1. Introduction

The repertoire of chemistry available to native nucleotides in DNA and RNA is limited to the purine and pyrimidine functional groups, along with the special role of the 2’-hydroxyl of RNA. The nucleobases have exocyclic amino groups and imines, neither of which is highly reactive or a good candidate for catalytic function. One might argue that the limited range of chemical reactivity is an evolutionary advantage in molecules whose functions are primarily to store (DNA) and process (RNA) genetic information. The most important attribute of both DNA and RNA is the molecular recognition through base pairing. The hydrogen bond pattern combined with the conformation of the ribose sugar gives rise to the specific B-form double helix in DNA and A-form helix with a range of additional standard folds in RNA, loops, bulges and pseudoknots. These structures are well known for their ability to interact with proteins to provide a scaffold for transcription (DNA → RNA) to create messenger RNA (mRNA), and processing of mRNA by enzymes that have active components composed of RNA molecules. The chemical action of RNA on other RNAs by means of the 2’-hydroxyl is an exception to the lack or reactivity of DNA and RNA. Self-splicing by action of the 2’-hydroxyl as a nucleophile was the process that broke the dogma that RNA is always a passive molecule that simply transmits information [1]. Indeed, RNA is very active in processing other RNAs using the 2’-hydroxyl as a nucleophile for hydrolysis of the phosphodiester bond leading to cleavage or to rearrangements of structure such as RNA splicing. Outside of this reactivity, the components of the purine and pyrimidine rings are largely inert. Aromatic amines are poor nucleophiles, and imines are even less reactive. The purine and pyrimidine rings are not particularly electrophilic because of the nitrogen heteroatoms and carbonyls.

Using in vitro selection (also known as SELEX) [2-4], and appropriate modification, RNA and DNA have been developed for numerous applications that transcend their biological functions. In this chapter we will consider the modifications of the nucleobase as a means to expand upon the native function. The extensive literature on modifications of the phosphodiester backbone and the ribose sugar will not be considered in this chapter due to space limitations. Base modifications may consist of expansions of the purine/pyrimidine ring, appended functional group or chemical modification to increase the stability of the backbone with respect to hydrolysis. Expanded DNA or xDNA has been developed as mimics of native DNA with potential biotechnology applications [5, 6]. In these molecules, the purine and pyrimidine rings are fused to phenyl or naphthyl rings to give rise to
extended nucleobases. RNA and DNA aptamers have been obtained through *in vitro* selection using both native nucleobases and modifications in the 5-position of uracil [7-10] or the 8-position and 7-deaza positions of adenine [8, 10, 11]. In both of these cases, the ability of polymerases to tolerate the modifications is crucial to the development of novel technologies. The additional feature that is omnipresent in the strategy for the development of new structure and function is the role played by divalent ions. We consider this as well since it is a technological modification and non-natural ion concentrations are routinely used in selections and in the technologies developed. One can divide technological applications into two structural categories. 1.) the interaction with nucleic acids and 2.) interactions with other classes of molecules, amino acids, or other ligands or substrates that are completely unnatural. This chapter is concerned with the role of modified nucleic acids in determining the structure of an oligonucleotide. The large number of modified nucleosides and nucleotides for clinical applications are not considered here [12].

The structure of RNA and DNA is defined to promote interaction with other nucleic acids. Both the base stacking and electrostatic repulsion of the phosphodiester backbone provides a specific driving force and ionic atmosphere that favors interaction with a complementary shaped molecule. The practical applications that involve nucleic interactions and molecular recognition include ribozymes [13] which can play a role in gene regulation similar to microRNA [14]. Therapeutic ribozymes catalytically hydrolyze transcripts and thereby regulate gene expression, often by translational repression. The therapeutic implications of the RNA regulation pathways are largely beyond the scope of this chapter, but one comment is in order. The development of modified nucleobases may play a significant role in the development of therapeutic RNA. However, the selections and functional testing must then be carried out under the condition of low divalent ion concentration that mimics intracellular conditions. This issue is discussed below further since it is relevant to the development of strategies in the laboratory. Secondly, modified RNA for in vivo application may also be fortified by alterations of the phosphodiester backbone to prevent degradation by hydrolysis. One further point is that RNA is most adapted to the modification of other RNA. RNA hybridization and the positioning of the 2'-hydroxyl are unique structural features that will likely be important in therapeutic strategies. Precisely, for this reason therapeutic RNA development is a separate topic from the thermodynamics of the modifications. Given the susceptibility of RNA to hydrolysis a variety of alternatives have been sought in the laboratory to permit the development of technologies that use the evolutionary advantage of RNA and DNA. Modified nucleosides are needed to extend the functional range of binding and catalysis beyond those of nucleic acids. For example, RNA and DNA catalysts have been developed through *in vitro* selection using bases modified in the 5-U and 8-A,G positions [8, 15, 16]. DNA catalysts have been also designed using intercalators that contain metal centers [17, 18]. Templated structures that use the advantage of recognition in chemistry have been employed to advantage in the development of novel hydrolysis catalysts using both divalent ions and embedded amino acid functionality [19-21].

Addition of chemical functionality that causes large changes in DNA or RNA properties requires consideration of the structural and chemical aspects of nucleic acids in applications ranging from binding interactions to enzymatic catalysis. In this chapter we consider the effect of nucleobase modification on the stability of RNAs and DNAs for applications as aptamers or enzymes. The relationship between the modified nucleobase and polyanionic phosphodiester backbone requires consideration in the design of new molecules for catalytic and binding applications. When DNA and RNA come into contact with proteins the electrostatic repulsion can reduce binding constants and specificity of binding. For this
reason the majority of (deoxy)ribozyme and aptamer applications to date involve the processing or binding of other RNA and DNA molecules. While the majority of the altered DNA and RNA structures involve hydrophilic groups that may be charged and may interact with the phosphodiester backbone, there has also been a concerted effort to develop hydrophobic groups for modification of DNA binding properties. The thermodynamics of the hydrophobic effect and the ramifications for structure and binding of amphipathic molecules will be applied to an understanding of the behavior of DNA in protein binding assays based on these new modifications.

