Free-Probe Fluorescence of Light-up Probes

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Abstract: The fluorescence enhancement of light-up probes (thiazole orange (TO) conjugated peptide nucleic acids (PNAs)) upon hybridization to target nucleic acid depends on the probe sequence, mainly due to large variations in free-probe fluorescence. Here we study three probes where the fluorescence in free state varies more than 50-fold. We find that this variation is due to a fraction that has TO intramolecularly "back-bound" to the PNA bases. The intramolecular affinity constant for this unimolecular interaction was determined by temperature titrations using absorption spectroscopy, and the fluorescence quantum yields of the probes in back-bound conformation were calculated. The molar ratio of probes in back-bound conformation was 0.70−0.96 at 30 °C and 0.40−0.73 at 60 °C, and the fluorescence quantum yield in back-bound conformation varied between 0.0020 and 0.077 at 30 °C, and 0.00065−0.029 at 60 °C. These data show that the variation in free-probe fluorescence depends mainly on the fluorescence quantum yield of the probe in back-bound conformation and to a much lesser extent on the tendency of the probe to adopt the back-bound conformation. With increasing temperature the free-probe fluorescence decreases owing to both reduced degree of back-binding and a decrease of the fluorescence quantum yield in back-bound conformation.

Introduction

As the advantages of homogeneous hybridization assays[1−4] are being realized the need for probes that generate large signal increase upon hybridization is growing. One such probe is the light-up probe, which is a peptide nucleic acid (PNA)[5,6] to which an asymmetric cyanine dye[7] is tethered. We have previously shown that light-up probes based on thiazole orange (TO) (Figure 1a) hybridize to DNA with high sequence specificity and with up to 50-fold increase in fluorescence.[8] The fluorescence increase observed depended on PNA sequence, which was mainly due to variations in free-probe fluorescence. The great potential of light-up probes in homogeneous hybridization assays[9] motivates a study of their fluorescence properties.

Here we characterize in detail three light-up probes. One is a homopyrimidine probe (TO−CCTCTTCCTC-lys$^+$), one is a mixed sequence probe (TO−TGTACGTCACAACTA-lys$^+$-lys$^+$) and one has two adjacent purines and then only pyrimidine bases (TO−TAGCCTCTTC-lys$^+$) (Figure 1a). The latter was designed to test the effect of purine bases, since we previously have shown that homopyrimidine probes have very low free-probe fluorescence while some mixed sequence probes have high free-probe fluorescence.[8] The positively charged TO dye (Figure 1a) binds with high affinity to single-stranded DNA,[10] and light-up probes are based on an uncharged analogue (PNA) to minimize internal complex formation.[8] Since TO fluoresces upon binding and free-probe fluorescence depends on the PNA sequence, TO is likely to "fold back" to some extent interacting with the PNA (Figure 1b) even though it is not attracted

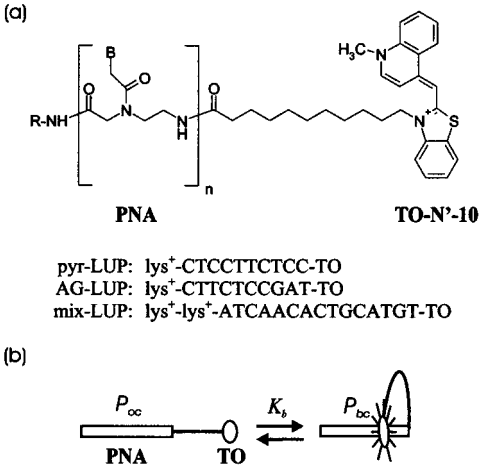

Figure 1. (a) Chemical structure and sequences of the studied light-up probes. B denotes nucleobases and R is either lysinyl amide or lys-lysinyl amide. (b) Schematic showing the intramolecular PNA−TO equilibrium where P_{oc} and P_{bc} denote the probes in open and back-bound conformation, respectively. K_b is the intramolecular affinity constant.

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electrostatically. It is this intramolecular interaction we study here by temperature titrations using absorption and fluorescence spectroscopy.

Materials and Methods

Light-up Probes. *N*-methyl-4-[(3′-carboxydecyl-1′,3′-benzothiazol-2′-yl)methylenyl]quinolinium iodide (TO−N′-10-COOH) was conjugated to the amino-terminal (N) of PNA as described previously.[8] Three light-up probes were synthesized: TO−N′-$(CH_2)_{10}$-CO−CCTCTTC-CTC-lys$^+$ (pyr-LUP), TO−N′-$(CH_2)_{10}$-CO-TAGCCTCTTC-lys$^+$ (AG-LUP), and TO−N′-$(CH_2)_{10}$-CO-TGTACGTCACAACTA-lys$^+$-lys$^+$ (mix-LUP). Thiazole orange (TO)[10] was synthesized as described.[11] Molar absorptivities of the probes at 260 nm were estimated as the sum of the contributions from PNA[12] and TO: $\epsilon_{260}/10^3 = 11.7n_G + 6.6n_C + 13.7n_A + 8.6n_T + \epsilon_{260}^{TO}$ M^{-1} cm^{-1}, where $\epsilon_{260}^{TO} = 7.1$ 10^3 $M^{-1}cm^{-1}$.[10] The concentrations of the probes were determined by absorption measured at 85 °C, where the PNA has lost its secondary structure.[12]

