Alloxazine as a ligand for selective binding to adenine opposite AP sites in DNA duplexes and analysis of single-nucleotide polymorphisms†

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Received 2nd January 2008, Accepted 7th January 2008
First published as an Advance Article on the web 18th January 2008
DOI: 10.1039/b719786a

Alloxazine can bind to adenine selectively over other nucleobases opposite an abasic site in DNA duplexes (5′-TCC AGX GCA AC-3′/3′-AGG TCN CGT TG-5′, X = AP site, N = A, T, C, G) with a dissociation constant of 0.82 μM (pH 7.0, *I* = 0.11 M, at 5 °C), and it is applicable to SNPs typing of PCR amplification products based on the binding-induced fluorescence response.

Studies on the chemistry of DNA-binding drugs and/or low molecular weight ligands are of on-going interest due to their promising functions and biological activities, including their anti-cancer properties and ability to regulate gene expression.[1,2] Of particular interest to us is the development of a class of ligands suitable for gene detection, especially for single-nucleotide polymorphisms (SNPs) typing.[3] One possible approach to this end is based on ligands capable of targeting double stranded DNAs by intercalation or groove binding.[4] Another promising, but still rare approach involves the use of ligands that are able to bind to non-Watson–Crick base-pairing sites in DNA duplexes, where the selective binding of ligands is promoted by a pseudo-base pairing along the Watson–Crick edge of target nucleotides.[5] Successful examples of this class of ligands are the mismatch-binding molecules developed by Nakatani and co-workers,[5] and a surface plasmon resonance (SPR) assay has been proposed based on these molecules for the detection of mismatched base pairs in heteroduplexes.

On the other hand, we have recently found a series of aromatic ligands that can bind to a nucleobase opposite an abasic (AP) site in DNA duplexes,[6–13] and have proposed a new strategy of ligand-based fluorescence assay for SNPs typing. As schematically illustrated in Fig. 1A, an AP site-containing probe DNA is hybridized with a target DNA so as to place the AP site toward a target nucleotide, by which a hydrophobic binding pocket is provided for aromatic ligands to bind to target nucleotides through a combination of stacking and hydrogen-bonding interactions. Highly selective and strong binding was indeed obtained toward target nucleotides by using a series of flat aromatic ligands, including cytosine-selective 2-amino-7-methyl-1,8-naphthyridine[6] and 2-amino-5,7-dimethyl-1,8-naphthyridine,[7] guanine-selective 2-amino-6,7-dimethyl-4-hydroxypteridine (diMe-pteridine),[8] and thymine-selective amiloride,[9] riboflavin,[10] or a naphthyridine-benzofurazane conjugate.[11] Binding selectivity for thymine has been enhanced further by controlling hydrogen-bonding motifs between nucleobase and ligand.[12] Our system was effectively applicable to the analysis of polymerase chain reaction (PCR) amplification products,[9] for which a binding-induced fluorescence signalling of these ligands was utilized to detect the single-base mutations. However, in order to analyze all kinds of single-base mutations, the need exists to develop ligands with a selectivity for adenine: in contrast to the other three nucleotides, the challenge is to achieve binding selectivity by the formation of two point hydrogen-bonds along the edge of adenine bases, keeping a deep insertion of the aromatic ligand within the AP site so as to effectively stabilize the ligand binding.

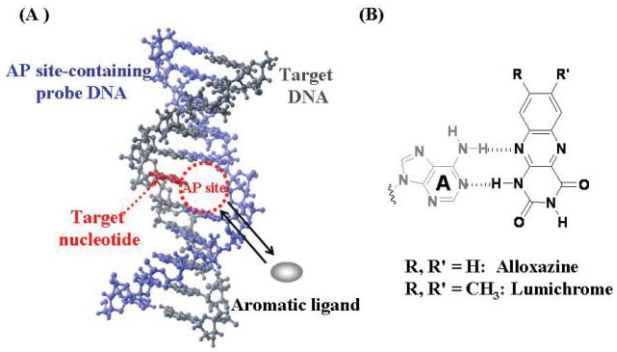

Fig. 1 (A) Schematic illustration of the ligand-based fluorescence detection of single-nucleotide polymorphisms, in combination with an AP site-containing probe DNA. (B) Possible binding mode of alloxazines with adenine in the AP site-containing DNA duplexes.

