EXCITED-STATE PROTON TRANSFER IN NUCLEIC ACID BASES, NUCLEOSIDES, AND THEIR ANALOGUES: A MINI-REVIEW

Dedicated to Professor David Shugar on his 95-th birthday

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Fluorescent nucleic acid base analogues and the corresponding nucleosides that reveal excited-state proton transfer, including phototautomerism, are briefly characterized, and their potential applications as fluorescent probes discussed. Among the most promising systems are formycins and some 8-azapurines as well as the corresponding nucleosides, like 8-azaxanthosine. Other, non-fluorescent systems exhibiting phototautomeric behavior are reviewed, with special emphasis on the G-C dimer.

INTRODUCTION

Fluorescent purines, the corresponding nucleosides and their analogues have found numerous applications in structural and kinetic investigations of nucleic acids and enzymes, including those related to nucleic acid metabolism (Sinkeldam, Greco & Tor, 2010; Rist & Marino, 2002). The most promising fluorescent probes are those which emitting parameters are strongly dependent on the micro-environment. Accordingly, fluorophores undergoing rapid excited-state physicochemical changes, which usually depend on the nearest neighborhood of the fluorophore, are of special interest. The aim of the present mini-review is to draw attention to one class of such fluorophores, which hitherto was rarely utilized in biological research, namely, the nucleic acid base and nucleoside analogues undergoing excited-state proton transfer.

The excited-state proton transfer (ESPT) is a wellknown phenomenon (Foerster, 1950; Ireland & Wyatt, 1975; Valeur, 2002), which may take place either intraor extra-molecularly, the former being frequently coupled with the electron transfer (Waluk, 2008). The inter-molecular proton transfer rate is critically dependent on the solvent, isotope content, and the presence of proton donors/acceptors (buffers), and consequently provides information on the microenvironment of the fluorophore (Klymchenko & Demchenko, 2008). Of special interest for the biological research are ESPT processes observed in green fluorescent protein (Remington, 2006; Meech, 2009), alloxazine (Song, Sun, Koziołowa & Kozioł, 1974; Sikorska et al., 2003), lumazine (Presidao et al., 2010), and luciferin (Erez & Huppert, 2010).

For many years, only a few examples of ESPT among the fluorescent nucleic acid base analogues were known, and the most frequently studied system was 7-azaindole dimer (7AI, Scheme 1) as well as complexes of 7AI with alcohols, both exhibiting phototautomerism (Taylor, Kasha & El-Bayoumi, 1969; for recent review see Sekiva & Sakota, 2008). The first nucleoside analogue for which the ESPT has been experimentally confirmed was etheno-adenosine cation (Takahashi et al., 1981), followed by formycin A (Wierzchowski & Shugar, 1978; Wierzchowski & Shugar, 1982). Later it was shown that some non-fluorescent nucleic acid bases or their complexes may also undergo ESPT (Srivatsava & Mishra, 1981; De La Harpe et al., 2009), but these are not directly applied in the biological fluorescence research, and will be only briefly discussed later.



Scheme 1. The 7-azaindole dimer biprotonic phototautomerism. In 3-methylpentane, the dimeric fluorescence is observed at \sim 480 nm, while that of the monomer has λ_{max} ca. 330 nm.

FLUORESCENCE OF INTERMOLECULAR ESPT SYSTEMS

The intermolecular excited-state proton transfer is typically manifested as the pH-, isotope-, and solventdependent dual fluorescence. This is, in particular, true for those cases when S₁ deactivation rate is comparable to that of the proton transfer reaction. But there are also examples of extremely rapid ESPT with femto-second kinetics (Tolbert & Solntsev, 2002; Solntsev et al., 2005), and in these cases only the fluorescence of ESPT product is detectable, leading to single-band emission with large Stokes' shift. Finally, in some cases when ESPT is energetically possible, kinetics of this process may be to slow and no product fluorescence visible. In these cases, however, ESPT may be promoted by introduction of strong proton donors/acceptors (e.g., buffers) in appropriate concentrations or geometrical arrangement.

The Foerster cycle (Foerster, 1950; Grabowski & Grabowska, 1976; Grabowski & Rubaszewska, 1977; Ireland & Wyatt, 1976; Valeur, 2002) may be used to assess a thermodynamic possibility of ESPT in a given system, expressed in terms of the excited state pK value, or pK*, on the basis of spectral analysis:

$$pK^* - pK = 2.1 \cdot 10^{-3} (\nu_{00B} - \nu_{00A}) \tag{1}$$

where v_{00A} and v_{00B} are 0-0 S_0 - S_1 transition energies (in cm⁻¹) of proton accepting (basic) and donating (acidic) species, respectively.

