Effect of substituents of alloxazine derivatives on the selectivity and affinity for adenine in AP-site-containing DNA duplexes†

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Received 29th April 2010, Accepted 15th July 2010
DOI: 10.1039/c0ob00057d

Using the DNA duplex containing an AP site (5′-TCC AG*X* GCA AC-3′/3′-AGG TC*N* CGT TG-5′, *X* = AP site, *N* = A, T, C, or G), we have found that 2-amino-4-hydroxypteridine (pterin) selectively binds to guanine (G), and that the enhanced binding affinity for G is obtained by its methylated derivative 2-amino-6,7-dimethyl-4-hydroxypteridine (diMe pteridine). Similarly, among the cytosine (C)-selective ligands, *i.e.* derivatives of 2-amino-1,8-naphthyridine, a trimethyl-substituted derivative (2-amino-5,6,7-trimethyl-1,8-naphthyridine) selectively binds to C with a strong binding affinity of 1.9×10^7 M^{-1}. In the case of lumazine derivatives, pteridine-2,4(1H,3H)-dione (lumazine) binds to adenine (A), and its methylated derivative, 6,7-dimethylpteridine-2,4(1H,3H)-dione (diMe lumazine) strongly binds to A with enhanced binding affinity, keeping the same base-selectivity. On the other hand, the benzo-annelated (with phenyl ring, 2.4 Å) derivative of lumazine, benzo[g]pteridine-2,4(1H,3H)-dione (alloxazine), can bind to A selectively, whereas its methylated ligand, 7,8-dimethylbenzo[g]pteridine-2,4(1H,3H)-dione (lumichrome) selectively binds to thymine (T) over A, C and G. Methyl-substituted lumichrome derivatives show moderate binding affinities for target nucleobases. The changes in the base-selectivity and binding affinities are discussed in detail with respect to the substituents of these ligands, considering hydrogen-bonding patterns, size of AP site and stacking interactions.

Introduction

The past few decades have witnessed some promising developments in the field of recognition of DNA by small molecules.[1–4] Studies on the chemistry of DNA-binding drugs and/or low molecular weight ligands are of ongoing interest due to their promising functions and biological activities, including their anticancer properties and ability to regulate gene expressions.[5] One possible approach to this end is based on the ligands capable of targeting double stranded DNAs by intercalation[6,7] or groove binding.[3,8,9] Another promising, but still rare approach involves the use of small ligands that are able to bind to intrahelical target bases by hydrogen bonding in DNA duplexes, where the selective binding of ligands is promoted by a pseudo-base pairing along the Watson–Crick edge of the target nucleobase.[10–12] In connection with this, we have recently developed a series of aromatic ligands that can bind to a nucleobase opposite an abasic site in DNA duplexes, and a new strategy of ligand-based fluorescence assay has been proposed for SNPs typing.[12–16] As shown in Fig. 1, an AP site-containing probe DNA is hybridized with target DNA so as to place the AP site toward a target nucleobase, by which a hydrophobic binding pocket is provided for aromatic ligands to bind to a target nucleobase through hydrogen-bonding.[12]

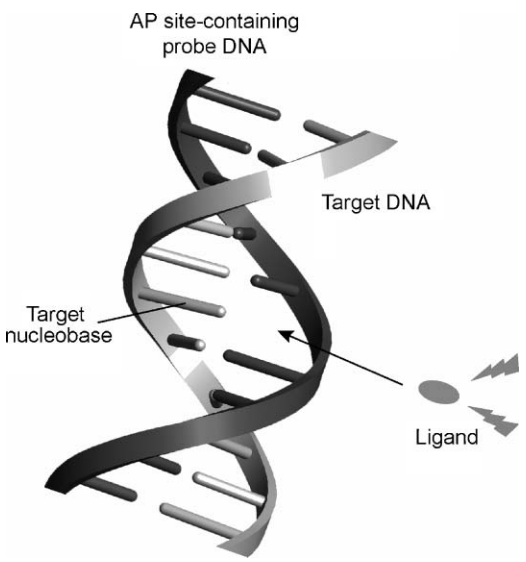

Fig. 1 Schematic illustration of the ligand-based fluorescence detection of a target nucleobase in combination with an abasic (AP) site-containing probe DNA.

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† Electronic supplementary information (ESI) available: Fluorescence titration curves (Fig. S1–S4), energy minimized complexes of alloxazine or lumichrome with adenine or thymine (Fig. S5 and S6), salt dependence of binding constants (Fig. S7 and Table S1). See DOI: 10.1039/c0ob00057d

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Though the selective binding of a ligand to a particular target base is achieved by our method, the binding affinities of some ligands are rather low. For example, the binding constant of 2-amino-4-hydroxypteridine (pterin) with guanine opposite an AP site in a DNA duplex was calculated to be 6.2×10^5 M^{-1}.[13] This low affinity is not sufficient for SNPs detection of the PCR amplified samples. Hence, it is important to improve the binding affinity. Introducing substituents into nucleotides and/or ligands has been proposed to improve the binding affinity of ligands and to stabilize DNA duplexes.[17,18]

The key idea to improve the affinity of a ligand can be derived from DNA itself. The methyl group of thymine increases the DNA stability when compared to RNA which lacks the methyl group for the uracil base. Wang and Kool[17] have explained the reasons that the methyl group in the major groove increases the polarisability, allowing thymine to gain more favorable van der Waals interaction with the neighboring base. When the neighbor is also methylated, the induced dipole–induced dipole attraction between bases would become larger.[17] Based on this we have successfully designed a series of methylated ligands and their binding affinity was greatly improved compared to their non-methylated parents.[19–21] For instance, the binding affinity of 2-amino-6,7-dimethyl-4-hydroxypteridine (diMe pteridine) for guanine is found to be 6.2×10^6 M^{-1}, which is 10 times higher than that of the non-methylated ligand.[18] The effect of the methyl group on the binding affinity is not just observed only for the introduction of a single methyl group, but the effect is largely increased upon the consecutive additions of methyl groups. The binding affinities (10^6 M^{-1}) of the cytosine-binding ligands 2-amino-1,8-naphthyridine (AND) and its methylated derivatives follow the order: AND (0.3) < 2-amino-7-methyl-1,8-naphthyridine (AMND, 2.7) < 2-amino-5,7-dimethyl-1,8-naphthyridine (ADMND, 6.1) < 2-amino-5,6,7-trimethyl-1,8-naphthyridine (ATMND, 19.0).[21] Although the binding affinity of ligands was improved by methylation, the binding selectivity to a particular base is not affected by the substitution.[18,21] In contrast to the previous studies, we explain here how the methyl substitutions alter the base selectivity and binding affinity.

The aim of this investigation lies not only in the effect of the substitutions on the binding affinity but also in development of ligands that can selectively bind to adenine at an AP site with high affinity. So far, no ligand has been reported, except our previous study,[15,16] for the selective detection of adenine with strong affinity. Even in organic media, the binding affinity for adenine based on the host–guest chemistry was reported to be in the order of 10^3–10^4 M^{-1}.[22] Hence, it is highly important to develop the ligands that can selectively bind to adenine.

