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PAPER

Emission properties of dihydropterins in aqueous solutions†

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Pterins belong to a class of heterocyclic compounds present in a wide range of living systems and accumulate in the skin of patients affected by vitiligo, a depigmentation disorder. The study of the emission of 7,8-dihydropterins is difficult because these compounds are more or less unstable in the presence of O_2 and their solutions are contaminated with oxidized pterins which have much higher fluorescence quantum yields (Φ_F). In this work, the emission properties of six compounds of the dihydropterin family (6-formyl-7,8-dihydropterin (H_2Fop), sepiapterin (Sep), 7,8-dihydrobiopterin (H_2Bip), 7,8-dihydroneopterin (H_2Nep), 6-hydroxymethyl-7,8-dihydropterin (H_2Hmp), and 6-methyl-7,8-dihydropterin (H_2Mep)) have been studied in aqueous solution. The fluorescence characteristics (spectra, Φ_F, lifetimes (τ_F)) of the neutral form of these compounds have been investigated using the single-photon-counting technique. Φ_F and τ_F values obtained lie in the ranges 3–9 $\times$ 10^{-3} and 0.18–0.34 ns, respectively. The results are compared to those previously reported for oxidized pterins.

Introduction

Pterins, heterocyclic compounds derived from 2-aminopteridin-4-(3*H*)-one,[1] are present in biological systems in multiple forms, and play different roles ranging from pigments to enzymatic cofactors for numerous redox and one-carbon transfer reactions.[2,3] The most common pterin derivatives are 6-substituted compounds, which have small substituents with 1 to 4 carbon atoms (unconjugated pterins) or larger substituents containing a *p*-aminobenzoic acid moiety (conjugated pterins). Pterins can also exist in different oxidation states: oxidized (or aromatic) pterins and reduced pterins (Fig. 1). Within the latter group, 7,8-dihydropterins and 5,6,7,8-tetrahydropterins (denoted throughout as dihydropterins and tetrahydropterins, respectively) are the most important derivatives due to their biological activity, *e.g.*, dihydroneopterin (H_2Nep) is secreted during the oxidative burst of stimulated macrophages,[4] dihydrobiopterin (H_2Bip) and tetrahydrobiopterin are involved in the metabolism of aminoacids,[5] and reduced derivatives of folic acid (PteGlu) participate in methionine and nucleotide biosynthesis.[6] Pterins exhibit several dissociation equilibria,[7] the neutral form (Fig. 1) being the predominant one at physiological pH for dihydropterins ($pK_a > 10$).

The interest in the photochemistry and photophysics of pterins has increased considerably during the last decade, as more evidence of their implication in photobiological processes became available.[8] In addition, H_2Bip, biopterin (Bip) and other pterin derivatives accumulate in the skin of patients affected by vitiligo, a depigmentation disorder, where the protection against UV radiation fails due to a lack of melanin.[9,10] Finally, pterins participate in biologically important photosensitization processes: pterins act as photosensitizers through different mechanisms[11,12] and are able to photoinduce the oxidation of DNA[13] and nucleotides.[14,15]

R	7,8-dihydropterins	oxidized pterins
-CH_3	6-methyl-7,8-dihydropterin (H_2Mep)	6-methylpterin (Mep)
-CH_2OH	6-hydroxymethyl-7,8-dihydropterin (H_2Hmp)	6-hydroxymethylpterin (Hmp)
-CHO	6-formyl-7,8-dihydropterin (H_2Fop)	6-formylpterin (Fop)
-$(CHOH)_2$-CH_3	7,8-dihydrobiopterin (H_2Bip)	biopterin (Bip)
-$(CHOH)_2$-CH_2OH	7,8-dihydroneopterin (H_2Nep)	neopterin (Nep)
-CO-CHOH-CH_3	sepiapterin (Sep)	-
-OH	7,8-dihydroxanthopterin (H_2Xap)	-

Fig. 1 Molecular structures of the dihydropterins investigated at physiological pH; structures of corresponding oxidized pterins are also represented.