Absorption and Fluorescence Measurements. All spectroscopic measurements were performed in 10 mM phosphate buffer, 500 mM NaCl at pH 7.0. The samples were prepared in a standard 1 cm square quartz cell and degassed with helium. Absorption spectra were recorded on a Varian Cary 4 spectrophotometer using 1 nm bandwidth, and fluorescence was measured on a SPEX Fluorolog τ2 spectrofluorometer using a bandwidth of 3.6−5.4 nm. Fluorescence emission spectra were recorded using 470 nm excitation and fluorescence excitation spectra by monitoring the emission at 580 nm. All spectra were digitized with five data points per nanometer. Absorption at the excitation wavelength never exceeded 0.05, making the inner filter effect negligible.[13] The buffer and instrument contributions to the fluorescence spectra were corrected for. Fluorescence quantum yields (Φ_F) were determined relative to fluorescein in 0.1 M NaOH, assuming a quantum yield of 0.93 for the fluorescein dianion.[14] A cell compartment temperature block was used to control the sample temperature. The temperature of the samples was increased from 15 to 85 °C in steps of 5 °C and was allowed to stabilize for 5 min before spectra were recorded.

Results

Spectroscopic Properties of Light-up Probes. Three light-up probes (LUP) were synthesized with the same linker and dye. Their sequences are shown in Figure 1a. pyr-LUP is a pure polypyrimidine and was expected to exhibit very low free-probe fluorescence. mix-LUP has a mixed sequence and exhibits high free-probe fluorescence.[8] AG-LUP has purines in positions 2 and 3 and then only pyrimidine bases. The length of pyr-LUP and AG-LUP is 10 bases, while mix-LUP has 15 bases.

Figure 2 shows absorption and fluorescence excitation spectra of the light-up probes at 15 °C. The spectrum of free TO is shown for comparison. Free TO absorbs mainly in the vis region, having maximum absorption at 500 nm, but it has also significant absorption below 350 nm (Figure 2). The absorption spectra of the probes in the vis region are red-shifted compared to that of free TO, and they have a pronounced shoulder around 485 nm (Figure 2). For all three probes the excitation spectra are practically identical to the absorption spectra; around maximum and at long wavelengths there is essentially no difference, while around the shoulder the excitation spectra have somewhat higher intensity. Below 300 nm nucleobase absorption dominates the probe absorption spectra (Figure 2). Interestingly, also the probe excitation spectra exhibit significant intensity below 300 nm, having maximum at 265−270 nm, which

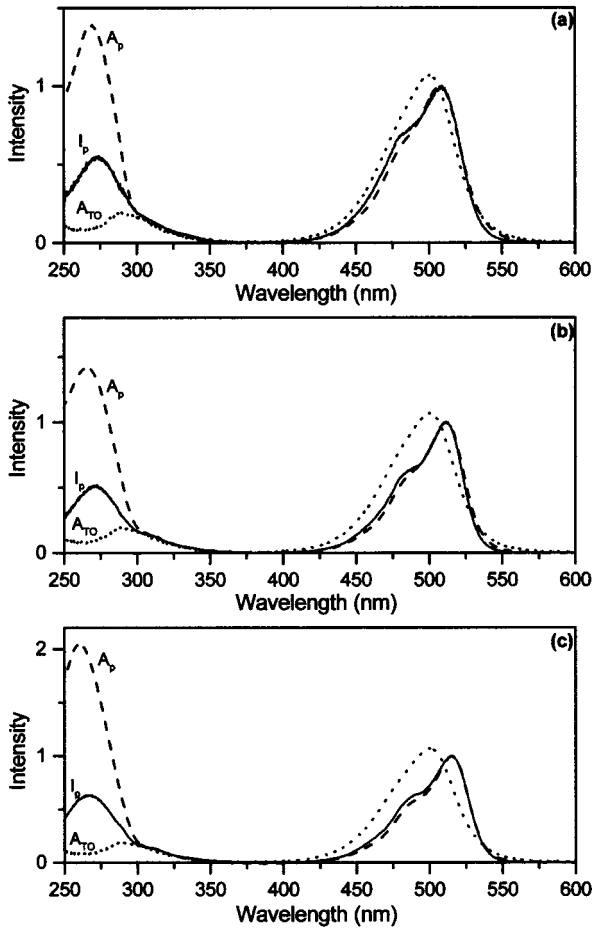

Figure 2. Absorption, A_p, (- - -) and fluorescence excitation, I_p, (—) spectra of (a) pyr-LUP, (b) AG-LUP, and (c) mix-LUP recorded at 15 °C. The absorption spectrum of free TO, A_{TO}, is shown for comparison (···). The spectra are normalized to have the same area in the interval 300−350 nm.

coincides with that of nucleobase absorption. In Figure 2 the spectrum of free TO and the probe excitation and absorption spectra are scaled to the same area in the region 300−350 nm. If the fluorescence quantum yields were independent of excitation wavelength, the probes' excitation spectra and the absorption spectrum of the free dye should be the same also at shorter wavelengths. This is not the case. Below 300 nm the probes' excitation spectra are much more intense than the TO absorption. Consequently, there must be an additional contribution to the probes' fluorescence than that caused by dye absorption. Since the probes' excitation maxima coincide with the probes' absorption maxima, excitation energy is most likely transferred from the bases to the dye. Such energy transfer has previously been observed for asymmetric cyanine dyes bound to the nucleobases of DNA.[15,16] Clearly, even though TO is not attracted to PNA electrostatically, the probes at low temperatures adopt a conformation where the dye interacts with the PNA bases (Figure 1b).