Here we report on various fluorescent ligands with a high binding affinity for adenine, such as lumazine (Lz) and its methylated derivative diMe lumazine (DMLz), and alloxazine (All) and its methylated derivative 7,8-dimethyl alloxazine (lumichrome (Lch)). Considering the structurez of previously reported ligands as a clue for the selective binding for adenine, we have found that lumazine and its derivative, diMe lumazine, possess suitable hydrogen-bonding sites with high complementarity for adenine (Chart 1C). Introduction of methyl groups to lumazine at the 6- and 7-positions is a key idea to improve the selectivity for adenine base; it is highly likely that introducing an electron donating group neighboring the hetero atoms (*N*-8 position of lumazine) will enhance the electron density at the binding site of diMe lumazine, by which the ligand can selectively bind to adenine with high affinity.[16]

Table 1 Binding constants, K_{11} (10^6 M^{-1}) at 5 °C for the binding of lumazine,[a] diMe lumazine,[b] alloxazine,[c] and lumichrome[c] with each target base

Target base	Lumazine	diMe lumazine	Alloxazine	Lumichrome
G	ND	0.02 ± 0.004	0.05 ± 0.02	0.28 ± 0.04
C	ND	0.02 ± 0.002	0.2 ± 0.04	1.7 ± 0.09
A	0.08 ± 0.002	0.83 ± 0.02	1.2 ± 0.03	1.9 ± 0.12
T	0.03 ± 0.003	0.07 ± 0.002	0.7 ± 0.04	16 ± 3.9

[a] [lumazine] = 10.0 μM, [DNA duplex] = 3.0–60.0 μM; [b] [diMe lumazine] = 5.0 μM, [DNA duplex] = 1.5–30.0 μM, [c] [alloxazine or lumichrome] = 1.0 μM, [DNA duplex] = 0.2–6.0 μM, [NaCl] = 100 mM, [EDTA] = 1.0 mM, [sodium cacodylate] = 10 mM, pH = 7.0. DNA duplex = 5′-TCC AG*X* GCA AC-3′/3′-AGG TC*N* CGT TG-5′; *X* = AP site (Spacer-C3) and *N* = target base.

Recently, it has been reported that base pairing using thymine and/or adenine benzo-annelated analogues is thermodynamically more stable than the Watson–Crick base pairing because of enhanced base stacking.[23–27] Taking this into consideration, adenine-selective lumazine expanded by a benzene ring, *i.e.* alloxazine, was used to improve the binding affinity for adenine. The expanded ligand, alloxazine, shows stronger binding affinity for adenine than the non-expanded ligand, lumazine. However, by further addition of methyl groups into alloxazine (7,8-dimethyl alloxazine which is commonly known as lumichrome), the base selectivity is changed towards thymine from adenine. The binding affinity between lumichrome and thymine reaches to 16×10^6 M^{-1} (see Table 1), which is the strongest binding among the T-selective ligands.[14,15,28] Here we discuss in detail the substituent effect on the base selectivity of ligands.

Experimental

Materials and methods

Lumazine, alloxazine and lumichrome were purchased from Sigma-Aldrich Chemicals Co. (Milwaukee, WI). diMe lumazine[16] and methyl-substituted lumichromes[29] were synthesized and details were described previously. Other reagents were commercially available analytical grade and were used without further purification. Water was deionized (≥18.0 MΩcm of specific resistance) by a Milli-Q system (Millipore Corp., Bedford, MA). All measurements were performed in 10 mM sodium cacodylate buffer (pH 7) containing 100 mM NaCl and 1.0 mM EDTA. The DNA samples used in the present study (custom synthesized and purified by HPLC) were obtained from Nihon Gene Research Laboratories Inc. (Sendai, Japan). For the synthesis of AP site-containing DNA duplexes, a propyl residue (Spacer phosphoramidite C3, Spacer-C3) was utilized. 11-meric DNA duplex has the sequence of 5′-TCC AG*X* GCA AC-3′/3′-AGG TC*N* CGT TG-5′ (*X* = AP site; Spacer-C3, *N* = target nucleobase). The molar extinction coefficient of the single stranded-DNA at 260 nm was calculated according to the literature.[30] The single stranded-DNA concentration was then determined from the calculated molar extinction coefficient. Before any experiment, the single-stranded

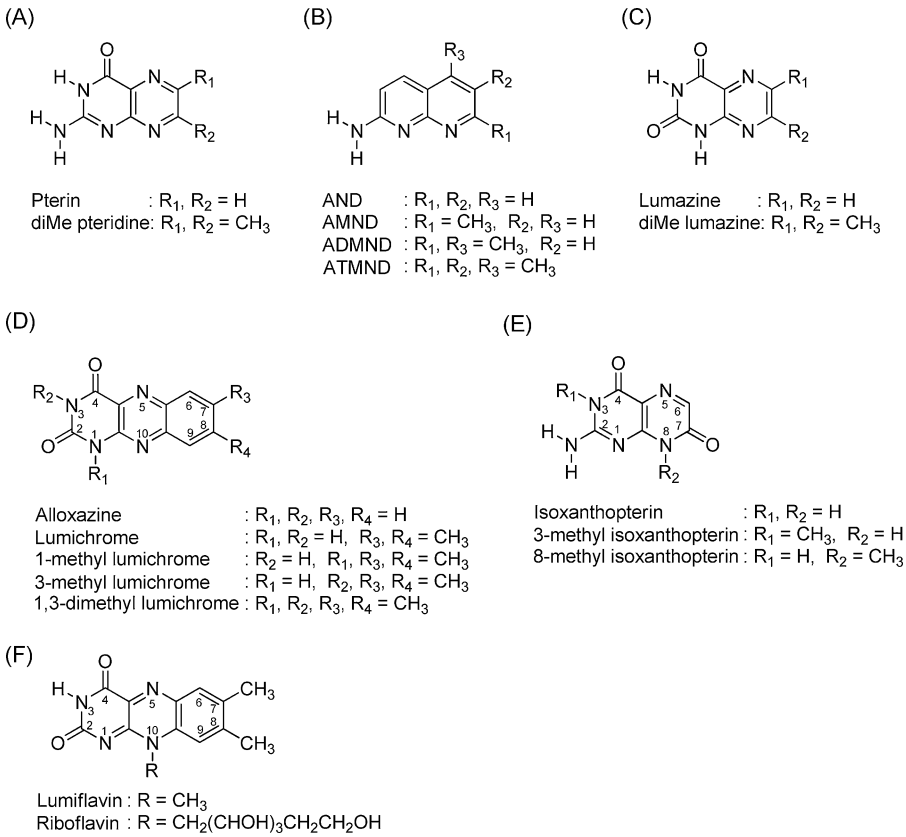

Chart 1 Structures and names of ligands used in this study. (A) Pterin and its methylated derivative diMe pteridine. (B) 2-Amino-1,8-naphthyridine (AND), and its methylated derivatives, 2-amino-7-methyl-1,8-naphthyridine (AMND), 2-amino-5,7-dimethyl-1,8-naphthyridine (ADMND), and 2-amino-5,6,7-trimethyl-1,8-naphthyridine (ATMND). (C) Lumazine and its methylated derivative diMe lumazine. (D) Alloxazine and its methylated derivatives. (E) Isoxanthopterin and its methylated derivatives. (F) Structure of flavins.

DNA samples were annealed to form the duplex as follows: heated at 75 °C for 10 min, gradually cooled down to 5 °C (1.5 °C min^{-1}), and finally the solution temperature was raised to 20 °C (1.5 °C min^{-1}). All DNA sequences are given in the 5′-3′/3′-5′ order throughout the article. We previously found that the binding affinity of pterin with G opposite an AP site increased more than 10 times at 5 °C compared to 20 °C using the same 11-meric DNA sequence and the AP site of Spacer-C3 as those used in this study.[18] Such increase in binding affinity was also observed for a T-selective ligand of riboflavin.[28,31] According to these previous results, fluorescence and ITC measurements were carried out at 5 °C.

