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Understanding Stacking Interactions between an Aromatic Ring and Nucleobases in Aqueous Solution: Experimental and Theoretical Study

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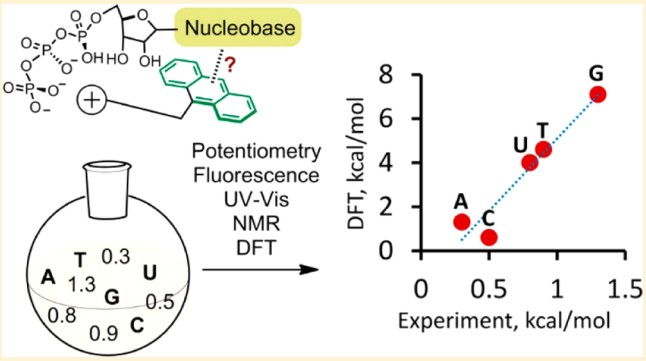
Supporting Information

ABSTRACT: Stacking interactions between aromatic compounds and nucleobases are crucial in recognition of nucleotides and nucleic acids, but a comprehensive understanding of the strength and selectivity of these interactions in aqueous solution has been elusive. To this end, model complexes have been designed and analyzed by experiment and theory. For the first time, stacking free energies between five nucleobases and anthracene were determined experimentally from thermodynamic double mutant cycles. Three different experimental methods were proposed and evaluated. The dye prefers to bind nucleobases in the order (kcal/mol): G (1.3) > T (0.9) > U (0.8) > C (0.5) > A (0.3). The respective trend of interaction free energies extracted from DFT calculations correlates to that obtained experimentally. Analysis of the data suggests that stacking interactions dominate over hydrophobic effects in an aqueous solution and can be predicted with DFT calculations.



INTRODUCTION

Noncovalent interactions between aromatic rings are ubiquitous in biological processes. They play an important role in DNA−protein, protein−protein and protein−ligand interactions, and determine the structure of duplex DNA.[1,2] Analysis of X-ray structures of DNA−protein complexes revealed that 40% of the structures involve either $\pi-\pi$ stacking, T-shape interactions or sugar−π contacts.[3] Aromatic amino acids build the population trend favoring phenylalanine (Phe > Tyr > Trp > His) in stacking with nucleobases. It was found that in a number of ATPases, regardless of fold, proteins form similar types of hydrogen bonds and stacking interactions. A key role in the process of ATP firing in phage T4 packaging motors was assigned to a specific adenine recognition, which involves cation−π and $\pi-\pi$ interactions with the base (Figure 1A).[4] Stacking interactions were found essential in a number of enzymes, e.g., in recognition of guanine in RNase T_1[5] and in aminoglycoside antibiotic kinase (Figure 1B). According to the experimental and theoretical investigations interactions between aromatic rings can supply up to one-third of the total nucleotide binding energy.[6] The idea to use stacking interactions for the recognition of nucleotides has been widely explored in recent years.[7,8] A number of receptors and fluorescent probes for nucleotides has been synthesized during the past decade utilizing metal-phosphate interactions together with stacking interactions between an aromatic residue and a nucleobase.[9−17] Little or almost no attention has been paid to quantification of stacking interactions between nucleobases and dyes in host−guest complexes.[3,18−23]

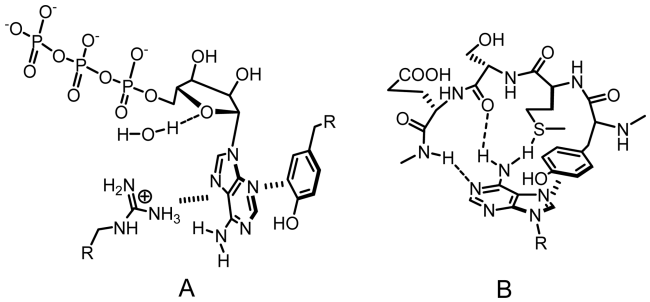


Figure 1. Structures of binding pockets in ATPase (A) and kinase (B) showing stacking interactions with adenine.

It is a challenge to detect and to quantify direct interactions between nucleobases and aromatic compounds because they are weak in such a highly competitive solvent as water.[24] There are two classical approaches for the detection and in some cases quantification of these interactions outlined in the literature.[25] The first approach rests on spectroscopic methods used to detect stacking interactions between molecules. Fluorescent properties of a number of dyes are sensitive to stacking interaction with nucleobases. For instance, Kubota and coworkers investigated fluorescence changes induced by an addition of different acridine dyes to nucleotides in a phosphate buffer at pH 7.[18] The authors observed static and dynamic

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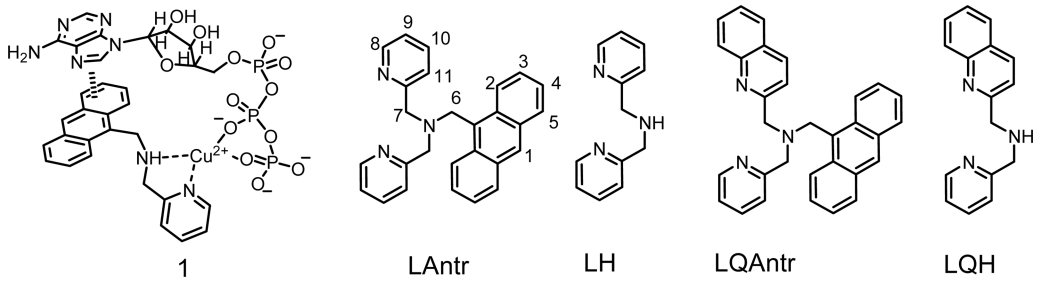

Figure 2. Complex with ATP (1) studied in the previous work. Structures of ligands used in this work to quantify stacking interactions between anthracene and nucleobases.

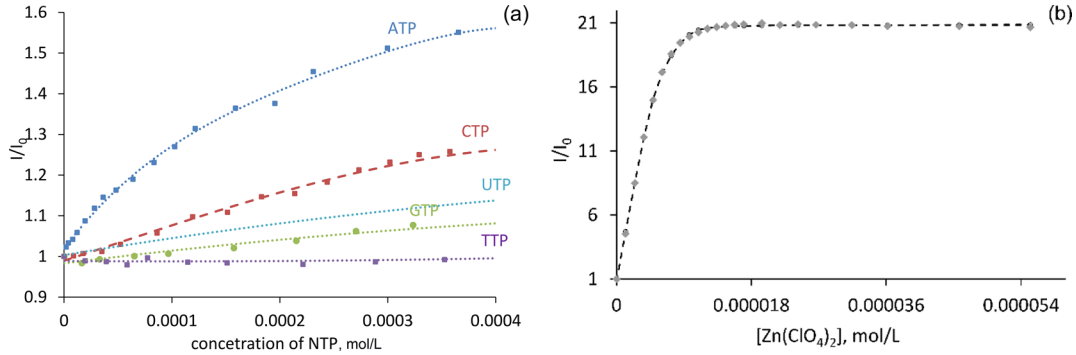

Figure 3. (a) Changes in fluorescence intensity of the ligand (10^{-5} M) at 415 nm induced by addition of five nucleoside triphosphates. (b) Fluorescence changes induced by addition of zinc(II) perchlorate to ligand LAntr (10^{-5} M). Conditions: 10 mM TRIS buffer (pH 7.4, 4% vol. MeOH, 0.1 M NaCl). Excitation at 370 nm.