Temperature Titration. The absorption in the vis region of the light-up probes was studied as a function of temperature. The probes' concentrations were 1.0−1.3 μM. Figure 3 shows the probes' absorption spectra recorded every 5 °C in the interval 15−85 °C. For all of the probes, absorption depended on

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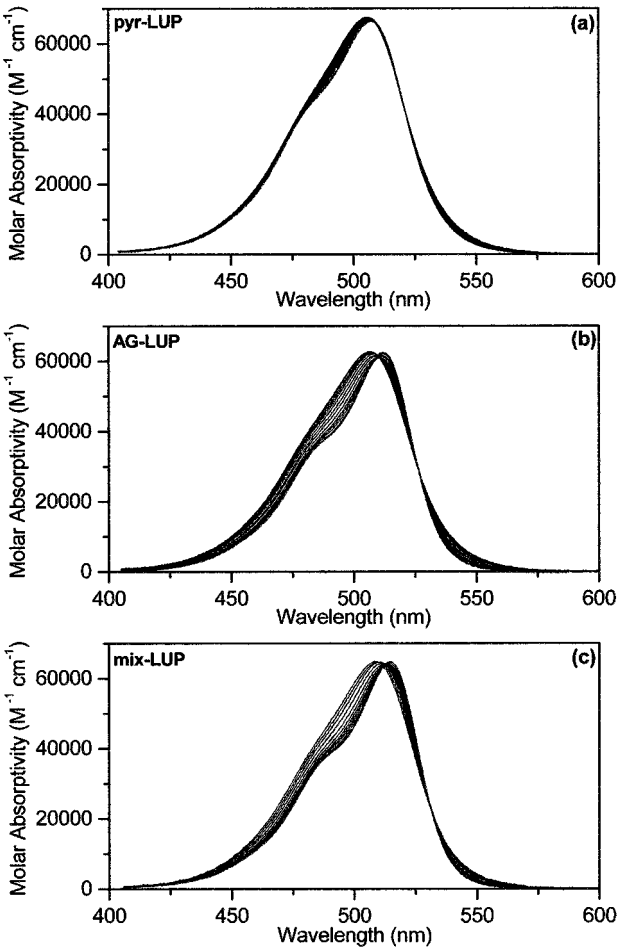

Figure 3. Absorption spectra of (a) pyr-LUP, (b) AG-LUP, and (c) mix-LUP recorded every 5 °C in the interval 15−85 °C. The probes' concentrations were 1.0−1.3 μM.

temperature. With increasing temperature the absorption shifts to shorter wavelengths, and the shoulder becomes less pronounced. Similar changes in absorption are observed for TO mixed with single-stranded DNA when the TO + DNA ⇔ TO−DNA equilibrium is shifted toward free species.[10] The wavelength of absorption maximum decreases from 507.2 to 505.0 nm for pyr-LUP, from 512.0 to 506.6 nm for AG-LUP, and from 514.6 to 508.2 nm for mix-LUP. In all titrations two isobestic points were observed (Figure 3); at 509.0 and 519.8 nm for pyr-LUP, at 510.2 and 526.4 nm for AG-LUP, and at 512.4 and 531.2 nm for mix-LUP, revealing the presence of two spectroscopic components in each titration. Two species were also identified by the statistical methods of Elbergali et al.[17] The obvious conclusion is that the two species are probe in open (P_{oc}) and back-bound (P_{bc}) conformation, respectively, that is, probe with the dye either free in solution or bound to the PNA (Figure 1b).

Determination of Equilibrium Constants. Assuming linear response the recorded spectra, $\mathbf{a}(\lambda)$ (vector variables are depicted as bold lower-case letters and matrix variables as bold capital letters), are linear combinations of the spectral responses of probe in open, $\mathbf{v}_{oc}(\lambda)$, and back-bound, $\mathbf{v}_{bc}(\lambda)$, conformation:

$$\mathbf{a}(\lambda) = c_{oc}\mathbf{v}_{oc}(\lambda) + c_{bc}\mathbf{v}_{bc}(\lambda) \tag{1}$$

where c_{oc} and c_{bc} are the concentrations of probe in open and back-bound conformation, respectively. The intramolecular TO−PNA equilibrium (Figure 1b) is described by

$$K_b(T) = \frac{c_{bc}}{c_{oc}} = \frac{x_{bc}}{x_{oc}} \tag{2}$$

where $x_{bc} = c_{bc}/(c_{oc} + c_{bc})$ and $x_{oc} = c_{oc}/(c_{oc} + c_{bc})$ are the molar ratios of probe in back-bound and open conformation, respectively. The intramolecular affinity constant $K_b(T)$ is assumed to depend on temperature according to the van't Hoff equation

$$\ln K_b(T) = \frac{1}{R}\left(\Delta S^\circ - \frac{\Delta H^\circ}{T}\right) \tag{3}$$

where ΔH° and ΔS° are the molar enthalpy and entropy changes, respectively, $R = 8.31$ J mol^{-1} K^{-1} is the universal gas constant, and T is the Kelvin temperature. The absorption spectra are digitized and arranged as rows in a matrix **A**. **A** is then decomposed into an orthonormal basis set using the NIPALS routine,[18,19]

$$\mathbf{A} = \mathbf{TP}' + \mathbf{E} \approx \mathbf{TP}' = \sum_{i=1}^{r} \mathbf{t}_i \mathbf{p}'_i \tag{4}$$