2. Thermodynamics of DNA and RNA structure

Calorimetric data and melting behavior have been used to determine the thermodynamic stability of DNA and RNA. Breslauer et al. have tabulated the free energies of base pair formation in DNA based on the calorimetric data [22]. Such analyses have led to accurate prediction of the stability of base pairs used in design of primers, polymerase chain reaction, and a host of other common applications that involve DNA. RNA thermodynamics and structure have likewise been studied [23], tabulated and used in RNA structure prediction programs such as mfold [24] and frabase [25]. The thermodynamic data do not account for the changes in stability that occur when altered nucleobases are incorporated into the sequence. There are three main contributions to formation of stable hybridized structures 1.) electrostatic repulsion of the phosphodiester backbone, 2.) hydrophobic interactions due to base stacking and 3.) hydrogen bonding interactions leading to molecular recognition in base pairing. In terms of the magnitude, contribution 3.) is clearly the weakest, although it is the essential feature of DNA and RNA recognition. Hydrogen bonding of the nucleobases in DNA or RNA must be referenced to hydrogen bonding in water. Since the hydrogen bond strength with water is significant, it is the molecular fit of the bases in the sequential base alignment rather than strength of the individual hydrogen bonds that dominates the hydrogen bonding contribution (effect 3). The hydrophobic effect, effect 2, is a major thermodynamic driving force for assembly leading to base stacking and self-assembly. The hydrophobic effect results primarily from the unfavorable entropy of solvation of hydrophobic molecules. The water molecules surrounding a hydrophobic solute do not have strong interactions with the solute and this leads to an altered structure, which can be described as an organized cage of hydrogen bonds around the solute. There is a driving force for aggregation of hydrophobic solutes in order to reduce the surface area-to-volume ratio, and hence to reduce the unfavorable entropy of solvation. The magnitude of this effect is sufficiently large that it is considered a dominant thermodynamic contribution in protein folding, membrane formation and DNA and RNA folding. The phosphodiester backbone is a polyanion (effect 1), which leads to unfavorable interactions that are overcome only when the ionic strength of the solution is sufficiently large to screen the charge on adjacent nucleic acid strands. Screening and specific structural interactions of divalent ions play a special role, particularly in RNA folding. RNA forms predominately A-form helices that are part of hairpins, bulges and pseudo-knots. DNA has a smaller conformational space than RNA. Hairpins form but the thermodynamic stability decreases dramatically with increasing loop size [26].

The thermodynamic contributions to the total folding or hybridization energy are affected by specific changes to DNA or RNA structure due to chemical modification or solvent conditions. For example, base stacking depends on base size, which is an important consideration in technologies based on expanded bases such as x- and yDNA shown in Figure 1. [27-29]
Modified nucleosides capable of photocrosslinking reactions include 4-thio and 4-halogeno derivatives of uridine of deoxyribouridine shown in Figure 2. Photocrosslinking with DNA or DNA/RNA strands depends on appropriate conformations, which are accessible transiently in DNA, and hence is dependent on the dynamics of the structure. Base modifications that consist of charged and hydrogen-bonding nucleophilic groups tend to increase solubility and stability as measured by melt temperature (Figures 3E and 3F). On the other hand, hydrophobic base modifications reduce solubility by increasing the tendency for aggregation and reducing the role played by base stacking in stabilizing the overall structure (Figure 3A-D and 3G). Hydrophobic pendant groups could conceivably induce formation of a hydrophobic core as observed in micelles or proteins. This possibility is considered in detail in this chapter. In view of the complex chemistry of DNA and RNA, this chapter strives to examine the thermodynamic studies available to explain the various modifications and their effects.

Fig. 1. The expanded 2'-deoxyadenosine used in the synthesis of x- and y-DNA.

Fig. 2. 4-thio and 5-halogeno modifications of uridine. The X substituent in the 5-position can be any of the halogens, F, Cl, Br or I. The 4-oxo position shown as Y can be substituted with a sulfur to produce the 4-thio derivative. Most frequently these modified uridines are synthesized as the 2’-deoxy (Z = H) or the 2’-O methyl (Z = OCH₃) derivatives.
3. Expanded DNA and modification of the base stack

The concept of modification of the nucleobase to create a new kind of genetic code has been implemented in x- and yDNA nucleobases shown in Figure 1. Quantum chemical calculations estimate that interactions in a double helix composed of xDNA are 10-15% stronger in expanded DNA compared to typical interactions native DNA [30]. The origin of the effect is not hydrogen bond strength, which should be nearly identical in xDNA and yDNA compared to native DNA [31]. Rather the base stacking interactions are the origin of the increased stability, which is manifest in the change in melting temperature from [27]. The melting or denaturation temperature is the temperature at which the duplex is in equilibrium with single-stranded DNA (ssDNA),

\[
(ssDNA_1): (ssDNA_2) \leftrightarrow ssDNA_1 + ssDNA_2
\]  

where the equilibrium constant

\[
K = \frac{[ssDNA_1][ssDNA_2]}{[(ssDNA_1): (ssDNA_2)]}
\]  