Fluorescence measurements

Emission spectra were recorded at 5 °C on a FP-6500 spectrofluorometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan), equipped with a thermoelectrically temperature controlled cell holder. Upon addition of DNA duplexes containing an AP site, the absorption intensity of each ligand decreases and the spectral width is broadened accompanied by the appearance of an isosbestic point. Thus, the wavelength at an isosbestic point was used for excitation of each ligand in fluorescence measurements. To avoid the volume effect on the spectroscopic experiments, a constant total volume of 250 µl of the samples was taken for the measurements. The binding constants were determined by nonlinear regression analysis of fluorescence titration curves based on a 1 : 1 binding isotherm model.

Isothermal titration calorimetry (ITC)

ITC measurements were carried out at 5 °C using a Microcal VP-ITC microcalorimeter (Microcal Inc., Northampton, MA). In a typical experiment, 1.42 ml of a 10 µM ligand in 10 mM sodium cacodylate solution (pH, 7.0) containing 100 mM NaCl and 1.0 mM EDTA was titrated by 10 µl of 11-meric DNA solution. The peaks produced during titration were converted to heat output per injection by integration and correction for the cell volume and sample concentration. The data thus obtained were best fitted to a model that assumed a single set of identical binding sites,[32] giving binding enthalpies and stoichiometries. The Origin software was used for data acquisition and analysis.

Molecular modeling

Molecular modeling (MM) was carried out using MacroModel Ver. 9.0 from a qualitative point of view. The MM optimizations were performed with the Amber* force field and GB/SA solvation model for water (with constant dielectric treatment for the electrostatic part) together with the default cut-off criterions,

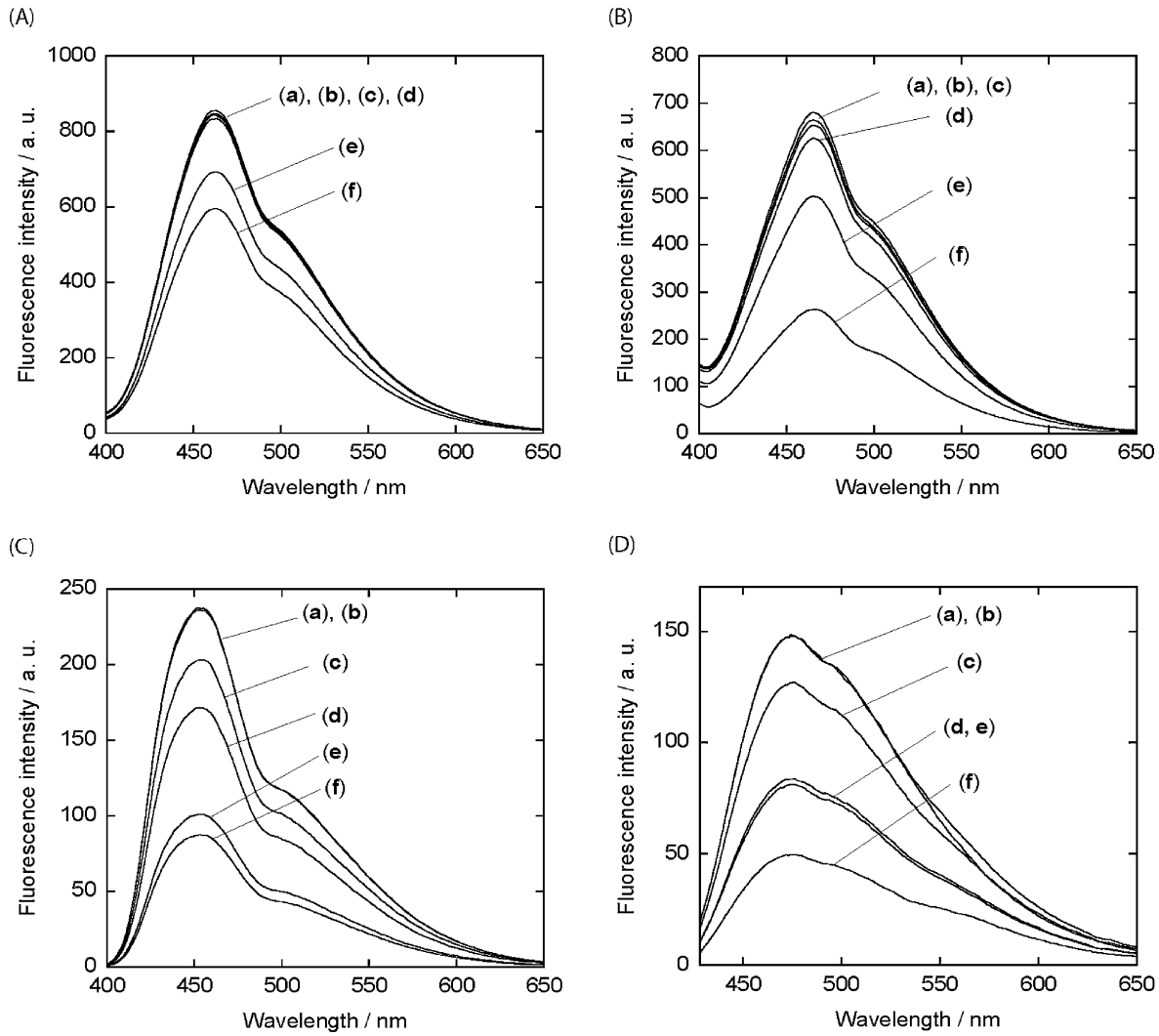

Fig. 2 Fluorescence spectra of (A) lumazine, a) free of DNA, b) fully-matched DNA, c) guanine, d) cytosine, e) thymine, f) adenine, (B) diMe lumazine, a) free of DNA, b) fully-matched DNA, c) guanine, d) cytosine, e) thymine, f) adenine, (C) alloxazine, a) free of DNA, b) fully-matched DNA, c) guanine, d) cytosine, e) thymine, f) adenine, and (D) lumichrome, a) free of DNA, b) fully-matched DNA, c) guanine, d) adenine, e) cytosine, f) thymine. Measurement conditions are (A) λ_{ex} = 335 nm, [DNA duplex] = 10.0 μM, [ligand] = 10.0 μM, (B) λ_{ex} = 350.5 nm, [DNA duplex] = 5.0 μM, [ligand] = 5.0 μM, (C) λ_{ex} = 385 nm, [DNA duplex] = 5.0 μM, [ligand] = 5.0 μM, and (D) λ_{ex} = 420 nm, [DNA duplex] = 1.0 μM, [ligand] = 1.0 μM. DNA duplex = 5′-TCC AG*X* GCA AC-3′/3′-AGG TC*N* CGT TG-5′; *X* = AP site (Spacer-C3) and *N* = target base. [NaCl] = 100 mM, [EDTA] = 1.0 mM, [sodium cacodylate] = 10 mM, pH 7.0, 5 °C.

except the gradient convergence threshold which had been set to be 0.005. Since the binding model is quite straight forward and follows the 1 : 1 binding isotherm, the docking processes were done by manually inserting the ligand into the DNA duplex. Although NMR studies revealed the structure of DNA duplexes containing an AP site as a slightly distorted B-type form,[33,34] intercalation of an aromatic group conjugated to the DNA strand into the AP site was reported to form B-type DNA.[35] An acridine derivative was also reported to bind to the AP site by threading intercalation stacked with the bases flanking the AP site.[36] We previously reported NMR results which confirmed the hydrogen bonding between ligands and nucleobases opposite an AP site by the appearance of imino protons in the ^{1}H NMR spectra.[31,37] Accordingly a B-type conformation was adopted in the molecular modeling.