The kinetic rate constant of the intermolecular ESPT in aqueous medium, k_{ESPT} , may be indirectly evaluated using formula (Weller, 1961):

$$pK^* = \ln k_{H^+} - \ln k_{ESPT} \tag{2}$$

where k_{H+} is the diffusion-limited rate constant for protonation in water at pH 0, $k_{H+} = 6 \cdot 10^{10} \text{ s}^{-1}$. It is evident that for pK* values between 1 and 3 the ESPT rate is in the nanosecond range, thus comparable to the S₁ state decay in most organic molecules.

In the polyfunctional heteroaromatic molecules like nucleic acid bases and their analogues, there are usually many possibilities of the ESPT, and usually no excitedstate prototropic equilibrium can be reached, so various ESPT processes may be characterized by the different *microscopic* pK* values. It is usually not easy to identify species which are responsible for the observed fluorescence, and possibility of the proton transfer further complicates this problem. Typical approach is by analysis of various alkyl derivatives, in which possibility of the ESPT (as well as of the ground-sate tautomerism) is reduced.

Double intermolecular or single intra-molecular ESPT

leads to the phototautomerism. The former process may be either concerted, as in the 7-azaindole dimer (Sekiya & Sakota, 2008) or sequential. There are many examples of phototautomerizable systems, including many bifunctional (or polyfunctional) aromatic and heteroaromatic compounds, e.g. hydroxy-benzoic or naphthoic acids, hydroxyquinolines etc. (Ireland & Wyatt, 1976).

FLUORESCENT BASE/NUCLEOSIDE ANALOGS SHOWING ESPT

The canonical purines, pyrimidines and corresponding nucleosides are characterized by very low fluorescence yields and short decay times in neutral aqueous environment (Peon & Zewail, 2001; Onidas et al., 2002). Two fluorescent purines, 2-aminopurine and 2,6diaminopurine, although useful as fluorescent probes, are spectroscopically rather insensitive to solvent changes, and therefore evidence of ESPT in these systems is weak (but see Santosh & Mishra, 1991, claiming contrary). The unambigous examples of ESPT in selected strongly fluorescent purines and purine analogues are described below.

$1, N^6$ -ethenoadenosine

This adenosine analogue, most frequently used as fluorescent probe (Leonard, 1984), exhibits intense fluorescence in neutral aqueous medium ($\lambda_{max} = 410$ nm, $\phi = 0.51$), ascribed to the neutral form of the compound. When the pH of the environment is lowered to \sim 3, this fluorescence persists, although the absorption and fluorescence excitation spectra are markedly changed, indicating protonation of the ground state. The simplest explanation of this fact is rapid deprotonation of the excited cation (Takahashi et al., 1981, Agbaria et al., 1994). The cationic fluorescence may be observed in non-aqueous solvents, appropriately acidified to ensure protonation of the ground state, or in the alkylated derivatives (Inoue et al., 1979). The pK* of the nucleoside protonation is probably <0, resulting in picosecond deprotonation rate of the excited cation, but this is difficult to confirm experimentally due to dynamic quenching of the 410 nm fluorescence band, observed at pH < 3.



Formycin A (8-aza-9-deazaadenosine)

Formycin A, its aglycone (See Scheme 3, below), and their N-methyl derivatives were subject of thorough spectroscopic examination due to many applications of this nucleoside in the biological research (Ward, Reich & Stryer, 1968, Wierzchowski, 1981, Wierzchowski & Shugar, 1982, Kierdaszuk et al., 2000; Włodarczyk et al., 2004). In the neutral aqueous medium this compound exhibits fairly intense fluorescence with λ_{max} 332 nm, thus characterized by small Stokes' shift (~5000 cm⁻¹), not indicative of ESPT. This fluorescence is ascribed to the two main tautomeric forms, N(7)H (major) and N(8)H (minor) in the purine numbering (Wierzchowski & Shugar, 1982).

When the formycin molecule becomes protonated ($pK_a = 4.4$), a dual fluorescence appears, showing maxima at 370 and 440 nm. Examination of N-methyl derivatives, and particularly N3-methylformycin A (Wierzchowski & Shugar, 1982) demonstrated that the long-wavelength band originates from the *neutral* N(3)H tautomer, generated via the ESPT from the excited formycin molecule, protonated at N(3), as depicted in Scheme 3. In the same time, N3-methylformycin in acidic media undergoes excited state deprotonantion from N(7)H and/or N(8)H, resulting in the presence of the 440 nm band well below the ground-state pK_a of this derivative (6.8). The pK* for N(7)H deprotonation has been estimated, on the basis of the Foerster cycle, to be ~1 (Wierzchowski & Shugar, 1982).