Fig. 3. Modification of 2’deoxyuridine in the 5-position. The modifications shown A) indolyl carboxamide, B) isopropyl carboxamide, C) phenyl carboxamide, D) naphthyl carboxamide, E) amino ethenyl, F) guanidinium ethenyl G) pyridinyl carboxamide H) imidazolyl ethenyl carboxamide and I) imdazolyl carboxamide ethynyl.
and double stranded DNA (dsDNA) is represented by \((ssDNA_1):(ssDNA_2)\). However, by measuring the absorbance change, \(\Delta A\), at the maximum wavelength of the combined purine and pyrimidine bases, one is measuring the difference in concentration, between the ssDNA and dsDNA forms. Although the individual nucleobases have small differences in the maximum wavelength, \(\lambda_{\text{max}}\), the average wavelength of 260 nm is used. The difference in concentration can be related to the fraction hybridized. If the initial concentration of dsDNA is \(C_o\) and the concentration of ssDNA is \(x\) then the fraction hybridized is

\[
\theta = \frac{\left[ (ssDNA_1):(ssDNA_2) \right]}{\left[ (ssDNA_1):(ssDNA_2) \right] + \left[ ssDNA_1 \right] + \left[ ssDNA_2 \right]} = \frac{C_o - x}{C_o + x}
\]

Since

\[
K = \frac{x^2}{C_o - x}
\]

Thus,

\[
x = \sqrt{\frac{K^2 + 4KC_o - K}{2}}
\]

Finally, the equilibrium constant contains the temperature dependence,

\[
K = \exp \left( -\frac{\Delta H^o}{RT} \right) \exp \left( \frac{\Delta S^o}{R} \right)
\]

The equilibrium constant approaches, \(K = 1\) at the the melt temperature, \(T_m\). This temperature is determined by the ratio of the enthalpy to the entropy,

\[
T_m = \frac{\Delta H^o}{\Delta S^o}
\]

Finally, we note that the steepness of the melt curve depends on the magnitude of the enthalpy and entropy in this ratio. Observation of a less steep melting curve is an indication that \(\Delta S^o\) is relatively small. It is noteworthy that xDNA melts at a significantly higher temperature than a native DNA with the same nominal sequence, however, both the enthalpy and the entropy of hybridization are significantly smaller than for native DNA. The basic analysis presented here complements analysis based on the van’t Hoff plot [27]. The smaller entropy change is attributed to “prestacking” of the nucleobases in xDNA. Since the nucleobases in xDNA are significantly larger they have greater exposed hydrophobic surface area and this leads naturally to a greater tendency of these bases to associate even in ssDNA. The greater thermodynamic stability of xDNA and yDNA may have application in a new genetic code for biotechnology applications. In summary, the hydrophobic modification appears to increase stability of hybridized x- and yDNA in a highly specific manner since they are in the base stack.
4. Photocrosslinking agents

Photocrosslinking is a known technique for structural biology applications. The crosslinking requires that pairs of nucleobases are in proximity, which is dependent on the range of their motion at a given temperature. Natural intrastrand crosslinking in DNA involves the formation of thymine dimers, which is facile when these two pyrimidines are adjacent in the base stack. However, photocrosslinking probes can also be introduced artificially into the base stack to provide information on three-dimensional structure. The naturally occurring modified nucleic acid 4-thiouridine (4-thioU) in RNA is capable of inducing crosslinks upon excitation with 330 nm light [32]. RNA or DNA made with 4-thioU provides a means to study the proximity of bases in a complex structure [33]. Photocrosslinking followed by digestion of the nucleic acid uses nucleases can be used to deduce the location of specific crosslinks. 5-halogenouridines have been used as artificial crosslinking agents to determine the interaction of polynucleotides with proteins or as the basis technologies based on photocrosslinking selections based on 5-bromouridine [34]. Based on this observation a recent innovation using 5-fluoro-4-thuouridine (FSU) has been demonstrated to induce crosslinks to thymidine, which result in the creation of a fluorescent molecule [35]. While 4-thiouridine forms a number of 6-4 and other crosslinks [33, 36], FSU forms a highly specific crosslink with thymidine that have a fluorescence quantum yield of ~0.5 for excitation at 370 nm and emission at 470 nm [35]. The thermodynamics of formation of the FSU fluorocrosslink has an analogy in the protein world. The formation of the chromophore in green fluorescent protein (GFP) involves ring formation (like the fluorocrosslink of FSU). In both cases the processes is apparently driven thermodynamically by a second step that involves loss of a stable molecule. In GFP there is an oxidative dehydrogenation step that results in the loss of water. In the fluorocrosslink, HF is lost in the process of formation of the fluorescent product [35]. The formation of covalent crosslinks has application in structural studies (4-thioU), detection of crosslinked binding partners (5-BrU) and fluorescent detection of DNA hybridization (5-fluoro- and 5-chloro-4-thioU). These crosslinks have the potential to inform on dynamic states of DNA and RNA since two nucleobases must be in proximity in order for photochemical crosslinking to succeed.

5. DNAzyme templates for enhanced nuclephilicity

Reactivity in DNA can be modulated using a templating approach that holds the catalytic nucleophile of non-native ribose sugar in an appropriate geometry can be positioned to accelerate hydrolysis [19]. This structural modification can be called templating since it brings two strands into proximity using the molecular recognition of the DNA binding arms to permit catalysis as shown in Figure 4. This idea has been tested using RNA-DNA hybrids containing either the 2’-OH and 3’-OH with an adjacent triphosphate [57], and more recently using the incorporation of amino acids serine and tyrosine into the DNA sequence [21]. The template approach is useful, but requires synthesis to prepare appropriate starting materials that can hybridize to the flanking regions shown in Figure 4. DNA-templated polymerization of peptide nucleic acid (PNA) aldehydes has been used to generate tetramer and pentamer PNA building blocks with one, two or three lysine side chains at various positions in the building block [38]. Controlled structural arrangement by hybridization
provides a method to immobilize co-factors or selected nucleic acid catalytic structures, which can serve as catalytic sites [39, 40]. The templating approach obviously has little or no effect on the thermodynamic stability of the DNA in the binding arms, which are the essential interaction for templating. This method is fairly conservative with regard to modification of structure, which is one of the reasons it is regarded as a robust method. However, the method also relies on high divalent ion concentrations for reactivity, but the identities of the ions required depends strongly on the DNA sequence. Based on the recent demonstration the a two-site mutant of 10MD5 DNAzyme switches from both Mg$^{2+}$ and Zn$^{2+}$ to a requirement of only Zn$^{2+}$, one may consider the DNAzyme as a kind of metalloenzyme [20]. However, the mechanistic role of the divalent ions in the DNAzymes is unknown at this time.