Here we report on alloxazine (Fig. 1B) as a candidate ligand for adenine with a useful binding affinity and selectivity in our fluorescence assay. From the examination of its binding to 11-mer AP site-containing DNA duplexes (5′-TCC AGX GCA AC-3′/3′-AGG TCN CGT TG-5′, X = AP site; Spacer-C3,[8b] N = target nucleotide) in solutions buffered to pH 7.0 (*I* = 0.11 M, at 5 °C), it is found that alloxazine selectively binds to adenine with a dissociation constant K_d of 0.82 μM. Comparison with the binding abilities of structurally-related riboflavin[10] or lumichrome (7,8-dimethylalloxazine, Fig. 1B) shows it is highly likely that the polar group along the edge of the alloxazine ring is involved in the adenine recognition (*cf.* Fig. 1B), and the size of ligand is crucial for the appearance of adenine selectivity. Potential use of

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† Electronic supplementary information (ESI) available: Fluorescence titration curves (Fig. S1 and Fig. S2), salt dependence of binding constants (Fig. S3 and Table S1), calorimetric isothermal titration (Fig. S4), effect of flanking nucleotides on fluorescence responses of alloxazine and dissociation constants (Table S2), and preparation and analysis of PCR products. See DOI: 10.1039/b719786a

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alloxazine is also presented for the detection of guanine/adenine transition present in PCR amplification products.

Fig. 2 shows fluorescence spectra of alloxazine (1.0×10^{-6} M) in a 10 mM sodium cacodylate buffer solution (pH 7.0) containing 100 mM NaCl and 1.0 mM EDTA. In the absence of DNAs, the ligand exhibits an emission band with a maximum at 453 nm, and almost no response is observed even in the presence of normal duplexes containing no AP site (1.0×10^{-6} M, 5′-TCC AGC GCA AC-3′/3′-AGG TCG CGT TG-5′). By contrast, as shown in Fig. 2, alloxazine shows significant quenching of its fluorescence upon addition of DNA duplexes containing adenine opposite the AP site (5′-TCC AG<u>X</u> GCA AC-3′/3′-AGG TC<u>A</u> CGT TG-5′, <u>X</u> = AP site; <u>A</u> = target). The resulting titration curve can be explained by the formation of a 1 : 1 complex with K_d of 0.82 μM (inset of Fig. 2). The observed binding affinity is comparable to the affinities previously reported for the C-, T-, and G-selective ligands,[6–12] and alloxazine would be applicable to the analysis of the order of sub-micromolar DNA samples, which is reasonably provided by standard PCR amplification. In addition, alloxazine is capable of selectively binding to A over T, C and G in AP site-containing DNA duplexes (*cf.* ESI, Fig. S1†). While the A/T selectivity is relatively moderate, the binding affinity for A is indeed one-order of magnitude higher than those for C and G (K_d/μM: T: 1.4; C: 5.0; G: 20.0). Alloxazine therefore has an ability to recognize adenine with a useful affinity and selectivity, and the binding is accompanied by effective fluorescence signaling.