The phototautomeric band of formycin A can be also visible in neutral media, but only in special conditions: in 90% methanol with 1 M ammonium acetate the 440 nm band was clearly visible, while in aqueous buffer solution of the same strength only quenching of the short-wavelength fluorescence occurred. As expected, no such phototautomerism can be observed in formycin alkylated at N7 or N8 positions.

No phototautomerism was observed in the enzymeformycin complexes thus far, although ground-state tautomerism was visibly affected by binding of the nucleoside to the active site of purine-nucleoside phosphorylase (Kierdaszuk et al., 2000). But it would be interesting to check other formycin congeners, particularly the transition-state analogue 8-azaimmucillin (Schramm, 2005), in complexes with various glycolytic enzymes like nucleoside phosphorylases or hydrolases.

8-aza-7-deazaadenine (UPAC name 4-aminopyrazolo [3,4-d]pyrimidine)

This is an adenine isomer (Scheme 3), which, in contrast to formycin A, is only very weakly fluorescent in neutral aqueous medium. But in a weakly acidic media (that is, when the ground state is protonated, pK_a \sim 4.6) its behaviour is somewhat similar to that of formycin A: a strong, dual emission is observed with maxima at 360 and 430 nm, the latter corresponding to the emission of the neutral form of the N(3)-methyl derivative (in purine numbering), and the former to the cationic species. The proposed scheme of phototautomerism in this system is presented below (Scheme 3). As in the case of formycin A, the longwavelength band is absent in the acidified alcoholic media, but it reappears in the presence of buffer ions (Wierzchowski et al., 1980).

An interesting case is a N⁶-methyl derivative of the title compound, which reveals very weak fluorescence both in neutral and acidic medium, and no indication of ESPT. But in a concentrated (up to 1 M) acetate buffer, pH \sim 4, the phototautomeric fluorescence becomes clearly visible at 430 nm (Wierzchowski, 1981), thus confirming the mechanism proposed in Scheme 3.



Scheme 3. Phototautomerism in the *aglycone* of formycin A (8-aza-9-deazaadenine, X = N; Y = CH) and 8-aza-7-deazaadenine (X = CH; Y = N) – generalized view. This type of phototautomerism is observed when the title compounds are protonated in the *ground* states. Note that purine numbering is maintained throughout.

N^{6} -imidazolium-purine

This highly fluorescent adenine derivative has been investigated in the Poznan group (Wenska et al., 1997). Using appropriate N-methyl derivatives it has been demonstrated that weakly fluorescent at ~340 nm, relatively short-lived N(9)H tautomer undergoes rapid double ESPT leading to appearance of an intense, long-lived ($\tau \sim 8$ ns) fluorescence from the resultant N(3)H excited tautomer, with maximum at 410 nm. The phototautomer fluorescence is visible over a broad pH range, since the title compound, being cationic, does not undergo further protonation at pH ~3.5 as does adenine.



Scheme 4. 3-methyl-N6-imidazoliumpurine.

8-azaxanthine and 8-azaxanthosine

8-azaxanthine, a known inhibitor of the urate and xanthine oxidases, and a precursor of many other biologically active substances (Albert, 1986; Giorgi & Scratoni, 2009) is nonfluorescent at pH 7, but highly fluorescent in weakly acidic media, where the compound is in its neutral form (ground-state $pK_a \sim 4.9$), with fluorescence maximum at 420 nm, resulting in the unusually large Stokes' shift of ~16000 cm⁻¹ (Wierzchowski et al., 2006). By contrast, the 1,3dimethyl derivative of the 8-azaxanthine, known as 8azatheophylline, exhibits also intense fluorescence in similar conditions, but in this case its maximum is shifted to ~355 nm. Similar, but virtually pHindependent fluorescence is displayed by 1.3.8trimethyl-8-azaxanthine, a biologically active isomer of 8-azacaffeine (Medza et al., 2009). These facts strongly suggest rapid excited-state transformation of the 8azaxanthine molecule.