Fig. 4. Templating strategies that use DNA to hold modified structures in place to promote catalytic efficiency. A.) a hydrolysis reaction is shown. B. a ligation reaction is shown.
The major targets for templated DNA catalysis are hydrolysis reactions involving phosphate esters, glycosidic, or amide bonds. One can compare the control over reactivity with serine prostates, in which the reactive hydroxyl group of serine is partially negative in charge because of controlled hydrogen bonding interactions in the Asp-His-Ser catalytic triad [41]. In fact, modifications that incorporate serine into the DNAzyme function very well. The charge relay concept involves the partial deprotonation of the hydroxyl group on serine by its hydrogen bond to imidazole. Imidazole in its turn is partially negative in charge because of a hydrogen bond to anion aspartate. The hydroxyl group has an alkoxide character and hence is a better nucleophile. Divalent metal ions may play a similar role interacting with an alcohol, such as serine or the 2'-hydroxyl. This role is well-known in RNA, but in DNAzymes it is less clear how divalent metal ions help to lower the barrier for phosphodiester cleavage. The key thermodynamic consideration in the design of such catalysts is the reactivity of the target amide, glycosidic or phosphodiester bond relative to that of the nucleophile. Amides and DNA phosphodiester bonds are ~20 and ~400 times less reactive than RNA phosphodiester bonds, respectively, making RNA the least stable biopolymer [42, 43]. The high thermodynamic stability of the DNA phosphodiester backbone relative even to protein amide bonds is a key motivation for their development as tools for catalysis of hydrolytic reactions [37].

6. Metal ions as cofactors: enhanced reactivity of chiral DNA scaffolds

Metal ions are a double-edged sword in RNA chemistry [44]. Divalent ions, usually Mg$^{2+}$, are needed for the function of catalytic RNA. Monovalent ions, such as Na$^+$ and K$^+$ are often sufficient for screening the charge of the phosphodiester backbone (vide infra), but divalent ions can be required for specific function of the RNA catalyst. For example the group I intron X-ray crystal structure shows two crucial Mg$^{2+}$ ions required for function [45]. Yet, the the increased nucleophilicity of the 2'-OH leads to hydrolysis of RNA itself. High divalent metal ion concentrations are therefore useful for structure mapping of RNA, but clearly they are detrimental to ribozyme stability. As discussed above, this reactivity and the need to modify the 2'-hydroxyl has resulted in a shift in the field of in vitro selection towards the use of DNA for aptamers and catalysts.

DNA can be regarded as a scaffold that can hold chelating agents and create active sites for substrate binding. DNA is less sensitive to hydrolysis than RNA, in the presence of metal ions, because it lacks the 2'-OH group. DNA can also be a metalloenzyme that binds a cofactor that is capable of chelation of a metal. Examples include Cu$^{2+}$-dependent, stereospecific Diels-Alder catalysis [46, 47], carbon-fluorine bond formation [48] and the Friedel-Crafts reaction [49]. This approach is not limited to common metals in biology, as observed in an Ir$^+$-diene-dependent allylic substitution catalyst [18]. Cofactors such as bipyridines [17], polyaza crown ethers [50], dienes [18], and metalloporphyrins [51] have been introduced by intercalation or clever use of the G-quadruplex motif of DNA. Chelation of metal ions in proteins is the essence is much of biological catalysis. By designing binding sites for metals in DNA and RNA, the number of catalytic reactions accessible to the nucleic acids is greatly increased. Chelation obviates the problems associated with free metals that can catalyze hydrolysis of the phosphodiester bonds.
7. Thermodynamics of RNA and DNA aptamer binding

The stability of aptamers and the thermodynamics of their binding can be measured by calorimetry and CD to reveal that the melting temperature and structural changes associated the binding to a target. One key conclusion from such studies is that electrostatic interactions are a dominant force in binding to amino acids, peptides and proteins. For example, L-argininamide binds to its aptamer with a $\Delta G^o = -5.1$ kcal/mol and $\Delta H^o = -8.7$ kcal/mol [52]. The unfavorable entropy of binding, $T\Delta S^o = +3.6$ kcal/mol, arises from the ordering of the loop region when L-argininamide binds to the hairpin structure. The melting temperature is 50.1 °C for this monomeric aptamer, but this value increases proportional to L-argininamide concentration indicative of the stabilizing effect of the bound cognate ligand [52]. The stabilization of DNA and RNA by cationic ligands and proteins is a consequence of the interaction of the negatively charged phosphodiester backbone. In the general case ligands such as L-tyrosinamide bind by electrostatic, hydrophobic and hydrogen bonding interactions [53]. Typically, a conformational change in the RNA or DNA aptamer is a hallmark of the small molecule binding [54]. However, the driving force for binding by the small molecules has a large electrostatic component. This holds true for the most studied of aptamer protein complexes. The thrombin aptamer binds primarily by electrostatic binding at a surface fold that contains many exposed arginine and lysine residues [55, 56].

