the helices and does so with different sensitivity depending on the dangling base. We observe that stacking of pyrene, the largest and most nonpolar of the structures studied, is considerably more sensitive to added ethanol than are the four natural bases. Among the five aromatic structures studied, we note that ethanol sensitivity correlates well with surface area of overlap. This is consistent with ethanol decreasing the magnitude of the hydrophobic effect and is also consistent with a significant degree of hydrophobic effect contributing to the stacking of the natural bases.

Relevance to Protein−DNA interactions. Although primarily focused on DNA−DNA interactions, the present results also have significance in analysis of protein−DNA interactions. Two of the ring systems studied here (an indole and a benzene) are analogous to tryptophan and phenylalanine side chain substituents, and we find that benzene stacks as well as, or better than, three of the four DNA bases, while the indole stacks considerably more strongly than all four. This suggests that there may be significant energetic benefit from intercalation of such side chains into DNA, such as has been observed in the complex of transcription factor TBP with its recognition sequence.[58−60]

Applications and Design Principles. The present results show that it is quite easy to arrive at designed structures that

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stack more strongly than the natural bases. Indeed, the simple aromatic hydrocarbon pyrene, for example, stacks considerably more strongly than any of the natural bases, and we find that it raises thermal stability (as T_m) by a quite substantial 23 °C for two substitutions. We have shown in preliminary studies that such strongly stacking nonnatural analogues can be used in designed DNA structures to stabilize helices merely by placing such residues at unpaired positions at the end of a helix.

On a related note, recent studies have shown that extending the nonpairing side of pyrimidine bases by addition of one or two aromatic rings can also lead to increases in thermal stability of duplexes.[61,62] This may be a complementary strategy for stabilizing duplexes, since in those cases the analogues can be used in base-paired positions, while in the present case, the nonnatural analogues are efficient in nonbase-paired positions. Since thermodynamic studies of stacking with natural bases (in the absence of pairing) have not been carried out for those previous cases, it is not yet clear how stabilizing they are relative to some of the present analogues. We noted in the pyrene case a T_m increase of 12.6 °C per residue, while a tricyclic (and undoubtedly more polar) "C" analogue has been reported to give a T_m increase of 5.0 °C per residue in a different sequence context.[62] It might in this regard be useful to study some of those other analogues by the present dangling end method. Since those other analogues are generally more polar than the current ones, the comparison might serve as an additional test of the importance of hydrophobicity.

Acknowledgment. This work was supported by the National Institutes of Health (GM52956) and the Army Research Office.

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