Results and discussion

Base selectivity and binding affinity

First, fluorescence measurements were carried out to examine the effect of methyl substitution on the binding behavior of ligands with DNAs containing an AP site. As shown in Fig. 2A, lumazine exhibits quenching of its fluorescence upon binding with the target adenine base, and the quenching efficiency is in the order of G < C < T < A, while almost no quenching is observed in the presence of fully-matched DNA containing no AP sites. Upon binding with the target nucleobase, the fluorescence response of diMe lumazine shows higher quenching efficiency than that of lumazine, keeping the same selectivity order as lumazine (G < C < T ≪ A) as shown in Fig. 2B, and it can be seen that highly selective detection of A

over T, C and G is attained by using diMe lumazine as a ligand. From the non-linear regression analysis of fluorescence titration curves (ESI Fig. S1†), K_{11} for lumazine with adenine was 0.08×10^6 M^{-1}, and for diMe lumazine with adenine it was 0.83×10^6 M^{-1} (see ESI Fig. S2†) which is about 10 times higher than that of lumazine. Similarly, the binding constants of lumazine and diMe lumazine with thymine are 0.03×10^6 M^{-1} and 0.07×10^6 M^{-1}, respectively. As expected, it is clear that the difference in the binding constants for adenine against thymine is achieved with a large discrimination ratio using diMe lumazine.

Next, to attain further improvement in the binding affinity for adenine, binding behavior was examined by using the size-expanded lumazine with a benzene ring, *i.e.*, alloxazine. Upon binding with the target nucleobase, alloxazine shows significant quenching of its fluorescence with the target adenine base (Fig. 2C), and the quenching efficiency is in the order of G < C < T < A, and the binding affinity for adenine, 1.2×10^6 M^{-1}, is higher than for thymine, 0.7×10^6 M^{-1} (see ESI Fig. S3†). From the reported results, benzo-annelation of nucleobases enhances the stability of the duplex due to the increased π–π stacking interactions between the ligand and the neighboring nucleobases.[23–27] As described before, the methyl substitution to lumazine (diMe lumazine) increased the binding constant about 10 times for the recognition of adenine; however, the benzo-annelation of lumazine by a phenyl ring (alloxazine) increased the binding affinity only about 1.4 times (Fig. 2C, Table 1). Then, the binding behavior of an alloxazine derivative with two methyl groups, *i.e.*, lumichrome, was examined. As shown in Fig. 2D and Table 1, the fluorescence response of lumichrome upon binding with the target nucleobase shows a significant change in the selectivity, and the binding affinity is in the order G < A ~ C ≪ T. Compared to alloxazine, lumichrome binds to thymine with remarkably high affinity (16×10^6 M^{-1}) and shows a slightly improved affinity for adenine (1.9×10^6 M^{-1}) (see Table 1 and ESI Fig. S4†). Although the exact reason for the change in the selectivity by adding electron donating methyl groups to alloxazine is not clear at present, strong π–π stacking interactions between lumichrome and neighboring nucleobases might be favored when the target base opposite the AP site is thymine due to different hydrogen bonding patterns between lumichrome and target bases (see energy minimized structures, ESI Fig. S5 and S6†). The increase in the binding affinity by methyl substitution could be ascribed to the hydrophobic nature of the AP site which favors incorporation of hydrophobic methylated ligands.

It is noteworthy that the binding affinity of lumichrome with adenine is higher than that of riboflavin or lumiflavin.[28,31,37] It is highly likely that the polar group along the positions 1 and 10 of alloxazine ring (Chart 1D) is involved in the adenine recognition, due to the lack of substituents at the N-10 position that restricts the hydrogen bonding with adenine. Accordingly, alloxazine and lumichrome give higher affinity for adenine than riboflavin and lumiflavin. For the complexation with thymine, the binding affinities of lumichrome and lumiflavin are 16×10^6 M^{-1} (see Table 1 and ESI Fig. S4†) and 21×10^6 M^{-1},[31] respectively. These values are remarkably higher than the binding affinity, 1.8×10^6 M^{-1} of riboflavin for thymine.[37] In comparison with lumichrome, the binding modes of riboflavin and lumiflavin with thymine may be different due to the substituent at the N-10 position. In the case of riboflavin, the long side chain at the N-10 position may tend to decrease the stacking interaction between the ligand and flanking bases when compared to lumiflavin.

To get deeper insight into the binding modes of ligands with target bases, we further studied the binding modes using methyl-substituted lumichromes, *i.e.*, 1-methyl lumichrome, 3-methyl lumichrome, and 1,3-dimethyl lumichrome (Chart 1D). However, these methylated lumichromes show weaker binding affinities than lumichrome for all the target nucleobases (Fig. 3). It may be due to the bulky methyl group existing at both ends of the ligands and also the proximity of the methyl group to the hydrogen bonding sites of ligands. Apparently, methyl-substituted lumichromes show a different order of base selectivity compared to lumichrome (G < A ~ C < T), *i.e.*, 1-methyl lumichrome follows the base selectivity in the order A < G < C ~ T and 3-methyl lumichrome follows the base selectivity in the order G < C < A < T). Interestingly, the substitution by a methyl group at the N-3 position of lumichrome shows significant fluorescence quenching for adenine (Fig. 3B), whereas substitution of a methyl group at the N-1 position of lumichrome gives no significant fluorescence quenching for adenine (Fig. 3A). Substitution of methyl groups at both the N-1 and N-3 positions of lumichrome shows fluorescence quenching with target bases of pyrimidines (cytosine and thymine) over purines (Fig. 3C). As shown in Fig. 3, the quenching efficiency of lumichromes upon binding to adenine is in the order of 1-methyl lumichrome < 3-methyl lumichrome < lumichrome. These results apparently support the binding modes of lumichrome for adenine (N-1 and N-10, see Fig. S5†) and thymine (see Fig. S6†). To draw a clearer conclusion for the selectivity for 1,3-dimethyl lumichrome, we evaluated the binding behavior based on the structural aspects of the ligands. First, it could be due to the intercalation of ligands into the AP site. In the case of thymine or cytosine as a target base, for example, the size of the AP site or hydrophobic pocket would be larger than in the case of adenine or guanine target base. In fact, 1,3-dimethyl lumichrome does not possess any complementary hydrogen bonding sites for any nucleobase, though it shows selective fluorescence quenching for pyrimidine bases.

Here it is also interesting to mention another example to specify the binding mode for adenine: Isoxanthopterin (see Chart 1E) was reported to be selective for thymine,[19] although the ligand possesses a complementary hydrogen bonding array for adenine at the N-1 and N-8 positions. The binding affinity between isoxanthopterin and adenine was calculated to be 0.12×10^6 M^{-1}, while no binding constant could be obtained between its methylated derivative, 8-methyl isoxanthopterin that lacks the binding site for adenine.[20] On the other hand, methyl substitution of isoxanthopterin at the N-3 position, giving 3-methyl isoxanthopterin, enhances the binding affinity for adenine (0.3×10^6 M^{-1}).[20] These results apparently support the specificity of binding modes of ligands for adenine. So far as we observed, adenine binds to positions 1 and 8 of the ligands along the polar edge.