The change in conformation associated with aptamer recognition stands in contrast to the entropically-driven minor groove binding of many hydrophobic drugs to dsDNA. Dyes such as Hoechst 33258 bind with little change in DNA structure leading to a reduction in hydrophobic surface area [57]. Thus, the thermodynamics of minor groove binding is clearly driven by the hydrophobic effect. On the other hand intercalators, such as the anthracyclines have significant contributions from the hydrophobic effect by insertion into the base stack and an electrostatic effect due to the charge-charge interactions, and hydrogen bonding effects [58]. Hydrogen bonding effects are considered the smallest contribution, e.g. ~1 kcal/mol, in the case of the anthracyclines. Intercalators also lead to unwinding of DNA, which gives rise to an unfavorable entropy contribution that essentially cancels the hydrophobic effect. Thus, binding of intercalators is largely enthalpic. One can conclude that for both intercalators and cognate ligands such as L-arginamide, ionic effects are particularly important determinants of aptamer binding strength.

Since DNA and RNA are polyelectrolytes it is useful to include the effects of ion displacement in the measurement of free energies of binding by determination of the binding constant as a function of salt. Using the relation between the equilibrium binding constant, $K$ and the free energy,

$$\Delta G^o = -RT\ln K$$

(8)

The polyelectrolyte contribution to the binding free energy can be obtained from the equation,

$$\Delta G_{pe}^o = -\left( \frac{\delta ln K}{\delta ln [Na^+]} \right) R T \ln [Na^+]$$

(9)
\( \Delta G_{p}^{o} \) is the excess binding free energy relative that at \( [Na^+] = 1 \). The non-electrostatic free energy change of binding is

\[
\Delta G_{t}^{o} = \Delta G^{o} - \Delta G_{p}^{o}
\]  

\( \Delta G_{t}^{o} \) is useful for comparison of charged and uncharged ligands since it contains a minimal contribution due to the ligand charge. This particularly important in cases where the structure of phosphodiester backbone is altered. Any conformational change that brings the charged phosphodiester groups in proximity will be highly dependent on the ionic strength of the solution. Aside from hybridization itself, which requires an ionic strength of at least 0.1 M, conformational changes in DNA that will cause it to deviate from an extended B-helix must depend strongly on ionic strength.

These considerations lead to separate discussion of the major types of modification of DNA that are current in use. Hydrophilic modifications can also carry a positive charge and partially neutralize the phosphodiester backbone. This effect can mitigate the repulsion and permit different folds and can also enhance certain types of reactivity. The effect of hydrophobic modifications of nucleobases is more difficult to predict. However, since the short chemical linkers used for nucleobase modification (Figure 3) precludes intercalation, there is a competition between standard B-form helix and a potentially new fold of DNA.

8. Modification of aptamers and (deoxy)ribozymes with hydrogen-bonding groups

Polypeptides serve as one source for design of novel function in DNA and RNA. Nucleobase modifications that include polypeptide functional groups involve the use of pendant imidazoles, amines, or guanidinium groups, which mimic the amino acids lysine, histidine and arginine, respectively [8]. In addition to those shown in Figure 3 for modified uridine, there are also the modifications at the 8-position of adenosine and guanosine shown in Figure 5. These groups provide reactivity that is normally absent in nucleic acid chemistry. These modifications increase the range of catalytic activity of (deoxy)ribozymes. These are water-soluble groups that tend not to interact with the hydrophobic interior of the nucleic acid. They may have some tendency to interact with the anionic phosphodiester backbone since they tend to be cationic with pKa at ~7, ~9 and ~12, respectively. These types of modification have been successfully employed in strategies to make a new class of phosphodiesterases for \textit{in vitro} application [8]. The charged groups may enhance substrate binding for recognition or catalysis [15]. Changes in pendant groups in the 5-position of uridine or 8-position of guanine or adenine do not have a major impact on the thermodynamic stability of DNA or RNA. However, specific charge interactions of guanidinium and amine groups with the phosphodiester backbone can increase the stability of modified DNA. The utility of these modifications has been shown recently in the development of DNA phosphatases that function at physiological concentrations of Mg\(^{2+}\) [8]. There are numerous protein enzymes that function without a requirement for a metal ion. Acid-base catalysis and nucleophilic displacement reactions are two common types of mechanism that can be catalyzed using acidic or basic amino acids. By including these functions in DNA structure, new tools can be developed for therapeutic degradation of RNA sequences.
Fig. 5. Modified 2’-deoxyadenosine molecules A. modification by an amino group in the 7-deaza position. B. modification by an imidazole ring in the 8-position.

9. Hydrophobic modifications: thermodynamic effects in amphipathic systems

Hydrophobic pendant groups have been used to structurally modify nucleobases in a number of recent applications. While the initial concept for these groups was tested on RNA aptamers and *in vitro* selection for enzymes, the field moved to DNA aptamers because of their greater thermodynamic stability. The hydrophobic pendant groups shown in Figure 3A-3D in modified DNA and the pyridyl group in Figure 3G has been used in RNA various applications. The phenyl, indolyl, and isopropyl groups mimic the hydrophobic amino acids, phenylalanine, tryptophan and valine, respectively. The hydrophobic modifications
have the potential to dramatically change the properties of both RNA and DNA. It is essential to understand the thermodynamic properties of these amphipathic structures to make sense of the consequences of the structural changes. If a single base is modified, as is the case in most studies where each uridine carries the modification, then 25% of the sites on the DNA or RNA sequence will contain the hydrophobic modification on average. The naphthyl group is the most drastic change. It has a larger hydrophobic surface area than the RNA and DNA nucleobases themselves.

The question for the structural library that is intended with these modifications is whether the balance of forces leading to hybridization can be significantly altered by these modifications. One can envision that a sufficient number of such modifications leads to a change in the structure that deviates from the dominant B-form DNA. Alternatively, if the thermodynamics that lead to B-form helix dominate then the hydrophobic groups will be on the exterior and will present a hydrophobic surface. In this case, the exposed hydrophobic surface area has the potential to produce aggregation. In applications that involve evolution of aptamers the exposed surface area may lead to greater interaction with proteins. However, give the nature of hydrophobic effects, this is unlikely to be a specific interaction. In essence the hydrophobic modification of polynucleotides can be regarded as the creation of a kind of organized surfactant, and the same properties must be considered as one would consider for traditional surfactants including aggregation and protein denaturation.