where $\mathbf{t}_i$ are orthogonal target vectors, $\mathbf{p}'_i$ are orthonormal projection vectors, **E** is the error matrix and $r = 2$ is the number of spectroscopically distinguishable components. Equation 1 can be written in matrix form

$$\mathbf{A} = \mathbf{CV} + \mathbf{E} \approx \mathbf{CV} = \sum_{i=1}^{r} \mathbf{c}_i \mathbf{v}_i \tag{5}$$

where $\mathbf{c}_i$ are vectors containing the component concentrations at the different temperatures and $\mathbf{v}_i(\lambda)$ are the component spectra. Equations 4 and 5 are related by rotation[19,20]

$$\mathbf{C} = \mathbf{TR}^{-1} \tag{6}$$

$$\mathbf{V} = \mathbf{RP}' \tag{7}$$

where **R** is an $r \times r$ rotation matrix, which for a two-component system has the elements

$$\mathbf{R} = \begin{bmatrix} r_{11} & r_{12} \\ r_{21} & r_{22} \end{bmatrix} \tag{8}$$

Since a single sample is studied, the total probe concentration is constant,

$$c_{oc}(T) + c_{bc}(T) = c_{tot} \tag{9}$$

which is used to constrain matrix **R** [eq 8].[10] The remaining elements of **R** can be determined by finding the combination of ΔH° and ΔS° trial values that produce the best fit of $\ln K_b(T)$ versus $1/T$ [eq 3]. Such analysis is, however, quite unstable,[21,22] and when performed on the probe absorption data, no unique solutions were found. Stable analysis would be

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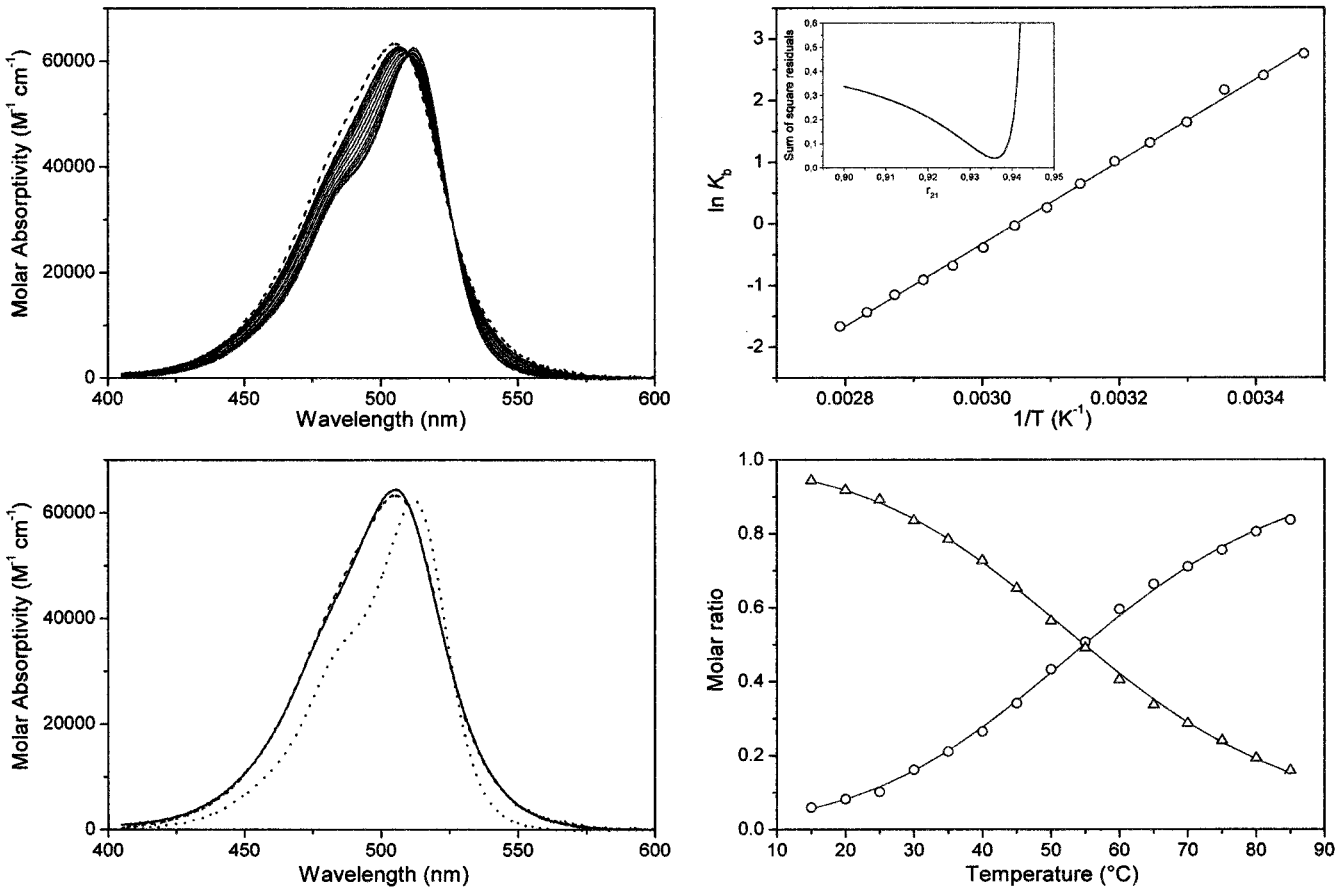