Scheme 5. 8-azaxanthine, shown in the fluorescent N(8)H form, and 8-azaxathosine ($R = \beta$ -D-ribosyl)

Examination of the solvent and isotope effects in 8azaxanthine, and the fluorescence properties of its Nmethyl derivatives, particularly the 8-methyl-8azaxanthine, has shown that the long-wavelength fluorescence of 8-azaxanthine is due to rapid excitedstate deprotonation of the N(3)H proton. This process generates a highly fluorescent anionic species, with negative charge located on N(3), while the ground-state deprotonation (pK_a 4.9) leads to the non-fluorescent anion with negative charge on the triazole ring (Wierzchowski et al., 2006; see also Albert, 1986). The Foerster cycle calculations for 8-methyl-8-azaxanthine $(pK_a = 7.2)$ gave values of $pK^* < -0.5$, indicating the deprotonation time below 10 ps, which explains apparent lack of the neutral molecule fluorescence in the emission spectrum of 8-azaxanthine in water. As expected, this fluorescence, centered at ~335 nm, has been found in the appropriately buffered alcoholic media (Wierzchowski et al., 2006).

8-azaxanthosine shows a dual fluorescence in the weakly acidic aqueous media, with maxima at 370 and 430 nm (Fig. 1). This fluorescence is different from that found in 8-azaxanthine (Medza et al., 2009), due to different tautomeric structure of the ground state (see Scheme 5), the situation somewhat analogous to this found in xanthine vs. xanthosine (Kulikowska et al., 2003). While the 370 nm band of 8-azaxanthosine fluorescence (Fig. 1) can be ascribed to the monoanionic emission (pK_a ~ 4.6, pK* < 1, based on the Foerster cycle), the observed pH effect (Fig. 1) and dependence on buffer concentration suggest that the appearance of the 430 nm band also may be result of the ESPT, possibly a keto-enol tautomerization.



Fig.1. Dual fluorescence of 8-azaxanthosine in aqueous medium (blue) pH 7; (red) pH 3; (green) pH 1.5. Excitation at 260 nm (adapted from Mędza et al., 2009). Ground-state pK_a is 4.6.

8-azaisoguanine

8-azaisoguanine (AIG, see Scheme 6) is a fluorescent analogue of isoguanine, the latter compound being a textbook example of solvent-induced ground-state ketoenol tautomerism (Sepioł et al., 1976), also investigated as a possible artificial "extended genetic code letter" (Benner & Sismour, 2005; Hirao, 2006). The polydeoxynucleotides of AIG have been recently synthesized (Seela et al., 2009) and shown to exhibit a measurable fluorescence, which was possible to apply in structural studies of the polynuclueotide complexes (Jiang & Seela, 2010; Seela et al., 2010).



Scheme 6. 8-azaisoguanine (shown in the one of the possible tautomeric forms)



Fig.2. Fluorescence of the neutral N8-methyl-AIG in water (blue), methanol (green) and in 99.5% D₂O (red).

8-azaisoguanine exhibits a solvent- and isotopedependent dual emission in its neutral form (pH ~ 3.5 – 4.5) in water (Wierzchowski et al., 2008). This emission has been interpreted on the basis of a similar behavior found for its more intensely fluorescent 8-methyl derivative (see Fig. 2). There is a rather strong evidence for the ESPT based on the solvent, isotope and buffer concentration effects (Figs. 2, 3), but the question remains whether it should be interpreted as a keto-enol or rather an annular N(3)H - N(1)H phototautomerism. The 3-methyl-8-azaisoguanine shows only the shortwavelength fluorescence band near 360 nm, as does the 3,8-dimethyl derivative (Mędza, unpublished data), pointing participation of N(3)H in the ESPT, and strong solvent effect suggest that this is probably intermolecular (double) ESPT, therefore the N(3)H-N(1)H phototautomerism is more likely.

It must be pointed out that neutral 8-methyl-AIG

exists in its ground state probably only as a N(3)H form, since the N(1)H species cannot be drawn without violating valence rules. By contrast, AIG probably exists as a mixture of many forms.



Fig. 3. Fluorescence of the neutral N8-methyl-AIG in aqueous medium – salt effect. Potassium acetate buffer, pH 4.8, was used at concentrations 3 (blue), 23, 73, 160, 300, 467 and 800 mM (green), respectively. Excitation at 290 nm.