The hydrophobic effect is considered the dominant effect in biological self-assembly ranging from membrane formation to protein folding. The role of hydrophobic amino acids is mainly to nucleate folding by providing a hydrophobic core for a protein. By analogy, extensive use of hydrophobic amino acids and other even more hydrophobic groups such as the naphthyl group can lead to some changes in structure and properties of nucleic acids. Based on the foregoing considerations, there are two significant alternatives that one can call refolding and hydrophobic surfactant formation, respectively. In the following we discuss the thermodynamic factors that govern the structural dichotomy.

The free energy that accompanies the transfer of a hydrophobic solute into aqueous solvent has been viewed as the key measurement to determine the relative contribution of a group to protein folding and other self-assembly phenomena. The magnitude of hydrophobic transfer can be quantified using the partitioning coefficient \( \gamma \), where,

\[
\gamma = \frac{C_w}{C_v}
\]  

(11)

In this definition \( C_w \) is the concentration in water and \( C_v \) is the concentration in the vapor phase, i.e. the vapor pressure of the organic solute [59]. Although benzene and naphthalene are considered less hydrophobic than isopropanol on the \( \gamma \) scale, their absolute solubilities in water are significantly lower.

DNA structures have a more limited repertoire than RNA structures, and consist mainly of hairpin structures. One can consider the effects of alterations of nucleobases in the stem and loop of a hairpin. In order to make a quantitative estimate of competing effects one can compare the thermodynamic stability of B-form DNA to the hydrophobic contribution of the added groups on modified nucleic acids. For DNA \( \Delta G^o \) per base pair is estimated to range from -0.9 to -3.6 kcal/mol [60]. This interaction energy, which is mostly driven by
hydrophobic interactions, can be compared with the gas phase dimerization energy for naphthalene, which is ca. 4 kcal/mol [61]. The structure of benzene differs significantly from that of naphthalene. While naphthalene tends to form cofacial dimers, benzene molecules interact in a T-conformation in H$_2$O with significant induced dipolar effects [62]. The solvation energy of benzene has been calculated to 1.5 kcal/mol less than that of naphthalene [63]. Sequestration of naphthalene dimers in hydrophobic surfaces has been demonstrated by the formation 2:2 complexes (two naphthalenes joining two cyclodextrins) with a free energy change of -11.8 kcal/mol [64]. This suggests that sequestration of naphthalenes in the loop region would certainly solidify the loop region by means of the hydrophobic effect.

Nucleobases interact with hydrophobic molecules, such as naphthalimide [65], leading to the possibility that these groups can either intercalate, or bind in the major groove on the hydrophobic surface of the double-helical base stack. Binding to the major groove is mainly enthalpic, while binding to the minor groove has a large entropic contribution due to the displacement of bound water in the A-T rich regions [66]. However, the pendant groups attached to nucleobases in the 5-uridinyl or 8-adenosynyl positions have limited mobility and can neither intercalate nor associate with the minor groove unless they significantly modify the double-helical structure of DNA. Given that there is an average of one hydrophobic group for every four nucleobases, this means that the average hydrophobic interaction would need to overcome a hybridization free energy in the range from 4.2 – 12.4 kcal/mol [60] in order to alter the structure from B-form helix to another kind of fold. The given range spans the possible sequence-dependent combinations of four nucleobases from weakest to strongest [60].

The basic considerations of loop and stem stability lead to the conclusion that hydrophobic base modifications will have the greatest effect on loop stabilization rather than modification of the double helix. Such stabilization is analogous to the binding of ethidium bromide to loops, which leads to increases in the free energy of formation of loops with n = 3, 5, 7 nucleotides [26]. Beyond these effects, the structural effects on the double helical regions are likely to be very small for single base modifications. Overall only a small structural effect is expected although there may be formation of a hydrophobic core in the loop regions by hydrophobic groups. Similar considerations apply to pyridine-modified RNA and have been examined structurally as discussed below.

9.1 Effect of hydrophobic interactions on ribozyme catalysis

A pyridyl-modified RNA, DA22, has been proposed to be capable of carbon-carbon bond formation [7]. The mechanism of the reaction can be understood in terms of hydrophobic surface presented by pyridyl-modified RNA (Figure 3G,Y = OH), which promotes binding of Diels-Alder reactants and thereby facilitates carbon-carbon bond formation as shown in Figure 6. Typical reactants in the Diels-Alder reaction are called the diene, a molecule containing a double bonded carbon, and the dienophile, a molecule containing two double bonds that reacts with the diene as shown in Figure 6. The diene and dienophile are themselves quite hydrophobic. For this reason the hydrophobic effect has a long history in acceleration of rates of Diels-Alder and related reactions in water using molecules like cycloextrin to provide a hydrophobic surface for the two participants in the Diels-Alder reaction to find one another in a confined space [67]. The pyridyl-modified RNA, DA22, behaves essentially like a cycloextrin in its ability to bind the
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diene and dienophile. Structure mapping using mung bean nuclease or lead acetate revealed that the pyridine modification had a minor effect on the structure of the RNA [68]. Kinetic experiments have shown that DA22 is not a metalloenzyme, and the claim the Cu$^{2+}$ participates in the reaction [7] has been disproven by both structure mapping and kinetic studies of DA22 [68]. One difference between the pyridyl-modified RNA and cyclodextrin is that there is no size discrimination in DA22. Therefore, relatively large substrates such as anthracycline derivatives were accepted as substrates by DA22 [68]. Given that the original selection was for a much smaller substrate, it is clear that DA22 does not have substrate specificity [7], but rather works based on a general hydrophobic effect [68].