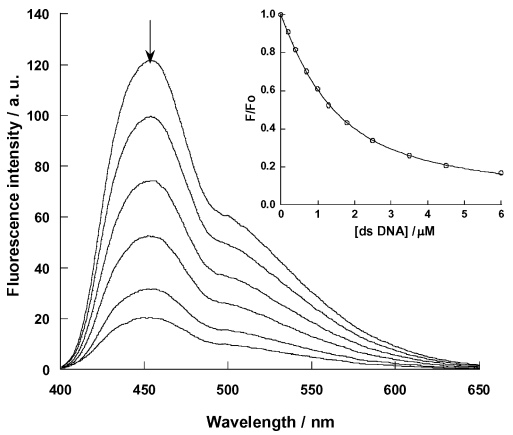

Fig. 2 Fluorescence responses of alloxazine (1.0 μM) to an AP site-containing DNA duplex (0, 0.4, 1.0, 1.8, 3.5, 6.0 μM; 5′-TCC AG<u>X</u> GCA AC-3′/3′-AGG TC<u>A</u> CGT TG-5′, <u>X</u> = AP site; <u>A</u> = target adenine) in solutions buffered to pH 7.0 (10 mM sodium cacodylate) containing 100 mM NaCl and 1.0 mM EDTA. Excitation wavelength: 385 nm. Temperature 5 °C. Inset: nonlinear regression analysis of the changes in the fluorescence intensity ratio at 453 nm based on a 1 : 1 binding isotherm model. F and F_0 denote the fluorescence intensities of alloxazine in the presence and absence of DNA duplexes, respectively.

Such a binding affinity and selectivity for adenine is not obtained by structurally-related riboflavin (K_d/μM: T: 0.56; C: 2.4; A: 29; G: 56),[10] under identical experimental conditions (pH 7.0, $I = 0.11$ M, at 5 °C). In analogy to alloxazine, riboflavin has a thymine-like hydrogen-bonding array, but has a substitution at the N(10) nitrogen. It is therefore likely that, in the binding event of alloxazine, the N(1)-H and N(10) groups form hydrogen-bonds to the Watson–Crick edge of the adenine base (*cf.* Fig. 1B), which allows an effective stacking of the alloxazine ring with nucleotides flanking the AP site.

Interestingly, substitutions at the 7- and 8-positions of the ring are found to significantly affect the binding selectivity. As revealed by fluorescence binding titrations (ESI, Fig. S2†), 7,8-dimethylalloxazine (lumichrome) does lose the selectivity for adenine, and shows a clear selectivity for thymine over other nucleobases: the binding affinity (K_d/μM) follows in the order of T (0.062) > A (0.53), C (0.59) > G (3.3). As compared to the binding of alloxazine, the affinity for thymine is particularly enhanced in the binding of lumichrome while the affinity for adenine is almost unaffected. The alloxazines–DNA interaction is thus strikingly sensitive to the subtle difference in the ligand structure, and the selective binding to adenine is assured by a combination of pseudo-base pairing and the size of ligand.

Further studies were done to obtain more details for the alloxazine–adenine interaction. According to the polyelectrolyte theory by Record *et al.*,[14] the examination of salt-dependence of binding constants (ESI, Fig. S3 and Table S1†) shows that the apparent charge Z on alloxazine is +0.05 when binding to adenine in AP site-containing DNA duplexes, from which a polyelectrolyte contribution ΔG_{pe} is calculated to be almost zero (−0.1 kcal mol^{-1}) at the 110 mM Na^+ concentration. Thus, in contrast to positively charged DNA binding molecules such as Hoechst 33258,[2] the binding of alloxazine does not accompany any release of counterions from DNA, and the overall free energy of alloxazine–adenine interaction (ΔG_{obs}, −7.7 kcal mol^{-1}) essentially results from a non-polyelectrolyte contribution (ΔG_t).

Isothermal titration calorimetry (ITC) experiments provided further thermodynamic character of the alloxazine binding. The addition of the duplex aliquots (2.0×10^{-4} M) into the solution containing alloxazine (2.0×10^{-5} M) causes a large exothermic heat of reaction, and the corrected binding isotherm is obtained after the heat of dilution is subtracted (ESI, Fig. S4†). The resulting titration curve can be best fitted using a model that assumes a single set of identical binding sites, giving the binding enthalpy ΔH_{obs} of −14.6 kcal mol^{-1} with a binding stoichiometry n of 1.1. The entropy change $T\Delta S_{obs}$ is then calculated to be −6.9 kcal mol^{-1}, using $T\Delta S_{obs} = \Delta H_{obs} - \Delta G_{obs}$.