quenching of dyes in the presence of nucleotides and the binding constants were in the order of 100 M^{-1}. 10-Methylacridine bound AMP with K_a = 40 M^{-1} and with the following selectivity: AMP ≈ GMP > TMP > CMP. The second approach involves an assessment of stacking interaction between aromatic components in a complex, held either by additional covalent or noncovalent bonds. Leonard connected indole with nucleobases with a linker, consisting of three and four carbon atoms and confirmed the presence of stacking interactions with the help of NMR and UV−vis spectroscopy.[26] In a number of publications it was reported that guanine and adenine form complexes with dyes better than other nucleobases.[27,28] Lhomme in 1987 showed that the hydrogen bonding between adenine and uracil is possible in water, but only if additional stacking interactions are present.[21] Rebek and co-workers were the first, who examined an effect of the structure of an aromatic ring on its stacking properties with adenine in water. The authors found from their work on receptors for adenine that the extension of the hydrophobic surface from phenyl to naphthyl corresponds to an increase in free binding energy of −1.5 kcal/mol.[23]

There are two main challenges facing the quantification of stacking interactions between aromatic compounds and nucleobases. First, binding affinities have small values so that it is difficult to measure them with standard analytical methods. Second, measurements in aqueous solution are usually impossible due to low solubility of organic compounds. In this work, we address these challenges by extracting stacking free energies from thermodynamic quantification of nucleotide binding to di(2-picolyl)amine-Zn(II) complexes. The overall stability constants for the complexes are high and allow one to measure binding affinities with high accuracy. With the help of quantum chemical calculation and designed experimental procedures, we have quantified stacking free energies between five nucleobases and anthracene.

RESULTS AND DISCUSSION

Design of Model Systems. Recognition and sensing of nucleotides is a rapidly growing field.[7,29−34] There are a number of selective receptors and fluorescent probes developed to date, whose structures are based on positively charged aromatic rings or metal complexes bearing an aromatic ring. Considering the recognition in an aqueous buffered solution, the affinity of single-charged aromatic systems for nucleotides are usually less than 100 M^{-1}.[35] Within the experimental error the affinities, e.g., for ATP and GTP, are the same. Thus, it is difficult to elucidate the effect of the structure of a nucleobase on the binding strength. On the other hand, dyes with Zn(II) complexes show much higher affinities (ca. 10^5 M^{-1}) for nucleotides because of strong electrostatic interactions between the Zn(II) site and the phosphate residue and are more suitable to detect small differences between nucleobases.[29,36] There are two general designs of Zn(II) complexes used for recognition and sensing of nucleotides. The first design consists of a rigid fluorescent scaffold with one or two Zn(II) sites. The selectivity of complexes for nucleotides (bearing different nucleobases) is usually low because electrostatic interactions dictate the overall affinity.[37,38] The second design consists of a Zn(II) site and a dye that is connected through a flexible linker. The dye is assumed to form π−π interactions with a nucleobase.[12] Hence, it was often expected that stacking interactions between the dye and a nucleobase introduce a selectivity into the complex for a certain nucleotide. However, such complexes still allow one to differentiate between nucleotides by using fluorescence spectroscopy because different nucleobases interact with an excited dye with different binding strengths. For example, adenosine

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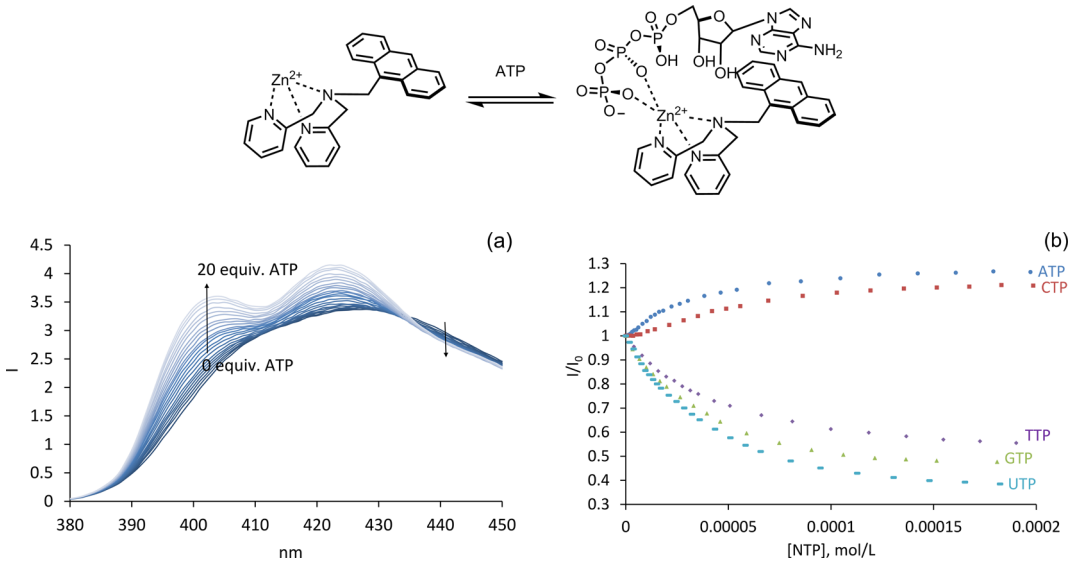

Figure 4. Coordination of ATP to complex LAntr·Zn(II) and (a) the observed changes in fluorescence during the titration experiment (excitation at 375 nm). (b) Fluorescence changes at 423 nm upon addition of five nucleoside triphosphates to complex LAntr·Zn(II). Conditions: 10 mM TRIS buffer (pH 7.4, 4% vol. MeOH 0.1 M NaCl), 10^{-5} M LAntr, 10^{-4} M zinc(II) perchlorate.

triphosphate often induces an increase in fluorescence of Zn(II) complex, while guanosine triphosphate leads to quenching of fluorescence.[7]

For our studies we used the first design and synthesized ligands LAntr, LH, LQAntr and LQH (Figure 2) according to the literature known procedures.[39−42] The Zn(II) complexes for these ligands are also known, but they have never been studied in complexation with nucleotides. In principle, any Zn(II) complex bearing free coordination sites for binding an anionic species can be a potential receptor for nucleotides. In our design, the Zn(II) site is responsible for electrostatic interactions with phosphate, while the anthracene dye can form $\pi-\pi$ interactions with a nucleobase. This concept—a combination of both types of interactions—rests on our previous investigation of Cu(II) complex **1** (Figure 2), which demonstrated selectivity for ATP (adenosine triphosphate) over ADP (adenosine diphosphate) and AMP (adenosine monophosphate).[43] Quantum chemical calculations and spectroscopic measurements provided an evidence of high complementarity in complex **1** (Figure 2), i.e., adenine forms $\pi-\pi$ interactions with anthracene. The proximity of two aromatic systems were impossible in cases of shorter nucleotides such as ADP or AMP.