Given that the original selection was for a much smaller substrate, it is clear that DA22 does not have substrate specificity [7], but rather works based on a general hydrophobic effect [68]. This type of interaction is quite distinct from a subsequent development of a true enzyme, J49, based on a fold of native RNA [69]. In fact the Diels-Alder reaction catalyzed by DNA, which may further indicate the general nature of the requirement for hydrophobic surfaces to bring the diene and dienophile together [46]. However, in the case of the RNA J49, the specificity of binding by the hydrophobic effect reveals a second major issue in the development of artificial ribozymes, namely product inhibition [70].

A. Enzymatic, multiple turnover

$$
\begin{align*}
S_2 & \xrightarrow{k_1} \text{ES}_1S_2 \xrightarrow{k_2} \text{products} + E \\
S_1 & \xleftarrow{k_{-1}} \text{ES}_1S_2
\end{align*}
$$

B. Non-Enzymatic, Second-order suicide inhibition

$$
\begin{align*}
S_2 & \xrightarrow{k_1} \text{ES}_1S_2 \xrightarrow{k_2} \text{products} \\
S_1 & \xleftarrow{k_{-1}} \text{ES}_1S_2
\end{align*}
$$

Fig. 6. Illustration of two kinds of transformations that involve the Diels-Alder reaction between a diene, $S_2$, and dienophile, $S_1$. A.) In the enzymatic transformation the enzyme E is unchanged during the course of the reaction. B.) In the non-enzymatic transformation the bimolecular reaction between $S_1$ and $S_2$ causes the enzyme to become inactivated.

Product inhibition is a major impediment to the development of enzymes with a well-developed binding site, i.e. those where there is significant sequestration from solvent water due to the hydrophobic effect. The first specific ribozyme-catalyzed Diels-Alder reaction (Figure 6A) used only naturally occurring nucleic acids [69, 71]. Extensive structural characterization reveals that the Diels-Alder product binds in a specific pocket in the RNA [72]. While this is an excellent example of the power of in vitro selection, the tight binding of the product, rather than the transition state, shows that product inhibition is an inherent
problem for the tethered substrate selection strategy [72]. It was later shown that product inhibition can be potentially overcome with appropriate selection design using an *in vitro* compartmentalization strategy [70]. In this strategy the reaction takes place under multiple turnover conditions in a single droplet so that selection of a true enzyme can be accomplished. However, pyridyl-modified RNA, DA22, cannot show product inhibition since the product is itself a suicide inhibitor as shown in Figure 6B. Given the large hydrophobic surface area in DA22 it is likely that there are many “active sites” on the hydrophobic surface that can promote the second order reaction of the tethered dienophile with a biotin labeled diene [68].

In considering the role of inhibition, one can use classical competitive kinetics to describe the inhibition of a single substrate.

\[
\frac{v}{v_0} = \frac{k_{\text{cat}} [E]_o [S]}{\alpha K_m + [S]} \\
K_I = \frac{k_{\text{cat}} + k_{\text{off}}}{k_{\text{on}}}
\]

(12)

Where

\[
\alpha = 1 + \frac{[I]}{K_I} , \quad K_I = \frac{[E][I]}{[EI]}
\]

(13)

The rate constants in this model are \(k_{\text{cat}}, k_{\text{on}}\) and \(k_{\text{off}}\) which are the catalytic rate constant, substrate on and off rates for binding, respectively. The competition of substrate, \(S\), and inhibitor, \(I\), is expressed in terms of the magnitude of the inhibitor dissociation constant, \(K_I\) relative to the inhibitor dissociation constant, which is related to the ratio of the off rate, \(k_{\text{off}}\) divided by the on rate, \(k_{\text{on}}\). While these equations are well-known textbook examples, they are derived for protein catalysis and have been used incorrectly in some treatments of ribozyme kinetics.

The equations should only be used for multi-turnover catalysts such as the J49. Eqns. 12 and 13 have no meaning for second-order reactions such as carbon-carbon bond formation by DA22. The original discovery of RNA “catalysis” required a proof that RNA can function as a true catalyst, which means that it is not changed structurally by the chemical transformation. A second caveat is that product inhibition requires a more involved treatment than Eqn. 13 above since the concentration of inhibitor \(I\) starts at zero concentration and increases with time in the case of product inhibition. It is absolutely incorrect to use methods such as Murrison’s quadratic equation which is only valid for tight-binding inhibitors when \([I] \sim [E]\) [7]. Finally, the maximum substrate concentration needs to be carefully checked since accurate fitting of the equation is only possible when \([S]_\text{max} > K_m\). In cases where data are limited due to lack of solubility of hydrophobic solutes, this should be noted and the accuracy of the result will clearly be less. Double reciprocal plots of the type used in Lineweaver-Burke analysis should be avoided since these plots mask this problem and can give rise to incorrect parameters [7].

9.2 Hydrophobic effect in RNA-mediated materials synthesis

RNA-mediated materials synthesis is a relatively new concept that extends the potential role of RNA processing to include inorganic materials. This challenging idea requires an
in vitro selection for a templating reaction or catalysis of starting materials that will lead to formation of a structure that is by definition much larger than the RNA itself. The first example of this type used two sequences of pyridyl-modified RNA. The sequences identified as Pd17 and Pd34 were reported to form Pd hexagons and cubes, respectively, in aqueous solution [73]. In this case the hydrophobic effect is a major contributor to the chemistry since the starting material in the synthesis is the zero-valent Pd complex trisdibenzylideneacetone dipalladium(0), Pd$_2$(dba)$_3$, which is insoluble in water [74]. For this reason approximately 50% THF was used in the experiments in Ref. 73, as was revealed later as the mechanism of the formation was considered in more detail [75]. The potential effect of such high concentrations of organic solvent on the hydrophobically modified RNA is likely to be quite large. Although the goal was the production of Pd nanoparticles [76], it is apparent from the data that aggregates of Pd$_2$(dba)$_3$ were formed instead [77-79]. These aggregates form spontaneously in hexagonal shaped crystalline form, but degrade quickly at room temperature. One can liken the phenomenon of particle formation to the formation of organometallic snowflakes composed of Pd$_2$(dba)$_3$ [78]. Given the increased hydrophobicity of pyridyl-labeled RNA, its solubility may be enhanced in THF, which may in turn accelerate the process of nucleation of crystalline aggregates of Pd$_2$(dba)$_3$, although no specific role for the modified RNA has been established in this process.