Salt-dependency of binding constant

According to the polyelectrolyte theory proposed by Record *et al.*,[38] the salt-dependent changes in the binding constant can be used to estimate the apparent charge on the ligands upon complexation with a DNA duplex. Further, the salt dependency can be used to dissect the observed binding free energy change

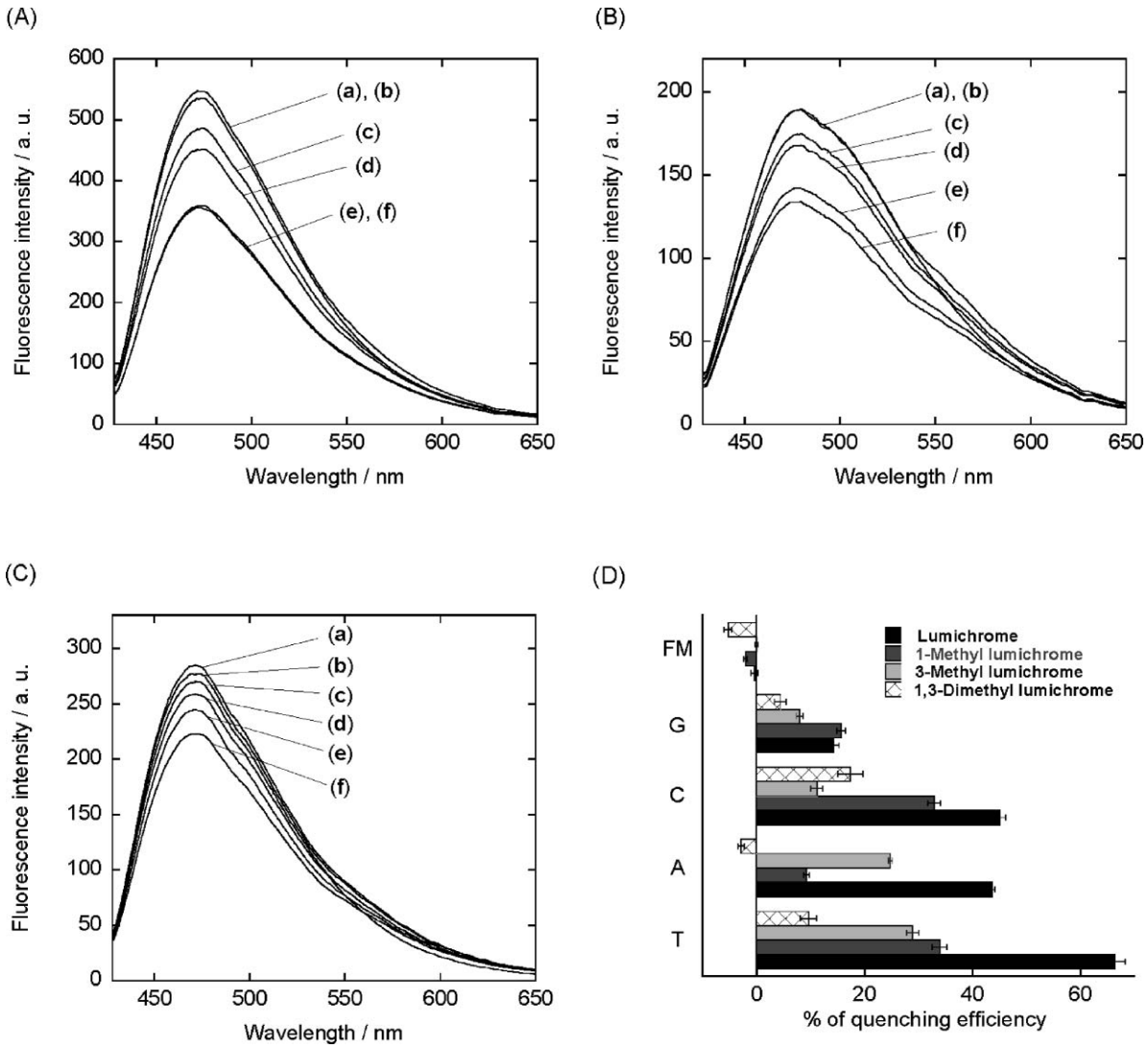

Fig. 3 Fluorescence spectra of (A) 1-methyl lumichrome, a) fully-matched DNA, b) free of DNA, c) adenine, d) guanine, e) cytosine, f) thymine, (B) 3-methyl lumichrome, a) free of DNA, b) fully-matched DNA, c) guanine, d) cytosine, e) adenine, f) thymine, and (C) 1,3-dimethyl lumichrome, a) fully-matched DNA, b) adenine, c) free of DNA, d) guanine, e) thymine, f) cytosine. (D) Fluorescence quenching efficiencies of lumichrome (black bars), 1-methyl lumichrome (dark gray bars), 3-methyl lumichrome (light grey bars) and 1,3-dimethyl lumichrome (black hatched bars). DNA duplexes = 5′-TCC AG*X* GCA AC-3′/3′-AGG TC*N* CGT TG-5′; *X* = AP site (Spacer-C3) and *N* = target base. λ_{ex} = 420 nm, [DNA duplex] = 1.0 μM, [ligand] = 1.0 μM, [NaCl] = 100 mM, [EDTA] = 1.0 mM, [sodium cacodylate] = 10 mM, pH 7.0, 5 °C.

(ΔG_{obs}) into polyelectrolyte (ΔG_{pe}) and nonpolyelectrolyte (ΔG_t) contributions. The effect of ionic strength on the binding constants of alloxazine and lumichrome with AP site-containing DNA duplexes having thymine or adenine targets was examined by fluorescence titration experiments at different salt concentrations ranging from 110 to 410 mM (ESI Fig. S7 and Table S1†). The apparent charge (Z) on the ligand can be estimated by double logarithmic plots of K_{11} values *versus* activity of Na^+ according to eqn (1).[38]

$$SK = \delta \ln K_{11} / \delta \ln[Na^+] = -Z\Psi \quad (1)$$

Where SK is the slope of the double logarithmic plots and Ψ is a constant which is equal to the fraction of counterions associated with each DNA phosphate (0.88 for B-type DNA[38]). From Fig. 4, the apparent charges on alloxazine and lumichrome were estimated to be 0.044, 0.017, 0.321 and 0.433 for alloxazine/A, lumichrome/A, alloxazine/T and lumichrome/T complexations, respectively. This suggests that the ligands of alloxazine derivatives interact with the DNA duplexes taking a neutral form, especially for the adenine target. The slope SK, estimated from the logarithmic plots of K_{11} values *versus* activity of Na^+, can be used to evaluate the electrostatic or polyelectrolyte contribution (ΔG_{pe}) of the free energy change to the observed binding free energy change (ΔG_{obs}) at a given Na^+ concentration using the following equation:[38]

$$\Delta G_{pe} = (-SK)RT \ln[Na^+] \quad (2)$$

Where R is the gas constant and T is the absolute temperature. The observed binding free energy ($\Delta G_{obs} = -RT \ln K_{11}$) is given

Table 2 Thermodynamic parameters for interactions between alloxazine or lumichrome and thymine or adenine in the 11-mer AP site-containing DNA duplex

Ligand/target base	ΔG_{obs}/kcal mol^{-1}	$-SK$	ΔG_{pe}/kcal mol^{-1}	ΔG_t/kcal mol^{-1}	ΔH_{obs}/kcal mol^{-1}	ΔS_{obs}/kcal mol^{-1}	$T\Delta S_{obs}$/kcal mol^{-1}
Alloxazine/A	−7.74	0.044	−0.10	−7.69	−14.60	−0.025	−6.98
Lumichrome/A	−7.98	0.017	−0.021	−7.96	−13.59	−0.021	−5.89
Alloxazine/T	−7.52	−0.321	−0.392	−7.12	−13.28	−0.021	−5.84
Lumichrome/T	−9.18	0.433	−0.528	−8.65	−18.18	−0.035	−9.84

ΔG_{obs} is the observed binding free energy obtained from fluorescence titration data (see ESI Fig. S5 and Table S1†). *SK* is the slope of the plot of $\ln K_{obs}$ *versus* $\ln[Na^+]$. ΔG_{pe} and ΔG_t are the polyelectrolyte and nonpolyelectrolyte contributions to the binding free energy. $T\Delta S_{obs}$ is calculated from $T\Delta S_{obs} = \Delta H_{obs} - \Delta G_{obs}$. DNA duplex = 5′-TCC AG*X* GCA AC-3′/3′-AGG TC*N* CGT TG-5′; *X* = AP site (Spacer-C3) and *N* = A or T. [NaCl] = 100 mM, [EDTA] = 1 mM, [sodium cacodylate] = 10 mM, pH 7.0, 5 °C.