In preliminary studies we investigated the interaction of free ligand LAntr with nucleotides in a 10 mM TRIS buffer (pH 7.4, 100 mM NaCl). The ligand has pK_a value of 5.25 and thus, only 0.4% of the ligand are singly protonated at pH 7.4. The ligand binds NTPs, but with relatively low affinities (less than 500 M^{-1}). The observed increase in fluorescence of the ligand during the titrations with ATP and CTP (Figure 3a) can be explained by the fact that the complexation favors protonation of the tertiary amine. This protonation hinders a photoinduced electron transfer (PET) between the dye and the amine leading to a fluorescence increase.[40]

Formation of complex LAntr·Zn(II) from the ligand and zinc(II) perchlorate accompanies with strong fluorescence increase (Figure 3b). The stability constant of LAntr·Zn(II) in a 10 mM TRIS buffer is log K_{11} = 8.2. However, according to fluorescence titrations complex LAntr$_2$·Zn(II) is also formed with stability constant log K_{21} = 14.3. To ensure the formation of a 1:1 complex, we performed all titrations with nucleoside triphosphates in the presence of 10 equiv of Zn(II). The Zn(II) complex shows a different profile of fluorescence changes in the presence of nucleotides compared to one of the free ligand. Adenosine and cytosine triphosphates increase the fluorescence of the complex further, while thymidine, guanosine and uridine triphosphates quench the fluorescence of the ligand (Figure 4). Increasing amounts of a nucleotide favor the formation of a ternary complex LAntr·Zn·NTP (NTP, nucleoside triphosphate), whose fluorescence has a structure characteristic for anthracene (Figure 4). According to De Silva[44] and Hamachi[37] coordination of an anion to a Zn(II) site suppresses the PET quenching of the photoexcited anthracene by the cationic pyridine leading to an increase of fluorescence. Such an increase we observed for ATP, CTP and pyrophosphate. On the contrary, UTP, TTP and GTP quench the fluorescence of the complex due to a different PET process, from nucleobases to anthracene, as revealed previously by Seidel and co-workers in dye-nucleobase complexes.[28]

An evidence of stacking between anthracene and nucleobases was obtained from UV−vis titration of LAntr·Zn(II) complex with nucleotides. Addition of nucleotides induced a red shift of the absorption spectra, while addition of a pyrophosphate anions induced a blue shift (Figure S4). Changes in both UV−vis and fluorescence induced by the interaction of the complex with nucleoside triphosphates are similar to those observed for the anthracene-containing DNA intercalators explored by Kumar.[45,46] Bathochromic shifts are considered as an evidence of stacking interactions and were reported for pure organic receptors for nucleotides,[35,47] as well as for metal complexes intercalating with DNA[48] and coordinating nucleotides.[20]

Interaction of two aromatic rings in the solution was additionally studied by ^{1}H−^{1}H ROESY measurements. As can be inferred from Figure 5, proton H8a (adenine) interacts with protons H3/H4 (anthracene) and H2′/H1′ (sugar), while proton H2a (adenine) interacts only with protons H3/H4 (anthracene). These interactions are in a good agreement with

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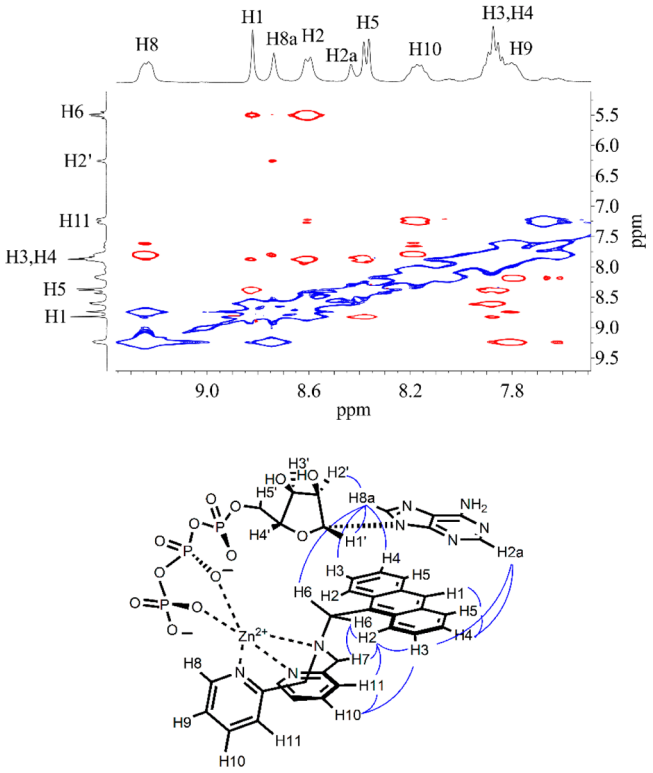

Figure 5. $^{1}H-^{1}H$ ROESY spectrum of complex LAntr·Zn(II) in the presence of 1 equiv of ATP. Lines between protons in the structure of the complex demonstrate correlations, as inferred from the spectrum. Conditions: concentration of the complex 8 mM, the pH of the solution was adjusted to pH 7.4 with NaOH, 3:2 CD_3CN:D_2O 298 K, 400 MHz.

the DFT optimized structures of ternary complexes (see below).