Figure 4. (Top) Left: Absorption spectra of AG-LUP recorded every 5 °C in the interval 15−85 °C (—), and of free TO red-shifted by 5.0 nm (- - -). Right: Linear regression of ln K_b with respect to $1/T$ for the r_{21} value that resulted in the smallest sum of square residuals (insert). (Bottom) Left: Calculated absorption spectra of AG-LUP in open (—) and back-bound (···) conformation. The spectrum of free TO red-shifted by 5.0 nm (- - -) is shown for comparison. Right: Molar ratios of AG-LUP in open (○) and back-bound (△) conformation compared to those predicted by the temperature dependence of the equilibrium constant (lines).

possible if the spectrum of the probe in open conformation, $\mathbf{v}_{oc}(\lambda)$, could be determined separately to further constrain the **R**-matrix.[10,21] The only chemical difference between TO and TO−N′-10 tethered to PNA is that the TO methyl group is replaced for an aliphatic linker (Figure 1a). This is not expected to affect the dye absorption appreciably. Absorption spectra of free TO and free TO−N′-10-COOH, when measured at low concentration (∼0.8 μM) and high temperature (85 °C) to avoid dimer formation,[21] are identical in shape but shifted relative to each other by 2.8 nm (not shown). We therefore assume that the spectrum of the probe in open conformation, $\mathbf{v}_{oc}(\lambda)$, can be modeled by the appropriately red-shifted and scaled spectrum of free TO. The shift and scaling factor are readily estimated from the two isobestic points observed in the titrations (Figure 4, upper left panel).

R can now be described by a single scalar, r_{21}, and four factors that are determined by the constraints,[21]

$$\mathbf{R} = \begin{bmatrix} f_{21} & f_{22} \\ r_{21} & f_{22} + (f_{21} - r_{21})\dfrac{f_{11}}{f_{12}} \end{bmatrix} \qquad (10)$$

where $f_{11} = (r_{22} - r_{12})/(r_{11}r_{22} - r_{12}r_{21})$, $f_{12} = (r_{11} - r_{21})/(r_{11}r_{22} - r_{12}r_{21})$, $f_{21} = r_{11}$ and $f_{22} = r_{12}$. r_{21} can be determined by requiring that matrix **R** should rotate the target vectors to give concentration vectors that produce an equilibrium constant whose natural logarithm is a linear function of $1/T$ [eq 3]. This is done by generating trial values of r_{21} and for each calculating a trial **R** matrix [eq 10], trial concentrations [eq 6], and trial affinity constants [eq 2]. Linear regression of ln K_b with respect to $1/T$ is then performed, and the r_{21} trial value that produces the best fit is considered correct. It is then used to calculate the spectroscopic profiles [eq 7][10] (the analysis is readily performed with the DATAN program available on the Internet: www.molbiotech.chalmers.se/research/mk/datan).[10]

Intramolecular Interaction of TO and PNA in Light-up Probes. The temperature dependence of the affinity constant for the intramolecular TO−PNA interaction for AG-LUP is shown in the top right panel of Figure 4, and the calculated absorption profiles and molar ratios of the probe in open and back-bound conformation are shown in the bottom panels. Best fit gave $\Delta H° = -55$ kJ mol^{-1} and $\Delta S° = -169$ J mol^{-1} K^{-1} and generated an almost perfectly linear van't Hoff plot. The absorption of the probe in back-bound conformation relative to the probe in open conformation is more red-shifted ($\lambda_{max}^{bc} = 512.0$ nm, $\lambda_{max}^{oc} = 505.4$ nm) and has somewhat lower absorptivity ($\epsilon_{max}^{bc} = 62\,300$ M^{-1} cm^{-1}, $\epsilon_{max}^{oc} = 64\,500$ M^{-1} cm^{-1}). The spectrum of the probe in back-bound conformation also exhibits a pronounced shoulder around 485 nm. The spectrum of free TO red-shifted by 5.0 nm, which was used to model the spectrum of the probe in open conformation (Figure 4, upper left panel), is practically identical to the spectrum of the probe in open conformation calculated from the fit (Figure 4, lower left panel). The pyr-LUP and mix-LUP absorption titrations were analyzed accordingly, and the results are summarized in Table 1. The mix-LUP spectra recorded at low temperatures (15−25

Table 1

light-up probe	$\Delta H°$ [kJ mol^{-1}]	$\Delta S°$ [J mol^{-1} K^{-1}]	K_b 30 °C	x_{bc} 30 °C	x_{bc} 60 °C	Φ_F 30 °C	Φ_F 60 °C	Φ_F^{bc} 30 °C	Φ_F^{bc} 60 °C	λ_{max}^{bc} [nm]
pyr-LUP	−32	−99	2.3	0.70	0.46	0.0014	0.00037	0.0020	0.00065	507.4
AG-LUP	−55	−169	5.2	0.84	0.40	0.022	0.0019	0.026	0.0046	512.0
mix-LUP	−62	−179	24	0.96	0.73	0.073	0.021	0.077	0.029	514.6

°C) were excluded in the analysis because they were identical to the spectrum recorded at 30 °C. The spectrum of pyr-LUP in open conformation was shifted by 4.0 nm relative to that of free TO, and that of mix-LUP was shifted by 5.8 nm. At 30 °C the highest affinity constant was observed for mix-LUP (K_b = 24), and the lowest, for pyr-LUP (K_b = 2.3) (Table 1).