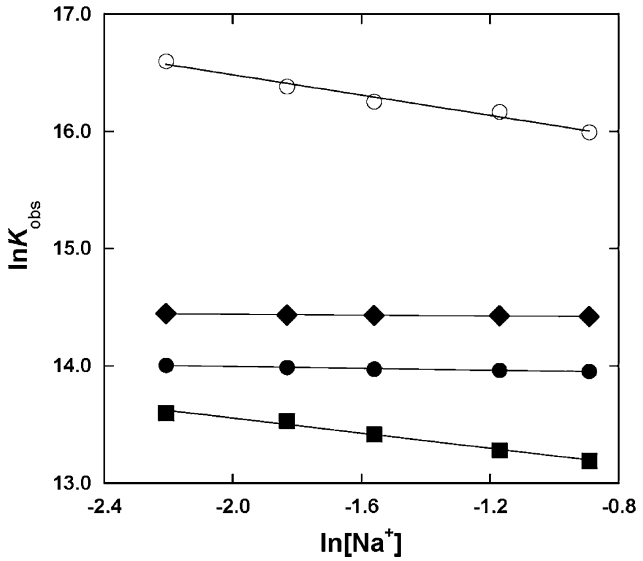

Fig. 4 Salt-dependence of binding constants for the lumichrome/A (◆), lumichrome/T (○), alloxazine/A (●) and alloxazine/T (■) interactions. The data are given in the ESI (Table S1†). DNA duplex = 5′- TCC AG*X* GCA AC-3′/3′-AGG TC*A* CGT TG-5′; *X* = AP site (Spacer-C3).

as the sum of the nonelectrostatic free energy change (ΔG_t) and ΔG_{pe}.[38]

$$\Delta G_{obs} = \Delta G_{pe} + \Delta G_t \quad (3)$$

Where ΔG_{obs} was obtained using K_{11} determined by fluorescence titration experiments according to the literature.[39] The polyelectrolyte and nonelectrolyte contributions to the total free energy are then calculated using eqn (2) and (3) and the values are summarized in Table 2. Since the neutral ligands bind to the DNA, the polyelectrolyte contribution to the ΔG_{obs} is expected to be negligible. As given in Table 2, the ΔG_{pe} values for the alloxazine derivatives are –0.10, –0.021, –0.392 and –0.528 kcal mol^{-1} for alloxazine/A, lumichrome/A, alloxazine/T and lumichrome/T interactions, respectively. As a result, the contributions of ΔG_{pe} to ΔG_{obs} are negligible and it is found that ΔG_t governs ΔG_{obs}.

Thermodynamics of the ligand–nucleobase interaction by ITC

We further evaluated the binding interaction of alloxazine and lumichrome with adenine or thymine by ITC, since ITC is a well-known method to obtain detailed thermodynamic parameters of interactions between small molecules and DNA.[6,40,41] The ITC curves and the plots of heat evolved per mole of ligand added against the molar ratio of the ligand to DNA are given in Fig. 5. The addition of the DNA duplex aliquots into the solution containing alloxazine or lumichrome causes a large exothermic heat of reaction, and the corrected binding isotherm is obtained after subtracting the heat of dilution for each titration. The ITC titration curve was best fitted using a model that assumed a single set of identical binding sites, giving the binding enthalpy (ΔH_{obs}). This value was then used to calculate the binding entropy ($T\Delta S_{obs}$) using $T\Delta S_{obs} = \Delta H_{obs} - \Delta G_{obs}$. Thermodynamic parameters for the interactions between alloxazine or lumichrome and thymine or adenine are summarized in Table 2. The binding constants calculated from ITC experiments were 0.94 (±0.03)×10^6, 1.12 (±0.04)×10^6, 0.72 (±0.05)×10^6 and 3.54 (±0.31)×10^6 M^{-1} with the stoichiometries of 1.11 (±0.003), 1.10 (±0.004), 1.14 (±0.01) and 1.04 (±0.005) for alloxazine/A, lumichrome/A, alloxazine/T and lumichrome/T interactions, respectively. These binding constants are comparable to the K_{11} values obtained by fluorescence titrations (Table 1). In all cases, the ligand–DNA interaction is found to be enthalpy driven, with the ΔH_{obs} of –14.60, –13.59, –13.28 and –18.18 kcal mol^{-1} for the interactions of alloxazine/A, lumichrome/A, alloxazine/T and lumichrome/T, respectively. For these interactions, the $T\Delta S_{obs}$ values are calculated to be –6.98, –5.89, –5.84 and –9.84 kcal mol^{-1} (Table 2). The observed free energies (ΔG_{obs}) are –7.74, –7.98, –7.52 and –9.18 kcal mol^{-1} for alloxazine/A, lumichrome/A, alloxazine/T and lumichrome/T, respectively at 5 °C. The thermodynamic feasibility of complexation is apparent from the negative values of the free energies.

The enthalpy terms for alloxazine and lumichrome upon binding to adenine are comparable; however, upon binding to thymine, the enthalpy term gains favorably for the lumichrome/T interaction over the alloxazine/T interaction, with a difference of –4.9 kcal mol^{-1}. The favored enthalpy term suggests that the stacking interaction between the lumichrome and the bases flanking the AP site increases upon binding to thymine. Such an increase in the enthalpy term was not observed in the case of adenine binding. As discussed earlier, this could be because the size of the AP site is larger when the target base is thymine rather than adenine. Lumichrome has two electron donating methyl groups that enrich the electron density of the aromatic moiety, resulting in the increase in the stacking efficiency. Moreover, the two methyl groups of lumichrome give a hydrophobic nature of the ligand compared to alloxazine, that has no methyl groups, and the hydrophobic ligand is effectively incorporated into the hydrophobic AP site. This hydrophobic nature is supported by