Experimental Quantification of Stacking Interactions. For the assessment of stability constants of complexes, potentiometric pH-titrations were performed. These titrations have several advantages (a) they are precise and the error can be easily derived from several repeating experiments; (b) chromophores in the structure of the ligand are not required in contrast to fluorescence or UV−vis titrations. We used the double mutant cycle depicted in Figure 6 for the calculation of stacking free energies between anthracene and nucleobases. The stabilities of complexes I−IV were obtained from potentiometric titrations. The R-group corresponds to a substituent, which shows negligible propensity to interact with nucleobases. The stacking free energy between, e.g., adenine and anthracene can be calculated as $\Delta G_{st} = \Delta G_{I,III} - \Delta G_{II,IV} = \Delta G_{III,IV} - \Delta G_{I,II}$. Sigel assessed some contributions of stacking interaction between nucleobases and phenanthroline-, bipyridine-,[49] and amino acid-based[50,51] metal complexes[19] by using a simpler scheme. His method is based on the calculation of only one mutation: $\Delta G_{I,II}$. Similar approach was used by Rebek and co-workers.[23] In this work, we assessed mutations $\Delta G_{I,III}$ and $\Delta G_{II,IV}$ for the calculation of interaction free energies. We used 2,2′-dipicolylamine as a ligand of comparison that does not bear any aromatic ring. In principle, *N*-ethyl-2,2′-dipicolylamine can also be used in the double mutant cycle. However, as appeared from potentiometric measurements, 2,2′-dipicolylamine and *N*-ethyl-2,2′-dipicolylamine yielded almost similar $\Delta G_{I,III}$ values. For instance, according to the potentiometric titrations, $\Delta G_{I,III}$ values (= $\Delta G_I - \Delta G_{III}$) for 2,2′-dipicolylamine are 0.21 ± 0.05 kcal/mol (ATP) and 0.69 ± 0.06 kcal/mol (UTP), while for *N*-ethyl-2,2′-dipicolylamine the values are 0.23 ± 0.05 kcal/mol (ATP) and 0.74 ± 0.06 kcal/mol (UTP), respectively. Thus, for further measurements we used 2,2′-dipicolylamine (LH).

Potentiometric titrations were carried out with ligands LH and LAntr in water containing 2.4% methanol and 0.1 M NaCl for the constant ionic strength. Since our solvent system contains small amount of methanol for a better solubility of ligands, we determined pK_a values for all compounds in question (Table S1). The ligands were titrated in the presence of zinc(II) perchlorate and nucleoside triphosphates. The pK_a values and stability constants of the complexes were calculated with the help of the Hyperqaud program.[52] Stability constants β and K are defined from eqs 1 and 2, respectively. The stability constant K in eq 2 can be also described as an affinity of the

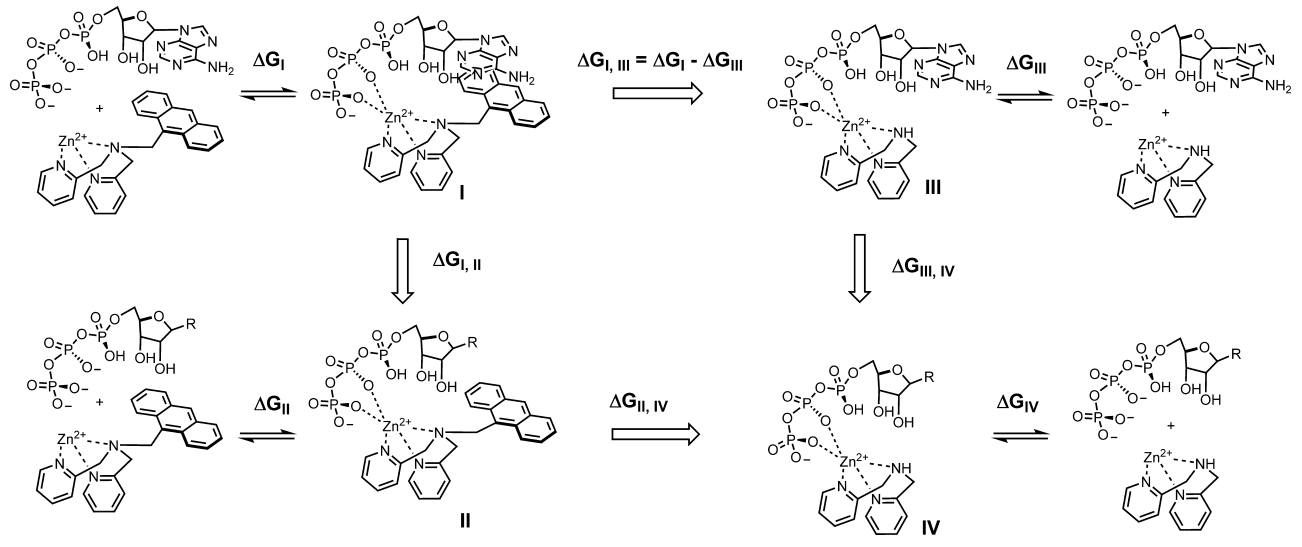

Figure 6. Double mutant cycle constructed for the calculation of the stacking free energy between adenine and anthracene and the corresponding equilibriums assessed in this work.

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Zn(II) complex for ATP. For simplicity the charges on zinc and nucleoside triphosphates (NTP) are omitted.

$$\mathrm{LH} + \mathrm{Zn} + \mathrm{ATP} \leftrightarrow \mathrm{LH{\cdot}Zn{\cdot}ATP}$$
$$\beta_{\mathrm{LH{\cdot}Zn{\cdot}ATP}} \frac{[\mathrm{LH{\cdot}Zn{\cdot}ATP}]}{[\mathrm{LH}][\mathrm{Zn}][\mathrm{ATP}]} \quad (1)$$

$$\mathrm{LH{\cdot}Zn} + \mathrm{ATP} \leftrightarrow \mathrm{LH{\cdot}Zn{\cdot}ATP}$$
$$K^{\mathrm{LH{\cdot}Zn}}_{\mathrm{LH{\cdot}Zn{\cdot}ATP}} = \frac{[\mathrm{LH{\cdot}Zn{\cdot}ATP}]}{[\mathrm{LH{\cdot}Zn}][\mathrm{ATP}]} \quad (2)$$

The results of potentiometric titrations are shown in Table 1. The data is shown only for four nucleobases, except thymine, for which we were not able to obtain reproducible results Interestingly, the affinities of LAntr·Zn(II) for nucleoside triphosphates (defined as log $K^{\mathrm{LAntr{\cdot}Zn}}_{\mathrm{LAntr{\cdot}Zn{\cdot}NTP}}$) do not correlate with $\Delta G_{\mathrm{I,III}}$. However, these affinities were often compared in the literature to speculate which nucleobase has the strongest stacking interaction with an aromatic ring in the complex. The correct answer give $\Delta G_{\mathrm{I,III}}$ values calculated as a difference between the free energies of the complex formations with and without the anthracene ring. Since the triphosphate with R = H (Figure 6) is scarcely accessible in sufficient quantities and purity, we suggested to calculate $\Delta G_{\mathrm{II,IV}}$ from stabilities of complexes with the pyrophosphate anion (PPi). $\Delta G_{\mathrm{II,IV}}$ is a constant value for all nucleobases and with this approximation the full double mutant cycle was calculated. As determined by potentiometric pH titrations, the affinities of complexes LAntr·Zn(II) and LH·Zn(II) for PPi are log $K^{\mathrm{LAntr{\cdot}Zn}}_{\mathrm{LAntr{\cdot}Zn{\cdot}PPi}}$ = 7.97 ± 0.02 and log $K^{\mathrm{LH{\cdot}Zn}}_{\mathrm{LH{\cdot}Zn{\cdot}PPi}}$ = 7.93 ± 0.02, respectively. The difference between these values (which is $\Delta G_{\mathrm{II,IV}}$) is 0.04 logarithm units or 0.05 kcal/mol. This value is even smaller than the experimental error. Therefore, in a rough approximation, $\Delta G_{\mathrm{I,III}}$ is equal to the ΔG_{st} considering pyrophosphate as a reference.