Figure 5 shows the temperature dependence of ln K_b and the molar ratios of probe in open and back-bound conformation for the three light-up probes. The temperature dependence of the affinity constants varies among the probes (Table 1). For pyr-LUP $\Delta H° = -32$ kJ mol^{-1} and $\Delta S° = -99$ J mol^{-1} K^{-1} are substantially higher than for AG-LUP ($\Delta H° = -55$ kJ mol^{-1}, $\Delta S° = -169$ J mol^{-1} K^{-1}) and mix-LUP ($\Delta H° = -62$ kJ mol^{-1}, $\Delta S° = -179$ J mol^{-1} K^{-1}) (Table 1). At 30 °C mix-LUP is almost entirely in back-bound conformation (x_{bc} = 0.96) while the molar ratios of AG-LUP and pyr-LUP in back-bound conformation are 0.84 and 0.70, respectively. Increasing the temperature decreases the degree of back-binding. At 55 °C the molar ratios of pyr-LUP and AG-LUP in back-bound conformation are about 0.5, while it is 0.8 for mix-LUP. Not even at 85 °C are any of the probes entirely in open conformation (Figure 5).

Figure 5 also shows the calculated absorption spectra of the probes in back-bound conformation. The spectra of AG-LUP and mix-LUP are quite similar, λ_{max}^{bc} = 512.0 and 514.6, respectively, while that of pyr-LUP is substantially different (λ_{max}^{bc} = 507.4 nm) (Table 1).

Fluorescence Properties of the Light-up Probes. Fluorescence emission spectra of the probes were measured every 5 °C in the interval 15−85 °C. The spectra measured at 15 °C are shown in Figure 6. At this temperature the back-bound conformation dominates in all of the probes (Figure 5). The probes' emission spectra vary both in shape and in the wavelength at emission maximum. From the combined fluorescence and absorption data fluorescence quantum yield as a function of temperature was calculated and is shown in Figure 7. It varies substantially among the probes (Table 1). At 30 °C it is only 0.0014 for pyr-LUP; it is more than 15-fold higher for AG-LUP (Φ_F = 0.022), and it is 50-fold higher for mix-LUP (Φ_F = 0.073). Increasing the temperature from 30 to 85 °C decreases the fluorescence substantially of all three probes (Figure 7). For pyr-LUP it decreases 8-fold, becoming as low as that of free TO ($\Phi_F^{pyr-LUP,85°C}$ = 0.00018, $\Phi_F^{TO,85°C}$ = 0.00014). The fluorescence of mix-LUP ($\Phi_F^{85°C}$ = 0.0018) and of AG-LUP ($\Phi_F^{85°C}$ = 0.00030) decreases 40 and 70-fold, respectively.

Discussion

Back-Folding of TO onto the PNA Gives Rise to Free-Probe Fluorescence. Analysis of absorption spectra recorded at various temperatures reveals the presence of two forms of the light-up probes that are in thermal equilibrium. The one dominating at low temperature is characterized by absorbing at longer wavelengths and by having a characteristic shoulder in the TO band (Figure 3, Figure 5). It has also substantial fluorescence (Table 1). These features are characteristic of the