Interestingly, the protonation of both 8-azaisoguanine and its 8-methyl derivative (ground-state pK_a 2.2 and 3.5, respectively) leads to a strong fluorescence similar to that of the phototautomeric band observed in the neutral medium (430 nm). This band is absent in the fluorescence of the cations of 3-methyl- and 3,8dimethyl derivatives, both intensely fluorescent with maximum at ~370 nm (Mędza, unpublished). It is therefore postulated that 8-azaisoguanine cation, bearing positive charge at N(1), also undergoes rapid deprotonation of the N(3)H in the excited state, producing the neutral N(1)H phototautomer.

NATURAL NUCLEIC ACID COMPONENTS SHOWING ESPT

The natural nucleic acid components are virtually nonfluorescent in the neutral aqueous media (Onidas, 2002), so putative ESPT processes cannot be studied by the standard room-temperature fluorescence methods. Nevertheless there are well documented examples of the phototautomerism occurring in the canonical purines and pyrimidines under special conditions.

Historically, the first experimental evidence of phototautomerism in purines and pyrimidines came as a surprising addition to the detailed study of the IR spectra of the natural pyrimidines recorded in inert gas matrices at 15 K (Szczesniak et al., 1983). It was found that after UV-irradiation of the samples the recorded IR spectra were markedly modified, by changing relative intensities

of the observed peaks. This phenomenon, occurring mainly in oxo-substituted compounds, was interpreted as an intramolecular keto-enol phototautomerism, and confirmed by ab-initio calculation of the vibrational spectra of the putative phototautomers. It has been documented in uracil (Szczepaniak et al., 1998; Shukla & Leszczynski, 2002), 9-methylguanine (Szczepaniak et al., 1988) and hypoxanthine (Gerega et al., 2006), as well as in some other oxo- and thio- substituted purine and pyrimidine analogs (Gerega et al., 2006; Wenska et al., 2006; Khvorostov et al., 2005; Chmura et al., 2008). Occurrence of such photon-induced tautomerism in cytosine in the gas phase was also proposed (Kosma et al., 2009). It is not certain, however, if an analogous mechanism may operate at the ambient temperatures and in solution.

The photo-induced double proton transfer in the G-C base pair was postulated many years ago based on semiempirical calculations (Srivatsava & Mishra, 1981), and recently reexamined using the *ab initio* methods (e.g., Sobolewski & Domcke, 2004; Zoete & Moewly, 2004; Kwon & Zewail, 2007; Jensen & Govind, 2009). This phenomenon was hypothesized to be responsible for the UV-induced mutations (Lowdin, 1963; Taylor et al., 1969; Strazewski & Tamm, 1990). First regarded as highly speculative, the double ESPT in G-C pair has been in recent years confirmed to occur in the gas phase, thanks to sophisticated experimental methods combining biphotonic UV-IR excitation with mass spectrometry (Nir et al., 2000; Schulz et al., 2004; Abo-Riziq et al., 2005; Sobolewski et al., 2005). Furthermore, the recently reported high deuterium isotope effect of S₁ state deactivation observed in double-stranded, but not single-stranded G-C polynucleotides (De la Harpe et al., 2009) is a next evidence for ESPT in this system, this time in solution. This process must be very rapid, since excite-state decay times of the canonical nucleosides are within low picosecond range (Onidas et al., 2002).

The analogous double ESPT in the A-T pair is still under debate (Perun et al., 2006; Santoro et al., 2009), but a simple inspection of the UV spectra of the model imino- derivatives of adenine (Dreyfus et al., 1977), shows that the latters in aqueous medium are characterized by low-energy excited singlet states, comparing to adenine, so the amino-imino phototautomerism should be at least energetically feasible. The incoming years will certainly add new information on these systems.

CONCLUSIONS

The intermolecular excited-state proton transfer seems to be a common phenomenon in the fluorescent purine analogues, and many other such systems are likely to be found (see, for example, McLaughlin et al., 2006; Kitamura et al., 2005). Their possible biophysical applications involve studies of nucleic acids (e.g., ribozymes) and enzyme-ligand complexes, primarily those related to the enzymes of purine metabolism. Particularly interesting seems to be spectral behavior of enzymes complexed with the transition-state analogues, where the hydrogen bond network plays key role (e.g., Schramm, 2005; Taylor-Ringia & Schramm, 2005). The ESPT probes, as particularly sensitive to the geometry of the neighboring proton donors/acceptor (Klymchenko et al., 2008), should help to resolve structural and dynamic aspects of enzyme and ribozyme active sites.

To gain maximum information from such systems as the ESPT probes, detailed spectral analyses of the presented analogs should be done, including the timeresolved spectroscopic studies with the use of up conversion (femtosecond domain) and streak camera based (sub picosecond and picosecond domain) techniques.

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