Fluorescence spectroscopy was the second method for determination of association constants because of its high sensitivity. For this purpose ligands LQAntr and LQH were synthesized. Both ligands bear a quinoline dye, which may allow one to compare stability constants of complexes with and without anthracene. Fluorescence titrations were carried out in 10 mM TRIS buffer (pH 7.4, 0.1 M NaCl) and zinc(II) perchlorate. Affinity constants were calculated by fitting the experimental data with the HypSpec program.[52] Analysis of stacking free energies obtained from fluorescence titrations (Table 2) reveals that (a) the values are smaller in comparison with those obtained from potentiometric measurements; (b) experimental errors are relatively high; and (c) the selectivity trend in interaction free energies calculated as $\Delta G_{\mathrm{I,III}}$ agrees with that determined from potentiometric titrations. Small differences in stability constants for complexes with LQAntr and LQH may be explained by the fact that nucleobases may form relatively strong stacking interactions with both quinoline and anthracene. The presence of both interactions substantially level the effect of anthracene present in ligand LQAntr.

Because the experimental values of stacking interactions are different in Table 1 and Table 2, we tested a different, third approach to calculate the contribution of stacking interactions to overall binding free energies. The strongest changes and precise stability constants were observed from fluorescence titrations of LAntr·Zn(II) complexes with nucleotides (Figure 3). In the previous work, we showed that nucleoside monophosphates do not form stacking interactions with anthracene because they are too short in comparison with nucleoside triphosphates.[43] Thus, the difference in affinities of the complexes for NTPs (nucleoside triphosphates) and NMPs (nucleoside monophosphates) can give stacking free energies for nucleobases, when corrected to electrostatic interactions. It is reasonable to assume that the mutation from structure I to III results in a loss of one negative charge. According to the potentiometric titrations, pyrophosphate and phosphate are present in monoprotonated forms at pH 7.4. The contribution of this charge loss can be calculated by the mutation from structure II to IV (Figure 7). The resulting double mutant cycle shown in Figure 7 was used to calculate ΔG_{st} values (Table 3). The affinities of the complex for the pyrophosphate anion and the phosphate anion are log $K^{\mathrm{LAntr{\cdot}Zn}}_{\mathrm{LAntr{\cdot}Zn{\cdot}PPi}}$ = 5.00 ± 0.05 and log $K^{\mathrm{LAntr{\cdot}Zn}}_{\mathrm{LAntr{\cdot}Zn{\cdot}PPi}}$ = 4.80 ± 0.05, respectively. It appeared that ΔG_{st} values have excellent agreement with the results obtained by potentiometric titrations.

Computational Analysis of Stacking Interactions. A parallel approach to assess stacking energies between anthracene and nucleobases was undertaken by using quantum chemical calculations. The computational analysis was based on the same double mutant cycle used in potentiometric titrations (Figure 6), but with protonated complexes to avoid charged structures. The obtained energies (E) are formally equivalent to free energies at 0 K, as they contain neither temperature nor entropic contributions. Since the term "stacking free energies" suggests to feature such contributions, especially as we compare computational and experimental results at 298 K, we will further use only the term "stacking energies" to avoid confusion. A comparison of the calculated stacking energies (ΔE_{st}) and the experimental values will allow us to reveal the origin of stacking interactions. The structure of ternary complex LAntr·Zn·ATP (Figure 5), constructed from ^{1}H−^{1}H ROESY measurements, was the starting point for the geometry

Table 1. Affinities (log K) of Zn(II) Complexes for NTPs and ΔG_{st} As Determined by Potentiometric pH Titration at 23°C, 2.4% vol. MeOH and I = 0.1M (NaCl)[a]

NTP	log $K^{\mathrm{LAntr{\cdot}Zn}}_{\mathrm{LAntr{\cdot}Zn{\cdot}NTP}}$	log $K^{\mathrm{LH{\cdot}Zn}}_{\mathrm{LH{\cdot}Zn{\cdot}NTP}}$	stacking free energies ΔG_{st} between anthracene and a nucleobase, kcal/mol
A	7.39 ± 0.02	7.20 ± 0.03	0.21 ± 0.06
C	5.11 ± 0.02	4.68 ± 0.02	0.53 ± 0.06
G	6.13 ± 0.04	4.86 ± 0.07	1.68 ± 0.13
U	6.43 ± 0.02	5.92 ± 0.02	0.64 ± 0.05

[a]Affinities are calculated as follows: e.g., log $K^{\mathrm{LH{\cdot}Zn}}_{\mathrm{LH{\cdot}Zn{\cdot}NTP}}$ = log $\beta_{\mathrm{LH{\cdot}Zn{\cdot}NTP}}$ − log $\beta_{\mathrm{LH{\cdot}Zn}}$.

Table 2. Affinities (log K) of Zn(II) Complexes for NTPs and $\Delta G_{\mathrm{I,III}}$ Values As Determined by Fluorescence Titrations at 23°C in a 10 mM TRIS Buffer (4% vol. MeOH, pH 7.4, 0.1 M NaCl)[a]

NTP	log $K^{\mathrm{LQAntr{\cdot}Zn}}_{\mathrm{LQAntr{\cdot}Zn{\cdot}NTP}}$	log $K^{\mathrm{LQH{\cdot}Zn}}_{\mathrm{LQH{\cdot}Zn{\cdot}NTP}}$	$\Delta G_{\mathrm{I,III}}$, kcal/mol
A	[b]	[b]	–
C	5.40 ± 0.05	5.29 ± 0.02	0.14 ± 0.09
G	5.95 ± 0.04	5.10 ± 0.05	0.85 ± 0.12
T	5.18 ± 0.05	5.07 ± 0.06	0.20 ± 0.15
U	5.04 ± 0.05	4.89 ± 0.06	0.14 ± 0.15

[a]Excitation wavelength 370 nm, emission region 380−460 nm. [b]Small changes of fluorescence were observed.