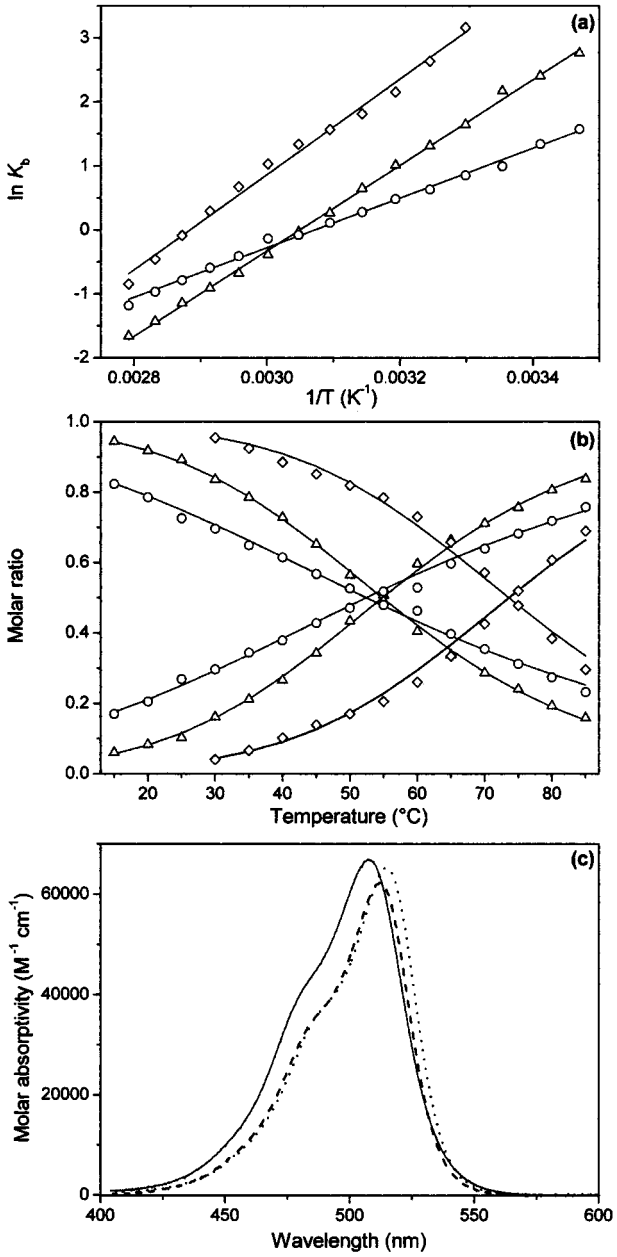

Figure 5. (a) Calculated values of ln K_b at some inverted temperatures for pyr-LUP (○), AG-LUP (△), and mix-LUP (◇) compared to the temperature dependence of ln K_b predicted by the van't Hoff equation (lines). (b) Molar ratios of probe in open and back-bound conformation for pyr-LUP (○), AG-LUP (△), and mix-LUP (◇) compared to those predicted by the temperature dependence of the equilibrium constant (lines). (c) Absorption spectra of pyr-LUP (—), AG-LUP (- - -), and mix-LUP (···) in back-bound conformation.

TO monomer bound to single-stranded DNA,[10] suggesting that the low-temperature form of the probes is a back-bound conformation where TO folds back onto the PNA interacting with its bases (Figure 1). All three probes exhibit unproportional fluorescence when excited in the UV region (Figure 2), evidencing energy transfer from the PNA bases to the dye. Such

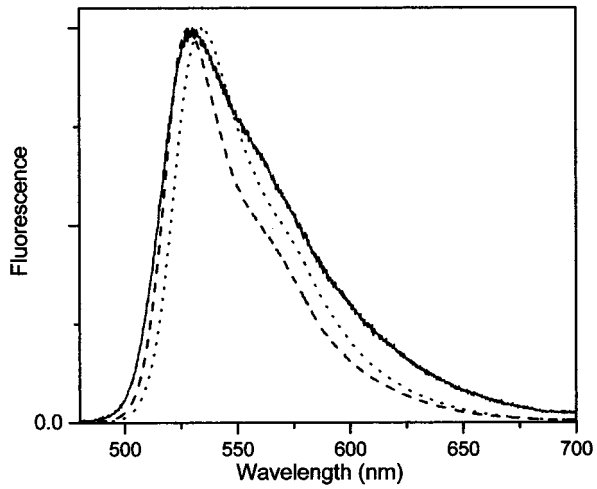

Figure 6. Normalized fluorescence emission spectra of pyr-LUP (—), AG-LUP (- - -), and mix-LUP (···) recorded at 15 °C.

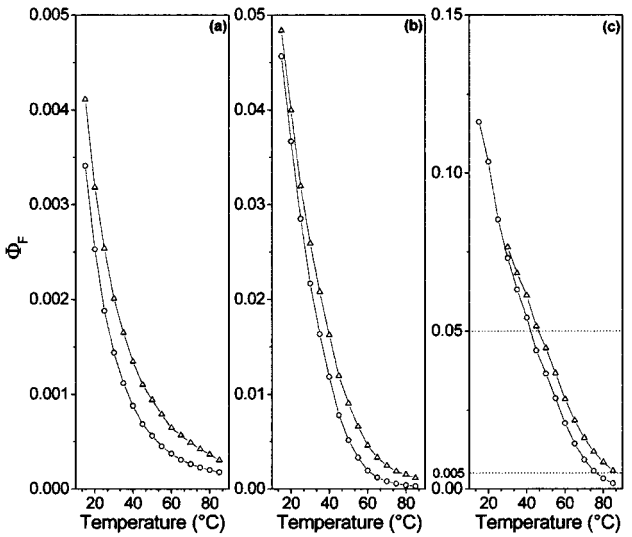

Figure 7. Effective fluorescence quantum yields of the free light-up probe (○) and the fluorescence quantum yield of the light-up probe in back-bound conformation (△) as function of temperature; (a) pyr-LUP, (b) AG-LUP, and (c) mix-LUP. The probes' concentrations were 1.0−1.3 μM. The lines are shown to guide the eye. In (c) the dashed lines indicate the maximum of the *y*-axes in the two other graphs.

close contact energy transfer[23] is efficient only over very small distances. Although it has been observed for groove-binding drugs,[24] it is usually taken as evidence for intercalation.[15,16,25] We conclude that a subpopulation where TO folds back onto the PNA interacting with its bases, probably by intercalation, causes the free-probe fluorescence.

Free-Probe Fluorescence Depends Mainly on the Quantum Yield of the Dye in Back-Bound Conformation. The free-probe fluorescence varies substantially among the three probes. Largest difference was between pyr-LUP and mix-LUP: it was 50-fold at 30 °C and 60-fold at 60 °C (Table 1). In the same temperature range the difference between the back-bound fractions of the same two probes was never more than 2-fold (Figure 5, Table 1). Clearly, the fluorescence quantum yields of the probes in back-bound conformation must be different. Assuming that the fluorescence quantum yield of the probes in open conformation is the same as for TO free in solution, the quantum yield of the probes in back-bound conformation (Φ_F^{bc}) can be calculated as

$$\Phi_F^{bc}(T) = \frac{\Phi_F(T) - x_{oc}(T)\Phi_F^{TO}}{x_{bc}(T)} \qquad (11)$$

where $\Phi_F^{TO} = 0.00014$, and $x_{oc}(T)$ and $x_{bc}(T)$ are the molar ratios of probe in open and back-bound conformation, respectively. At 30 °C the difference in Φ_F^{bc} for mix-LUP ($\Phi_F^{bc} = 0.077$) and pyr-LUP ($\Phi_F^{bc} = 0.0020$) is 40-fold, while the fraction in back-bound conformation differs by only 40% ($x_{bc}^{mix-LUP} = 0.96$, $x_{bc}^{pyr-LUP} = 0.70$). The difference in Φ_F^{bc} is even larger at 60 °C (Table 1). Hence, the variation in free-probe fluorescence is mainly due to the fluorescence quantum yield of the probe in back-bound conformation and to a much lesser extent to the tendency of the different probes to adopt the back-bound conformation.