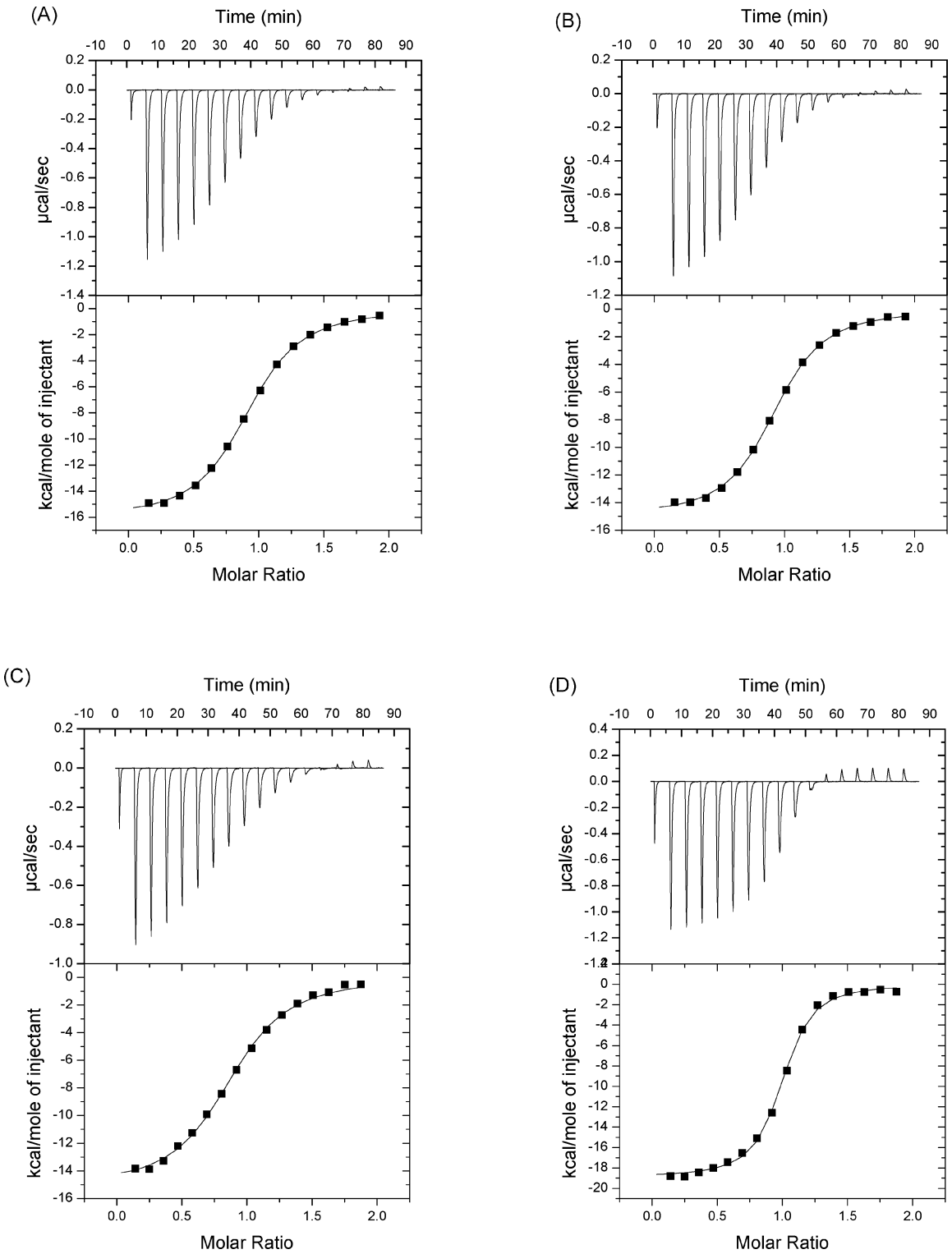

Fig. 5 ITC curves for the bindings of (A) alloxazine/A, (B) lumichrome/A, (C) alloxazine/T, and (D) lumichrome/T. DNA duplex = 5′-TCC AG*X* GCA AC-3′/3′-AGG TC*N* CGT TG-5′; *X* = AP site (Spacer-C3) and *N* = A or T. [DNA duplex] = 200 µM, [ligand] = 20 µM, [NaCl] = 100 mM, [EDTA] = 1.0 mM, [sodium cacodylate] = 10 mM, pH 7.0, 5 °C.

the favored ΔG_t for lumichrome/T complexation compared to alloxazine/T complexation. These could be the reasons for the increased binding constants of the methylated lumichrome to thymine.

Effect of flanking nucleobases on binding affinity

As we demonstrated, upon binding to thymine or adenine in the sequence of 5′-GTT GC*N* CTGGA-3′ (*N* = A or T) the ligands display changes in the fluorescence intensity. It can be expected that the fluorescence response is affected by the nucleobases flanking the AP site and the changes in the flanking environment are expected to influence the binding affinity between the ligands and target base. We further examined the binding affinity of alloxazine or lumichrome with adenine or thymine with all possible flanking nucleobases. As we expected, the binding affinity between the ligands and target base depends on the nucleobases flanking the AP site. Fig. 6 shows fluorescence quenching efficiencies (%) of alloxazine and lumichrome upon binding to adenine or thymine in the 23-mer AP site-containing DNA duplexes with 16 different possible combinations of the flanking nucleobases. The binding constants estimated from the observed fluorescence quenching are also given in Table 3. As can be seen from Fig. 6A and Table 3, fluorescence is strongly quenched for the adenine target base when a purine base (A or G) situates at the 5′-side of the AP site compared to the case of a pyrimidine base (C, T) at the 5′-side. For example, alloxazine shows effective quenching from 27% (5′-AAT-3′/3′-TXA-5′) to 61% (5′-GAC-3′/3′-CXG-5′), which corresponds to the range of K_{11} from 0.53×10^{-6} M^{-1} to 4.6×10^{-6} M^{-1}. Lumichrome shows stronger quenching for adenine than alloxazine from 35.4% (5′-AAT-3′/3′-TXA-5′) to 66% (5′-GAC-3′/3′-CXG-5′), which corresponds to K_{11} values ranging from 0.91×10^{-6} M^{-1} to 7.1×10^{-6} M^{-1}. By contrast, relatively weak responses are obtained for the duplexes containing a pyrimidine base at the 5′ side, and the quenching is reduced to 8% for 5′-CAA-3′/3′-GXT-5′ ($K_{11} = 0.1 \times 10^{-6}$ M^{-1}). As is well known, guanine and cytosine are very polar bases. Therefore, the GC base pair is also very polar. Thymine has a small dipole moment and adenine is even less polar. As a result, the whole AT base pair is much less polar than the GC one.[42] It is therefore likely that, as compared to pyrimidine bases, purine bases effectively stack with the alloxazine or lumichrome, and nucleotides at the 5′ side of the AP site are involved in the binding event more effectively than nucleotides at the 3′ side. It has been reported that the 5′ side guanine in B-form DNA effectively localizes on its neighboring base and shows strong π-stacking interactions.[43] In contrast to the case of the adenine target base, the quenching behavior of alloxazine and lumichrome seems to be complicated when the target base is thymine (Fig. 6B). The weakest quenching efficiency is observed for the 5′-ATA-3′/3′-TXT-5′ sequence. The quenching efficiency for alloxazine–thymine interaction, except for the 5′-ATA-3′/3′-TXT-5′ sequence, ranges from 13.1 to 36.4%, and the binding affinities are 0.18×10^{-6} M^{-1} to 0.93×10^{-6} M^{-1}. Similarly, the lumichrome–thymine interaction ranges from 44.5 to 79%, and the binding affinities are 1.6×10^{-6} M^{-1} to 26×10^{-6} M^{-1}. There seems to be a tendency that high quenching efficiency is obtained for both alloxazine and lumichrome when a GC base pair located at the 5′-side of the AP site. The details about the sequence-dependent fluorescence quenching are not clear at present, and further study is necessary to clarify the quenching mechanism.