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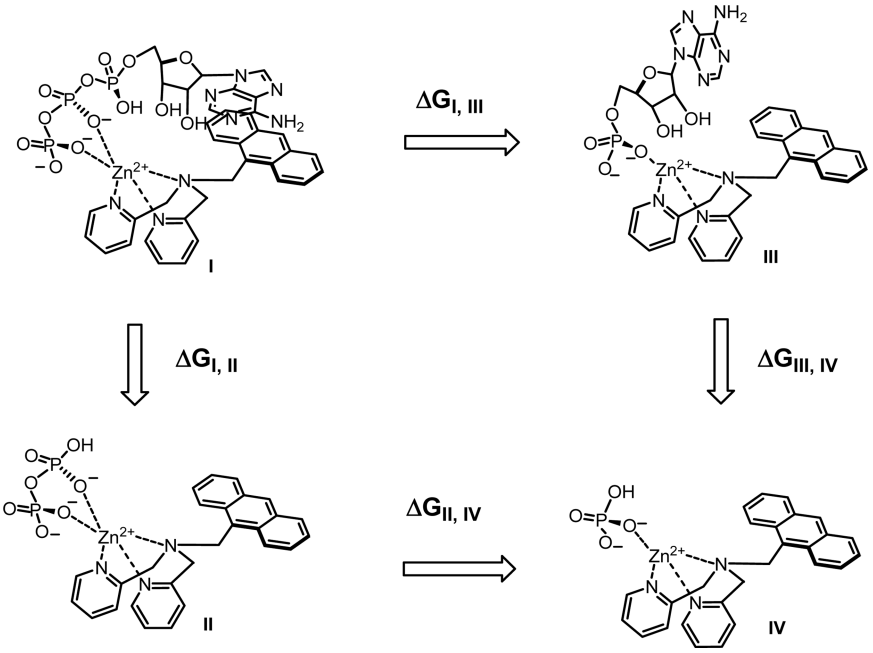

Figure 7. Double mutant cycle constructed for the assessment of the binding free energy between adenine and anthracene. Mutations take into account contribution of electrostatic interactions.

Table 3. Affinities (log K) of LAntr·Zn(II) Complex for NTPs and NMPs and ΔG_{st} Values As Determined from Fluorescence Titrations at 23°C in a 10 mM TRIS Buffer (4% vol. MeOH pH 7.4, 0.1 M NaCl)[a]

NTP	log $\beta^{LAntrZn}_{LAntrZnNTP}$	log $\beta^{LAntrZn}_{LAntrZnNMP}$	stacking free energies between anthracene and a nucleobase ΔG_{st}, kcal/mol
A	4.67 ± 0.01	4.22 ± 0.03	0.34 ± 0.05
C	4.28 ± 0.01	3.70 ± 0.03	0.52 ± 0.05
G	4.53 ± 0.01	3.40 ± 0.04	1.27 ± 0.07
T	4.44 ± 0.01	3.54 ± 0.02	0.94 ± 0.05
U	4.45 ± 0.01	3.63 ± 0.02	0.84 ± 0.05

[a]Excitation wavelength −370 nm, emission region 380−460 nm. $\Delta G_{st} = \Delta G_{I,III} - \Delta G_{II,IV}$.

optimization. There are two possible orientations of the adenine ring over the anthracene ring. These two conformation were generated by rotating the nucleobase ring by 180° over the sugar-nucleobase bond. The corresponding structures were optimized and the resulting geometries are shown in Figure 8. Configuration 1 (conf. 1) always represents the most stable configuration and the energetic differences between both configurations (conf. 2 relative to conf. 1) are shown in Table 4. To eliminate the errors in calculations of stacking energies, the conformations of the nucleobase in complexes I and III were kept the same (Figure 6). As can be inferred from the side-views of the complexes, Zn(II) cation coordinates two oxygen atoms from first two phosphate residues, i.e., similar to the coordination of pyrophosphate. Sigel and co-workers observed this coordination mode in a number of different complexes with nucleotides.[19] The average distance between nucleobases and the anthracene ring is 3.3 Å. interestingly, adenine prefers conformation 1, while guanine forms more stable complex in conformation 2. A close analysis of structure "LAntr·Zn·GTP conf. 1" reveals that the amino-group of guanine forms a hydrogen bond (N···O is 2.8 Å) with the oxygen of the phosphate residue. This hydrogen bond is not possible in case of adenine.

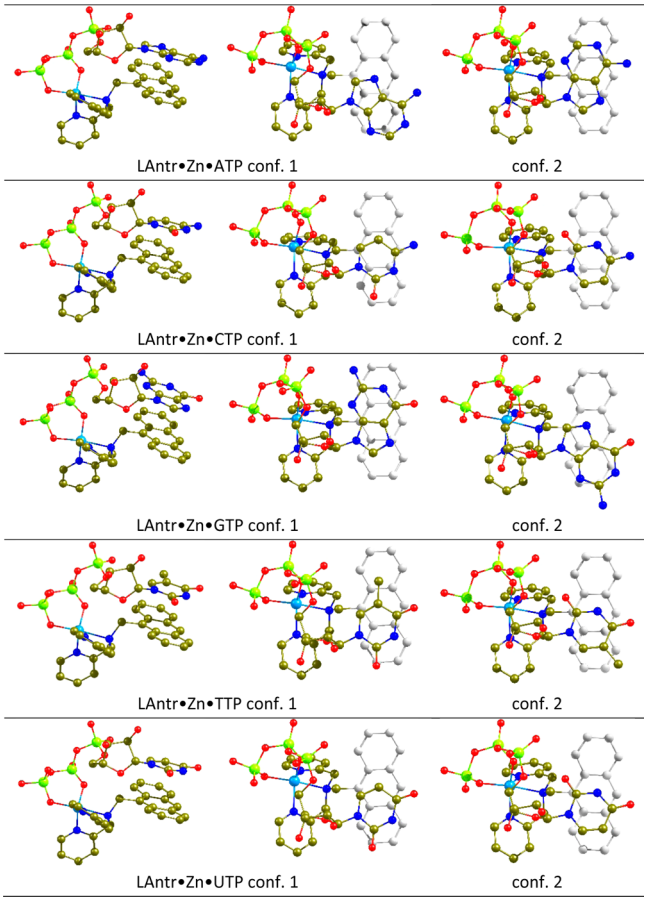

Figure 8. Side- and top-views of ternary complexes with five nucleotides. For top-view figures of configurations 1 and 2 the anthracene ring is shown in gray color for best view of the rings.

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Table 4. Calculated Stacking Energies $\Delta E_{st} = \Delta E_{I} - \Delta E_{III} - (\Delta E_{II} - \Delta E_{IV})$ for Nucleobases by Using a Double Mutant Cycle Shown in Figure 6[a]

nucleobase	ΔE_{st}, conf. 1	ΔE_{st}, conf. 2	$E_{I,conf.2} - E_{I,conf.1}$
A	1.3	5.4	2.6
C	0.6	−2.6	6.1
G	7.1	−0.9	2.5
T	4.6	1.5	5.7
U	4.0	1.3	4.4

[a]The energies were calculated for two configurations conf. 1 ($E_{conf.1}$) and conf. 2 ($E_{conf.2}$); Conf. 1 always corresponds to the configuration of LAntr·Zn·NTP with the energetically preferred minimum. All calculated energies are in kcal/mol and contain zero-point vibrational energies (ZPEs).