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Table 1 Wavelengths of fluorescence maxima (λ_F), quantum yields (Φ_F), and lifetimes (τ_F) of pterin derivatives in air-equilibrated aqueous solutions (pH = 5.5–7.0). All the values listed were obtained by excitation into the lowest-energy band of each compound. Inpurities: Molar percentage of oxidized analogues (Pt) and H_2Xap calculated from HPLC chromatograms in aqueous solutions of dihydropterins prepared from the standards commercially available

				Impurities (% molar)	
Compound	λ_F/nm (±5)	τ/ns (±0.07)	Φ_F	Pt	H_2Xap
H_2Fop	528	0.34	$8.7(\pm0.3) \times 10^{-3}$	—	—
Sep	533	0.28	$7.0(\pm0.6) \times 10^{-3}$	—	—
H_2Bip	425	0.30	$9(\pm2) \times 10^{-3}$	1.3	0.7
H_2Nep	425	0.31	$5(\pm1) \times 10^{-3}$	0.3	1.2
H_2Hmp	425	0.21	$3(\pm1) \times 10^{-3}$	0.6	3.1
H_2Mep	410	0.18	$3(\pm1) \times 10^{-3}$	2.7	<0.5

The fluorescence emission of some aromatic pterins has been known for several decades and used for analytical purposes.[16] These compounds are responsible for the characteristic fluorescence emitted upon Wood's light examination of the white skin patches of patients suffering vitiligo,[9] and their emission properties have been studied in detail.[17–19] Upon UV-A excitation (320–400 nm), unconjugated oxidized pterins present broad emission bands centered at approximately 450 nm, relatively high fluorescence quantum yields ($\Phi_F > 0.10$) and fluorescence lifetimes (τ_F) in the range 2–14 ns (Table 1). On the other hand, conjugated pterins, such as PteGlu, show weak fluorescence ($\Phi_F < 0.01$) because the substituent acts as an "internal quencher" and efficiently deactivates the singlet excited states of the pterin moiety. In addition, fluorescence properties of oxidized pterins have been used in different studies in the field of molecular interactions, *e.g.* some pteridine-based fluorophores that are chemical analogues of the nucleosides of DNA have been developed[20,21] and interactions between nucleotides and pterins were investigated.[22]

Despite the biological interest of the photophysical behaviour of pterins, to the best of our knowledge, except for a work on reduced PteGlu derivatives,[23] no systematic study has been performed on the fluorescence of dihydropterin derivatives. Technical drawbacks are probably responsible for this. In the first place, dihydropterins are unstable in air-equilibrated solutions, undergoing oxidation with a rate depending on the chemical nature of the substituent.[24] Besides, preliminary experiments revealed two facts, which together constitute a problem to be solved: (i) the emission of dihydropterins is very weak and, (ii) the solutions prepared from chemicals of the highest purity commercially available are contaminated with small concentrations of oxidized pterins. Therefore, taking into account that the absorption spectra of dihydropterins and oxidized pterins are in general more or less superimposed (Fig. 2), the emission of the latter is a main interference in the analysis of the fluorescence of the former.

Notwithstanding the technical difficulties described in the previous paragraph, the photophysical properties of dihydropterins deserve to be investigated from two main points of view: (i) these heterocycles belong to a family of compounds involved in photobiological processes and, in particular, some of them are present in the skin of humans under pathological conditions (*vide supra*); (ii) the comparison of the photophysical properties of the aromatic pterins previously reported[17–19] and those of dihydropterins is essential for assessing the influence of the ring reduction on the relative importance of the various pathways following excitation of these compounds.