Conclusions

In summary, the results presented here clearly indicate that a notable increase in the binding affinity was observed for adenine when methyl substitutions are neighboring the hydrogen-bonding site of a ligand (lumazine *vs.* diMe lumazine). Whereas methylation of a ligand is not very effective to keep the same base selectivity when the methyl substitutions are far from the binding sites, increased binding affinity was obtained for thymine instead of adenine. Studies on the salt-dependency of the binding constants revealed that the neutral ligands interacted with adenine target base and partially charged ligands interacted with thymine target base. The thermodynamics of the ligand–DNA interaction were

Table 3 Effect of flanking nucleobases on the binding constants ($K_{11}/10^6$ M^{-1}) estimated from the fluorescence quenching efficiency[a]

5′-*N*′-3′/3′-*X*′-5′	Alloxazine/T	Lumichrome/T	Alloxazine/A	Lumichrome/A
5′-TNC-3′/3′-AXG-5′	0.32	3.6	3.6	3.6
5′-GNC-3′/3′-CXG-5′	0.50	8.0	4.6	7.1
5′-CNC-3′/3′-GXG-5′	0.93	26	1.7	1.2
5′-ANC-3′/3′-TXG-5′	0.40	9.1	1.4	2.5
5′-TNT-3′/3′-AXA-5′	0.22	1.6	1.3	1.9
5′-GNT-3′/3′-CXA-5′	0.27	2.6	2.3	2.8
5′-CNT-3′/3′-GXA-5′	0.42	4.3	0.77	0.83
5′-ANT-3′/3′-TXA-5′	0.18	3.0	0.53	0.91
5′-TNG-3′/3′-AXC-5′	0.63	8.6	0.23	0.43
5′-GNG-3′/3′-CXC-5′	0.58	11	0.33	0.50
5′-CNG-3′/3′-GXC-5′	0.54	8.1	0.21	0.26
5′-ANG-3′/3′-TXC-5′	0.49	14	0.20	0.30
5′-TNA-3′/3′-AXT-5′	0.26	2.4	0.14	0.28
5′-GNA-3′/3′-CXT-5′	0.29	2.7	0.20	0.42
5′-CNA-3′/3′-GXT-5′	0.31	2.5	0.10	0.22
5′-ANA-3′/3′-TXT-5′	0.01	0.27	0.12	0.24

[a] Experimental conditions are the same as those given in Fig. 2. The binding constants are roughly estimated from the observed fluorescence quenching efficiency (alloxazine at 453 nm, lumichrome 475 nm), using a 1 : 1 binding isotherm model. The DNA sequences are 5′-GTG TGC GTT G*N*′T GGA CGC AGA-3′/3′-CAC ACG CAA C*X*′A CCT GCG TCT-5′, where X′ denotes the AP site (X) and its flanking nucleotides and N′ denotes the target base and its flanking nucleotides. In N′, "**N**" denotes A base or T base.

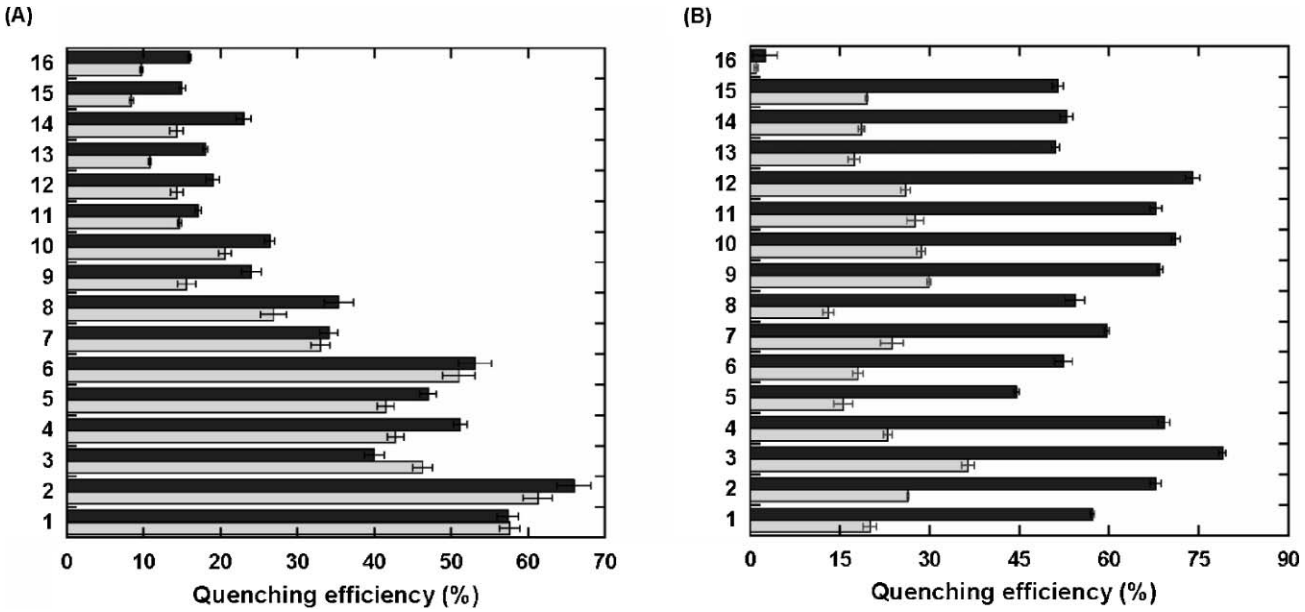

Fig. 6 Effect of flanking nucleobases on the fluorescence quenching efficiency of (A) alloxazine/A (gray bars) and lumichrome/A (black bars), (B) alloxazine/T (gray bars) and lumichrome/T (black bars) with different flanking nucleotides (5′-GTG TGC GTT G*N*′T GGA CGC AGA-3′/3′-CAC ACG CAA C*X*′A CCT GCG TCT-5′, and *N*′/*X*′ are: 1) TNC/AXG; 2) GNC/CXG; 3) CNC/GXG; 4) ANC/TXG; 5) TNT/AXA; 6) GNT/CXA; 7) CNT/GXA; 8) ANT/TXA; 9) TNG/AXC; 10) GNG/CXC ; 11) CNG/GXC; 12) ANG/TXC; 13) TNA/AXT; 14) GNA/CXT; 15) CNA/GXT; 16) ANA/TXT. N′ denotes a target base and its flanking bases, and the target base is A in (A) and T is (B); X′ = AP site and its flanking bases). Quenching efficiency (%) is calculated by $(F_0 - F)/F_0 \times 100$, where F_0 and F denote fluorescence intensities of alloxazine (at 453 nm) or lumichrome (at 475 nm) in the absence and presence of a DNA duplex, respectively. [DNA duplex] = 1.0 μM, [ligand] = 1.0 μM, [NaCl] = 100 mM, [EDTA] = 1.0 mM, [sodium cacodylate] = 10 mM, pH 7.0, 5 °C. λ_{ex} = 385 nm (alloxazine), λ_{ex} = 420 nm (lumichrome). Error bars denote the standard deviation obtained by 3 independent repeated measurements.

investigated by ITC measurements. As a result, clear evidence was obtained for the increased stacking interaction at the AP site upon lumichrome/T complexation from the increase in the enthalpy term. In all the cases, including previous reports and current studies, the interaction was found to be enthalpy driven when methylated ligands were used. The effect of the flanking bases on the ligand–DNA interaction was studied by fluorescence measurements. Benzo-annelation and methyl group substitution yielded an increase in the binding constants. We anticipate that these studies would be very useful for the understanding and future design of efficient ligands with respect to the substituent effects on the sequence specific or base selective binding.

Acknowledgements

MS is grateful for the financial support of Grant No. N N204 2659 33 for the years of 2007–2010 from the MNiSW, Poland, and for the support from Poznań Supercomputing and Networking Centre (PCSS) in calculations.

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