The affinities of Zn(II) complexes for nucleotides were calculated similar to the method used in experimental assessments. Analysis of Table 4 reveals a general trend of strong interactions between anthracene and guanine. Interestingly, the calculated values of stacking energies are higher than those obtained experimentally. The calculated values are in the range of 0.6−7 kcal/mol, while the experimental values are in the range of 0.1−1.3 kcal/mol. There are several reasons that can cause this deviation. First, experimental measurements were carried out in a buffered solution with a constant ionic strength. Second, additional approximations are the average description of solvation by the COSMO model, as well as the use of neutral instead of charged complexes in calculations. Third, the errors of the quantum chemical methods might not be negligible, especially when considering the small relative energies.[53] However, our calculations are able to indicate trends in binding energy and they are in a good agreement with the experimental data in terms of selectivity of noncovalent interactions between nucleobases and the anthracene ring. Taking into consideration complexes LAntr·Zn·NTP with the energetically preferred minimum, the following selectivity trend can be ruled out: G > T > U > A > C.

Comparison of the Measured and Computed Data. In literature, the selectivity of a receptor for a certain nucleobase is often ruled out from the selectivity for a certain nucleotide.[54−58] This can be in principle correct, when we do not take into account the processes, which are individual for each nucleotide, such as conformational changes and solvation/desolvation upon binding. For example, in theoretical calculations, where we do not consider reaction entropy, the selectivity of LAntr·Zn(II) complex for guanine, thymine and uracil can be directly derived from energies of the complexes with nucleotides. In particular, in the theoretical calculations the binding selectivity of complex LAntr·Zn(II) for nucleotides is GTP > TTP > UTP > ATP > CTP. This relationship correlates to the ΔG_{st} pattern: G > T > U > C > A (Table 4). On the contrary, according to the experiment, the affinity of LAntr·Zn(II) for ATP is slightly higher than the affinity for GTP (ATP > GTP). Hence, there is no correlation between affinities of the complex for nucleotides and the experimentally determined ΔG_{st} values (G > A). These facts underline the importance of calculating stacking free energies by using the double mutant cycle. It is conceivable to suggest that the easier to desolvate a nucleobase, the stronger is the stabilization of a stack. However, guanine as a highly solvated nucleobase has the highest stacking free energy. The selectivity pattern for the nucleobase binding determined in our studies does not correlate with solvation energies of nucleobases.[59] This fact indicates that the contribution of hydrophobic interactions between anthracene and nucleobases into the overall binding energy is relatively low in comparison with the stacking interactions, which determine the observed selectivity pattern. A support for this conclusion can be found in literature.[60,61] For instance, Inoue and co-workers analyzed binding parameters of thymidine and uridine derivatives to cyclodextrins and positively charged hosts in an aqueous solution.[62] The entropically driven interaction of nucleobases with cyclodextrins in a buffered aqueous solution was much lower than the interaction of nucleobases with the hosts able to form stacking interactions.

The experimental values for stacking free energies between aromatic compounds and nucleobases have relatively low values, in the range of 1 kcal/mol, but they perfectly agree with the experimental results obtained previously by Rebek[23] and Sigel.[63] Interestingly, the measured and calculated stacking free energies have still excellent agreement in selectivity pattern (G, T, U > C, A). This fact supports the proposed structures of the complexes and reliability of the experimental methods.

In the recent literature more attention has been paid to understanding the selectivity of nucleobase recognition by stacking with aromatic compounds. For example, Garcia-Espana reported on polyammonium receptors bearing anthracene.[20] DFT calculations of stacking free energies

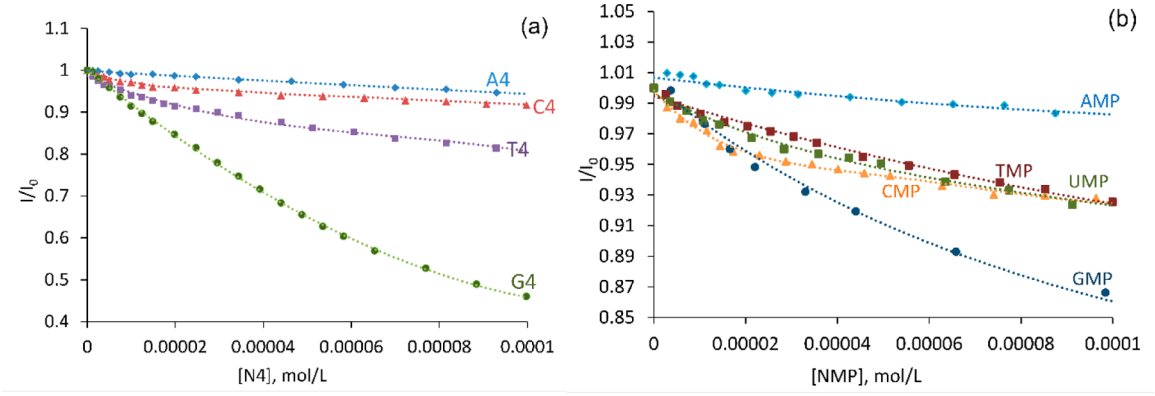

Figure 9. (a) Coordination of DNA oligonucleotides and (b) nucleoside monophosphates to complex LAntr·Zn(II) and the observed changes in fluorescence intensity at 423 nm during titrations. Conditions: 10 mM TRIS buffer (pH 7.4, 4% vol. MeOH, 0.1 M NaCl) at 10^{-5} M concentration of the ligand in the presence of 10^{-4} M zinc(II) perchlorate.

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between anthracene and nucleobases in a gas phase resulted in a pattern, which agrees with our results: GTP > UTP > ATP. The preference of anthracene-based Zn(II) complexes to bind gunanine, thymine and uracil nucleotides were reported by Fabbrizzi and co-workers. The authors attached two anthracene arms to 2,4,6-triamino-1,3,5-trimethoxycyclohexane and investigated its Zn(II) complex as a receptor for nucleotides. While addition of AMP and CMP induced very small changes in fluorescence, GMP, TMP and UMP induced strong quenching. Apparent binding affinities of the Zn(II) complex for nucleotides decreased in the order: TMP > GMP > UMP.[64] Grimme and co-workers carried out theoretical investigations of interaction of free nucleobases with graphenes and obtained stacking free energies.[65] The reported sequence for the interaction energies between nucleobases and, e.g., graphene $C_{96}H_{24}$ (G > A > T > C > U) is different from that obtained in this work. This difference presumably indicates that the structure of a stacking component also has an influence on the selectivity of nucleobase binding.