The thermodynamic parameters obtained for the alloxazine–adenine interaction are summarized in Table 1. The magnitude of the binding affinity (1.2×10^6 M^{-1}) is almost comparable to values for typical intercalators such as ethidium bromide (~10^6 M^{-1}),[2] and overall, the observed nature of the thermodynamic profile is similar to that of intercalators.[2] However, the loss of binding entropy (−6.9 kcal mol^{-1}) is significant in the present case, *i.e.*, the favorable gain in binding enthalpy (−14.6 kcal mol^{-1}) is lost by as much as ~50%, which is responsible for the relatively low binding affinity (−7.7 kcal mol^{-1}) despite the considerable gain in binding enthalpy.

It should be noted here that the binding affinity of alloxazine depends on the nucleotides flanking the AP site. Fig. 3 shows fluorescence quenching efficiencies (%) of alloxazine (at 453 nm, 1.0×10^{-6} M) upon binding to adenine in 16 kinds of 23-mer AP site-containing DNA duplexes (1.0×10^{-6} M). The dissociation constants (K_d/μM, pH 7.0, $I = 0.11$ M, at 5 °C), as estimated from the observed fluorescence quenching, are also given in Table S2.† As can be seen from Fig. 3, fluorescence responses of the ligand are strongly governed by nucleotides at the 5′ side of the AP site, and

Table 1 Thermodynamic parameters for alloxazine binding to adenine in the 11-mer AP site-containing DNA duplex[a]

K_{obs}/M^{-1}	ΔG_{obs}/kcal mol^{-1}	$-SK$	ΔG_{pe}/kcal mol^{-1}	ΔG_t/kcal mol^{-1}	ΔH_{obs}/kcal mol^{-1}	$T\Delta S_{obs}$/kcal mol^{-1}
1.2×10^6	−7.7	0.044	−0.1	−7.6	−14.6	−6.9

[a] K_{obs}/M^{-1}, determined by fluorescence titration experiments, is the 1 : 1 binding constant in 110 mM Na^+ at 5 °C ([sodium cacodylate] = 10 mM, [EDTA] = 1 mM, [NaCl] = 100 mM, pH 7.0). ΔG_{obs} is the observed binding free energy calculated from $\Delta G_{obs} = -RT \ln K_{obs}$. *SK* is the slope of the plot of log K_{obs} *versus* log a_{Na+}. ΔG_{pe} and ΔG_t are the polyelectrolyte and non-polyelectrolyte contributions to the binding free energy ($\Delta G_{pe} = SKRT \ln a_{Na+}$) evaluated at 110 mM Na^+. ΔH_{obs} was determined by ITC at 5 °C. $T\Delta S_{obs}$ was calculated from $T\Delta S_{obs} = \Delta H_{obs} - \Delta G_{obs}$. DNA duplex: 5′-TCC AGX GCA AC-3′/3′-AGG TCA CGT TG-5′, X = AP site; A = target adenine.