9.3 Amphiphatic effects in the design of aptamers for proteomic applications

Aptamers are structured RNA or DNA molecules selected by in vitro selection methods to bind to certain targets. Since RNA and DNA are polyanions, the binding of these molecules is limited to certain regions of surface charge. Although polynucleotides are amphipathic there is little evidence to date to suggest that the hydrophobic effect plays a major role in the interaction of aptamers of native RNA or DNA with their cognate targets. The hydrophobic modifications shown in Figure 3A-3D increase the amphipathic nature of DNA aptamers. In order to understand the thermodynamics of amphipathic modes of binding we can compare the possible modes of binding to the class of amphipathic transcription activation domains, which bind to kinase-inducible (KIX) domain of histone acetyl transferase CREB binding protein [80]. The KIX-transcription regulators are amphipathic proteins consisting of alternating regions of negatively-charged aspartates/glutamates and the range of hydrophobic amino acids. These amphipathic proteins have multiple binding sites on their target CREB, which underscores the lack of specificity of the binding of hydrophobic groups [80]. Moreover, competitive inhibition by hydrophobic isooxazolidene molecules considerably smaller than the transcription activator peptides strongly suggests that the mode of binding is hydrophobic. This mode of interaction stands in contrast to the known modes of aptamer binding to proteins such as the electrostatic binding of the thrombin aptamer (vide supra) [55, 56]. The electrostatic mode of binding is a common mode of binding in proteins such as cytochrome c, which docks via positive charged lysines to negative patches on its electron transfer partners. In fact, the KIX-transcription regulators represent a relatively unusual amphipathic mode of binding. The point of this comparison is that hydrophobic or amphipathic binding is possible, but it is difficult to obtain specificity as reported in a study of the binding affinity of these aptamers [81]. Studies of the binding of 1-anilino-8-naphthalene sulfonate (ANS), a small amphipathic molecule that is widely used as a probe of protein structure, show that it
has a predominantly electrostatic mode of binding [82, 83]. Thus, one might interpret this as a general observation that electrostatic interactions superecede hydrophobic interactions. While this statement is relatively clear for small molecules, it is harder to ascertain for typical aptamers with 40 nucleotides, of which 10 are modified on average. The novel concept of using hydrophobic groups to increase binding affinity can clearly lead to high affinity binding. The question that needs to be addressed is what structures are produced and can they be made to confer high specificity?

The binding of the hydrophobically modified DNA aptamers designed for proteomic analysis has recently been measured using gel electrophoresis and competition binding assays [81]. These experiments demonstrated non-specific binding to the protein targets both in the multiple bands in the gel and in the non-exponential dependence of binding in the competition assay for a generic anionic substrate. The lack of specificity for the target may be due to the amphipathic nature of the modifications introduced, which could lead to several modes of binding similar to the multiple modes of binding of KIX-domain transcription regulatory proteins [80]. These experiments demonstrate the need for a comprehensive examination of the functional groups used from the point of view of the structural folds accessible to DNA. Approaches using multiple modifications that mimic the exterior electrostatic properties of proteins more closely may increase binding specificity [8].

10. Conclusion

This chapter discusses the effects of base modification from a thermodynamic perspective. The use of modifications of nucleic acids must strike a balance between the opposing aspects that determine DNA and RNA structure, the polyanion backbone and the hydrophobic base stack. Base modification can be an advantage for design of catalysts because of the intrinsic reactivity of the functional group attached to the base. The functional groups provide the nucleophilicity of an amine, acid-base catalysis of an imidazole or the charge-withdrawing ability of guanidinium. Based on these chemical contributions to nucleic acids, properties well known in enzymatic catalysis are now being exploited to advantage in DNAzymes. The data available suggest that the melting temperature is increased by these modifications, which also means implicitly that DNA B-form structure is conserved. Less is known about hydrophobic modifications. The thermodynamic consequences could be a change in structure that would create new fold of DNA. However, no melting or spectroscopic data have been published, so even the most basic aspects of the structural and energetic consequences of the modifications remain unknown. There is a major difference between the formation of a hydrophobic core in a modified DNA and a protein. DNA has a strong propensity to form linear strands due to base stacking and electrostatic repulsion. The hydrophobic effect of each modification would need to overcome free energy of stabilization in the range of 4.2-12.4 kcal/mol for each 4 nucleobases in stem regions in order dramatically alter the structure from B-form DNA to another kind of structure. Loop regions of DNA may be stabilized by hydrophobic interactions, which may lead to formation of larger loops. Whether they form a hydrophobic core or remain exposed on the surface of a linear B-form helix, the hydrophobicity of the structure may increase binding interactions with proteins either by presentation of negative charge or by denaturation of proteins onto the exposed hydrophobic surfaces. Hydrophobic groups tend to have low specificity as exemplified by the Diels-Alderase case, where DA22 plays only a role a non-specific
environment that permits the the diene and dienophile to react without providing a specific binding pocket for catalysis. We conclude judicious use of hydrophobic groups with an appropriate mixture of charged or hydrophilic groups may provide a great repertoire of functional DNAzymes and DNA aptamers in the future.

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