Interaction with Tetranucleotides. Understanding the relationship between the structure of an aromatic ring and its binding affinity/selectivity toward a certain nucleobase can be useful for the design of new DNA binders.[66−70] Thus, we were interested to understand whether the binding selectivities and fluorescence response observed for nucleotides can be translated to the binding of DNA oligonucleotides. To answer this question we examined the interaction of complex LAntr·Zn(II) with DNA oligonucleotides A4 (5′-AAAA-3′), C4, T4 and G4, each of them carrying three phosphate residues and three negative charges. Addition of oligonucleotides to complex LAntr·Zn(II) resulted in quenching of fluorescence and the quenching pattern was similar to that observed for nucleoside monophosphate (NMPs) (Figure 9). The curves were fitted to a 1:1 interaction model, which was extracted from the Job's plot analysis. Interestingly, the stability constants between the complex and tetranucleotides were in most cases lower than those for nucleoside triphosphate (Table 5). The major difference in binding of oligonucleotides and nucleoside monophosphate was the fact that LAntr·Zn(II) has a selectivity for C4 and T4 oligonucleotides, while it binds nucleoside monophosphate with the preference for CMP and AMP. Additionally, G4 quenches the fluorescence of the complex much strongly than GMP does. This observation can be explained in terms of more guanines in the binding molecule and they all participate in the interaction with anthracene. Similar behavior was observed by Fox for pyrenyl-*N*-alkylbutanoamide end-labeled oligonucleotides.[71] The results of the fluorescence titrations show that although the quenching pattern for tetranucleotides and nucleoside monophosphate have similarities, the interaction mode of LAntr·Zn(II) with tetranucleotides is likely more complex. To obtain more information about these interactions in solution the complexes with more sensitive dyes are required, e.g., the dyes, which efficiently report on stacking interaction with a nucleobase.

Table 5. Affinities (log *K*) of LAntr·Zn(II) Complex for Oligonucleotides

Tetranucleotide N4	$\log \beta^{\text{LAntr·Zn}}_{\text{LAntr·Zn·N4}}$
A4	3.10 ± 0.05
C4	4.60 ± 0.01
G4	3.97 ± 0.01
T4	4.29 ± 0.01

CONCLUSIONS

Stacking interactions between aromatic compounds and nucleobases are essential in recognition of nucleotides and nucleic acids. In this work, we designed and studied different approaches to assess stacking free energies between anthracene and nucleobases. We used Zn(II) complexes with dipicolylamine-based ligands to bind nucleoside triphosphates. The receptors bearing the anthracene dye bind nucleotides by a combination of electrostatic and stacking interactions. For the first time, stacking free energies between five nucleobases and anthracene were experimentally determined. The anthracene ring prefers to bind nucleobases in the following order G (1.3 kcal/mol) > T (0.9 kcal/mol) > U (0.8 kcal/mol) > C (0.5 kcal/mol) > A (0.3 kcal/mol). The double mutant cycle based on the comparison of binding free energies of complexes with nucleoside monophosphate and triphosphates appeared to be the best in terms of accuracy and simplicity. The values obtained by this methods perfectly correlate to the values obtained by potentiometric titrations. Analysis of the experimental data and quantum chemical calculations suggest that stacking interactions dominate over hydrophobic effects in aqueous solution. These forces presumably determine the selectivity of aromatic compounds for nucleotides in aqueous solution. Fluorescence studies of DNA tetranucleotides revealed that their behavior resembles the behavior of nucleoside monophosphates rather than triphosphates. The methods reported here may set the stage for the evaluation of highly selective aromatic dyes for stacking with nucleobases, as well as new fluorescent probes for nucleotides and nucleic acids.

EXPERIMENTAL SECTION

General Remarks. All solvents were reagent grade and purchased commercially. All commercial reagents were used in the quality as purchased without further purification. NMR spectra were recorded on a 400 MHz spectrometer. Chemical shifts (δ) are reported in ppm relative to the residual solvent signal. Fluorescence measurements were recorded on a spectrofluoremeter. UV−vis measurements were recorded using an UV−vis−NIR spectrometer. DNA Oligonucleotides were purchased from Metabion International AG.

UV−Vis and Fluorescence Titrations. The general procedure for the UV−vis and fluorescence binding studies involved preparation of a stock solution with the host (ca. 10^{-5} M) and a stock solution with the guest (ca. 10^{-3} M). The guest is usually dissolved in the stock solution of the host. A typical titration experiments involves sequential additions of the titrant (guest) to a 1.6 mL sample of the host stock solution in the spectrometric cell and monitoring the changes in the spectral features. For the 1:1 binding stoichiometry one requires ca. 10 additions before 1 equiv of the guest and ca. 10 points after 1 equiv. The total number of data points in both UV−vis and fluorescence experiments were between 20 and 40, depending on the stoichiometry of complexation and binding affinity. The data points were then collated and combined to produce plots that, in turn, were processed by HypSpec computer program.

Potentiometric Titrations. Titrations were carried out using a titrating device at 23 °C. The pH scale was calibrated prior to each experiment with the help of three standard buffers: pH 4.0, 7.0, and 9.0 (Roth). For titrations ca. 20−25 mg of the ligand (10^{-3} M) were dissolved in 1.2 mL of MeOH, 2−5 equiv 1 M HCl were added and the solution was diluted with 0.1 M NaCl solution until the total volume reached 50 mL. All titrations were carried out using 0.1 M standard NaOH solution. Each titration was repeated at least 3−6 times to minimize the error. To determine the binding constants with

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zinc(II) salts and nucleotides, 0.5−1 equiv of $Zn(ClO_4)_2$ and 1 equiv of a nucleotide were added. Refinement of the potentiometric data was carried out using the Hyperquad program, which minimizes a least-squares function.

Theoretical Calculations. All calculations were performed by means of density functional theory (DFT)[72] with the RI approximation[73,74] as implemented in TURBOMOLE V6.5.[75] For the geometry optimizations we applied the BP86 functional,[76−79] the def2-SVP basis set,[80,81] Grimme's D3 model for dispersion correction[82,83] as well as the COSMO solvation model with $\varepsilon = \infty$ for water.[84] The stationary points were characterized by analyzing the numerically vibrational frequencies, obtained from the Hessian matrix.[85] In order to get more accurate energies, we performed single-point calculations for the received geometries, using the PW6B95 functional,[86] def2-TZVP basis set,[81,87] the D3 correction and the COSMO model. Furthermore, we always added the zero-point vibrational energy (ZPE), received from the numerical frequency analyses at the BP86/def2-SVP level of theory, to the SCF energies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b01130.

Computational data, NMR data, titration data and analysis, fluorescence spectra and potentiometric data (PDF)

Cartesian coordinates of the optimized complexes (PDF)

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Notes

The authors declare no competing financial interest.

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