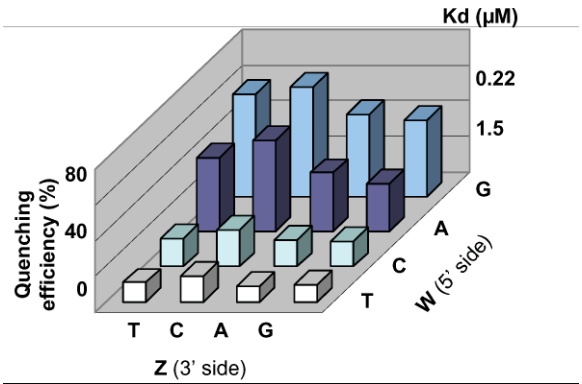

Fig. 3 Effect of flanking nucleotides on fluorescence responses and dissociation constants of alloxazine (1.0 μM) to adenine bases in 23-mer DNA duplexes (1.0 μM, 5′-TCTGCGTCCAwXz-CAACGCACAC-3′/3′-AGACGCAGGTw′Az′GTTGCGTGTG-5′, X = AP site; Spacer-C3). Sample solutions were buffered to pH 7.0 with 10 mM sodium cacodylate, containing 100 mM NaCl and 1 mM EDTA. Excitation 385 nm; detection and analysis 453 nm. Temperature 5 °C. See also Table S2 in the ESI.†

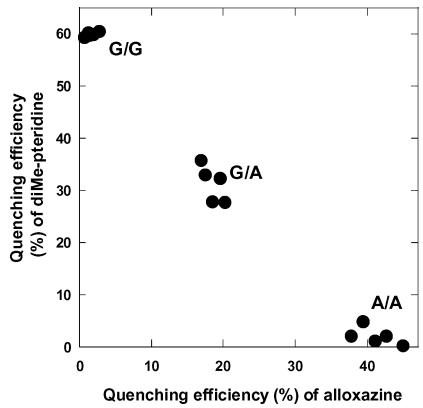

Fig. 4 Scatter plot for G>A genotyping (*K-ras* gene, codon 12, sense strand, 15 samples) based on fluorescence responses of adenine-selective alloxazine and guanine-selective diMe-pteridine. After PCR, the product was divided in half, and each aliquot was analyzed independently by alloxazine (0.1 μM) or diMe-pteridine (0.1 μM) in a buffer solution (pH 7.0, 100 mM sodium cacodylate) containing 1.6 mM EDTA, and 5.0 μM AP site-containing probe DNA. Excitation and detection wavelength: alloxazine: 385 and 453 nm; diMe-pteridine: 343 and 436.5 nm. Temperature 5 °C. See also the ESI.†

are roughly classified into two groups. When the DNA duplexes contain purine bases at the 5′ side of the AP site, 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_d from 1.9 μM to 0.22 μM. By contrast, relatively weak responses are obtained for the duplexes containing pyrimidine bases at the 5′ side, and the quenching is reduced to 8% for 5′-CAA-3′/3′-GXT-5′ (K_d = 9.6 μM). It is therefore likely that, as compared to pyrimidine bases, purine bases effectively stack with the alloxazine, and nucleotides at the 5′ side of the AP site are involved in the binding event more effectively than nucleotides at the 3′ side.

Finally, alloxazine was applied to the analysis of G>A present in 107-mer DNAs (*K-ras* gene,[15] codon 12, sense strand) obtained by asymmetric PCR (ESI†). Here, 15 samples (5 A-homozygous, 5 G-homozygous, and 5 heterozygous) were analyzed, for which guanine-selective diMe-pteridine (K_d = 0.16 μM)[8b] was also utilized. As shown in Fig. 4, SNP genotype of samples, A/A, A/G, G/G, can be clearly distinguished based on fluorescence responses of alloxazine and diMe-pteridine. The analysis requires no time-consuming steps such as purification of PCR products and careful control of temperature, and the result is readily obtained after PCR.

In summary, we have successfully discovered a class of AP site-binding fluorescence ligands, alloxazines, with useful affinity and selectivity for the base adenine. We are now undertaking further studies on the design and synthesis of this class of adenine-selective ligands, with improved binding selectivity and affinity.