Free-Probe Fluorescence Depends on Which Bases TO Interacts with in Back-Bound State. The absorption spectra of the probes in back-bound conformation (Figure 5) and the fluorescence emission spectra of the probes at 15 °C (Figure 6) differ both in shape and in the wavelength at intensity maximum (Table 1). This suggests that the dye is bound differently in the three probes, probably between different bases.[10] Also, the affinity constant of the intramolecular $P_{oc} \leftrightarrows P_{bc}$ equilibrium (Figure 1) varies: at 30 °C $K_b = 2.3$ for pyr-LUP, $K_b = 5.2$ for AG-LUP, and $K_b = 24$ for mix-LUP (Table 1). Since all probes have the same linker and dye, the PNA sequence must determine the dye affinity. Most likely, it depends on the particular bases the dye interacts with. This is reasonable, considering that the TO affinity is very different for the various single-stranded homopolymers.[10] The affinity for polypurines is much higher than for polypyrimidines, owing to more efficient stacking.[10] $\Delta H° = -32$ kJ mol^{-1} for the back-binding of pyr-LUP, while for AG-LUP and mix-LUP it is about −60 kJ mol^{-1} (Figure 5, Table 1). This suggests that TO in the latter probes folds back stacking with purine bases. Further, the fluorescence quantum yield of the probes in back-bound conformation (Φ_F^{bc}) varies. Again, pyr-LUP deviates from the other probes by having Φ_F^{bc} as low as 0.0020 (30 °C). For AG-LUP and mix-LUP Φ_F^{bc} is 0.026 and 0.077, respectively (Table 1). This suggests that TO in the latter probes is contacting guanine, which is the base giving rise to the highest fluorescence.[10]

In summary, the variations in back-binding and spectroscopic properties suggest that free-probe fluorescence depends on which bases the dye is interacting with in the back-bound conformation. In AG-LUP and mix-LUP TO is likely to contact purine bases, while in pyr-LUP it interacts with pyrimidine bases.

Free-Probe Fluorescence Decreases with Temperature Due to the Temperature Dependence of K_b and Φ_F^{bc}. The fluorescent back-bound conformation is favored at low temperature (Figure 5), resulting in a decrease in free-probe fluorescence with increasing temperature (Figure 7). At 30 °C mix-LUP is almost completely in back-bound conformation ($x_{bc}^{mix-LUP} = 0.96$, Figure 5), and still, its fluorescence is substantially lower than at 15 °C (Figure 7). Also for pyr-LUP and AG-LUP does the fluorescence increase considerably when temperature is lowered below 30 °C (Figure 7), even though the fraction in back-bound conformation does not change appreciably (Figure 5). This means that the fluorescence quantum yield of the probes in back-bound conformation must

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depend on temperature. Figure 7 shows the calculated [eq 11] fluorescence quantum yield of the probes in back-bound conformation (Φ_F^{bc}) as a function of temperature. Indeed, Φ_F^{bc} decreases with increasing temperature. This is typical of asymmetric cyanine dyes bound to DNA and is due to increased thermal motion of the nucleobases, which allows for more internal rotation in the bound dye.[10,16] The temperature dependence of Φ_F^{bc} varies among the probes. Φ_F^{bc} for AG-LUP decreases 22-fold when the temperature is increased from 30 to 85 °C ($\Phi_F^{bc,30°C} = 0.026$, $\Phi_F^{bc,85°C} = 0.0012$) while the decrease is 14-fold for mix-LUP ($\Phi_F^{bc,30°C} = 0.077$, $\Phi_F^{bc,85°C} = 0.0057$) and only 7-fold for pyr-LUP ($\Phi_F^{bc,30°C} = 0.0020$, $\Phi_F^{bc,85°C} = 0.00030$) (Figure 7). We do not know the reason for this variation, but it may reflect the energy barrier for rotating the benzothiazole and quinoline rings about the ethylene bond in the bound dye.[10] The temperature dependence of K_b for the $P_{oc} \Leftrightarrow P_{bc}$ equilibrium also varies among the probes (Figure 5, Table 1), and as a consequence the temperature dependence of the fraction in back-bound conformation (x_{bc}) is different for the three probes. For all probes the decrease in x_{bc} was less than that of Φ_F^{bc} in the temperature interval 30−85 °C. For AG-LUP it was 5-fold, while for mix-LUP and pyr-LUP it was 3-fold (Figure 5). Since AG-LUP has the largest decrease in both x_{bc} and Φ_F^{bc} with temperature, it has the most extreme temperature dependence of free-probe fluorescence (Figure 7).

In conclusion, free-probe fluorescence of light-up probes decreases with temperature due to the temperature dependence of both K_b and Φ_F^{bc}. For the three probes studied here the effect of Φ_F^{bc} dominates in the temperature interval 30−85 °C.

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