This work was partially supported by Grants-in-Aid for Scientific Research (A), No. 17205009, and Scientific Research (B), No. 18350039, from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Notes and references

1 (*a*) J. M. Gottesfeld, L. Neely, J. W. Trauger, E. E. Baird and P. B. Dervan, *Nature*, 1997, **387**, 202; (*b*) S. White, J. W. Szewczyk, J. M. Turner, E. E. Baird and P. B. Dervan, *Nature*, 1998, **391**, 468.
2 (*a*) I. Haq, J. E. Ladbury, B. Z. Chowdhry, T. C. Jenkins and J. B. Chaires, *J. Mol. Biol.*, 1997, **271**, 244; (*b*) I. Haq and J. E. Ladbury, *J. Mol. Recognit.*, 2000, **13**, 188.
3 (*a*) M. Chicurel, *Nature*, 2001, **412**, 580; (*b*) B. W. Kirk, M. Feinsod, R. Favis, R. M. Kliman and F. Barany, *Nucleic Acids Res.*, 2002, **30**, 3295.
4 (*a*) S. Takenaka, K. Yamashita, M. Takagi, Y. Uto and H. Kondo, *Anal. Chem.*, 2000, **72**, 1334; (*b*) F. Pattyn, F. Speleman, A. De Paepe and J. Vandesompele, *Nucleic Acids Res.*, 2003, **31**, 122.
5 (*a*) K. Nakatani, S. Sando and I. Saito, *Nat. Biotechnol.*, 2001, **19**, 51; (*b*) A. Kobori, S. Horie, H. Suda, I. Saito and K. Nakatani, *J. Am. Chem. Soc.*, 2004, **126**, 557; (*c*) S. Hagihara, H. Kumasawa, Y. Goto, G. Hayashi, A. Kobori, I. Saito and K. Nakatani, *Nucleic Acids Res.*, 2004, **32**, 278.
6 (*a*) K. Yoshimoto, S. Nishizawa, M. Minagawa and N. Teramae, *J. Am. Chem. Soc.*, 2003, **125**, 8982; (*b*) S. Nishizawa, K. Yoshimoto, T. Seino, C.-Y. Xu, M. Minagawa, H. Satake, A. Tong and N. Teramae, *Talanta*, 2004, **63**, 175.
7 N. Li, L. Mei, A. Tong, S. Nishizawa and N. Teramae, *Anal. Chim. Acta*, 2007, **597**, 97.

8 (*a*) K. Yoshimoto, C.-Y. Xu, S. Nishizawa, T. Haga, H. Satake and N. Teramae, *Chem. Commun.*, 2003, **24**, 2960; (*b*) Q. Dai, C.-Y. Xu, Y. Sato, K. Yoshimoto, S. Nishizawa and N. Teramae, *Anal. Sci.*, 2006, **22**, 201.
9 C Zhao, Q Dai, T. Seino, Y.-Y. Cui, S. Nishizawa and N. Teramae, *Chem. Commun.*, 2006, **11**, 1185.
10 (*a*) S. Nishizawa, N. B. Sankaran, T. Seino, Y.-Y. Cui, Q. Dai, C.-Y. Xu, K. Yoshimoto and N. Teramae, *Anal. Chim. Acta*, 2006, **556**, 133; (*b*) N. B. Sankaran, S. Nishizawa, T. Seino, K. Yoshimoto and N. Teramae, *Angew. Chem., Int. Ed.*, 2006, **45**, 1563.
11 H. Satake, S. Nishizawa and N. Teramae, *Anal. Sci.*, 2006, **22**, 195.
12 B. Rajendar, Y. Sato, S. Nishizawa and N. Teramae, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 3682.
13 (*a*) W. Huang, K. Morita, N. B. Sankaran, S. Nishizawa and N. Teramae, *Electrochem. Commun.*, 2006, **8**, 395; (*b*) K. Morita, N. B. Sankaran, W. Huang, T. Seino, Y. Sato, S. Nishizawa and N. Teramae, *Chem. Commun.*, 2006, **22**, 2376.
14 M. T. Record, C. F. Anderson and T. M. Lohman, *Q. Rev. Biophys.*, 1978, **11**, 103.
15 J. L. Bos, *Mutat. Res.*, 1988, **195**, 255.