The aim of this study was to investigate the emission properties of dihydropterins in their neutral forms (Fig. 1), in aqueous solutions upon UV-A irradiation. Since photophysical and photochemical properties of pterins strongly depend on the chemical nature of the 6-substituent,[8] we have chosen for this study a series of 6 pterin derivatives exhibiting different absorption spectra (Fig. 2) and electronic density distributions on the pyrazine ring of the pterin moiety:[25] 6-formyldihydropterin (H_2Fop), sepiapterin (Sep), dihydrobiopterin (H_2Bip), dihydroneopterin (H_2Nep), 6-hydroxymethyldihydropterin (H_2Hmp) and 6-methyldihydropterin (H_2Mep). In particular, we have investigated the emission spectra and determined the Φ_F and τ_F values. The effects of the substituents on the emission properties are discussed in the context of the general photochemical behaviour of dihydropterins. Finally, the results are compared with those previously published for oxidized pterins.

Experimental

General

Pterins were purchased from Schircks Laboratories (Switzerland) and used without further purification. Other chemicals were from Sigma Chemical Co. The pH measurements were performed with a pH-meter PHM220 (Radiometer Copenhagen) and a combined pH electrode pHC2011-8 (Radiometer Analytical). The pH of the aqueous solutions was adjusted by adding drops of HCl or NaOH solutions from a micropipette. The concentrations of the acid and base used for this purpose ranged from 0.1 to 2 M. The ionic strength was approximately 10^{-3} M in all the experiments. Electronic absorption spectra were recorded on a Shimadzu UV-1800 spectrophotometer. Measurements were made using quartz cells of 1 cm optical pathlength.

Analysis by high performance liquid chromatography (HPLC)

Chromatography of the solutions was performed using an instrument from Shimadzu (Prominence, solvent delivery module LC-20AT, on-line degasser DGU-20A5, auto sampler SIL-20A HT, column oven CTO-10AS VP and photodiode array detector SPD-M20A). A Synergi Polar-RP column (ether-linked phenyl phase with polar endcapping,

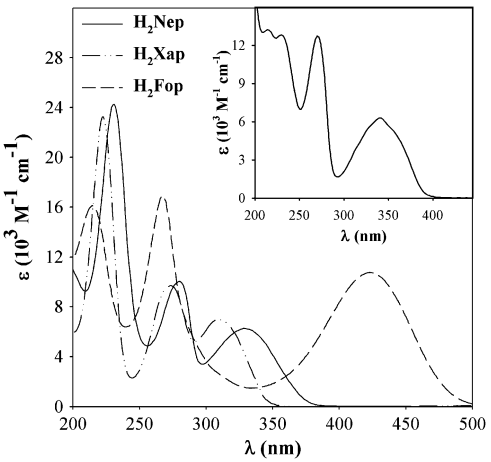

Fig. 2 Absorption spectra of H_2Nep, H_2Xap and H_2Fop in H_2O at pH 7.0. Spectra of H_2Bip, H_2mp, H_2Mep, and H_2Dmp are almost identical to that shown for H_2Nep, and the spectrum of Sep is almost identical to that shown for H_2Fop. Inset: Absorption spectrum of pterin.

150 × 4.6 mm, 4 μm, Phenomenex) was used for product separation, the elution being achieved with: (i) 4% methanol, 96% aqueous solution of ammonium acetate (NH_4OAc) (10 mM, pH = 7.0) or (ii) 5% acetronitrile, 95% aqueous solution of NH_4OAc (10 mM, pH = 7.0). Aqueous solutions of commercial standards were employed for obtaining calibration curves.

Emission experiments

Fluorescence measurements were performed at room temperature using a single-photon-counting equipment FL3 TCSPC-SP (Horiba Jobin Yvon).

Steady-state experiments. The sample solution in a quartz cell was irradiated with a CW 450W Xenon source through an excitation monochromator (FL-1004). The luminescence, after passing through an emission monochromator (iHR320), was registered at 90° with respect to the incident beam using a room-temperature R928P detector. The fluorescence quantum yields (Φ_F) were determined from the corrected fluorescence spectra using the following equation:

$$\Phi_F = \Phi_F^R \frac{IA^R}{I^R A} \quad (1)$$

where I is the integrated intensity, A is the absorbance at the excitation wavelength and the superscript R refers to the reference fluorophore. In our experiments, fluorescein in aqueous CO_3^{2-}/HCO_3^- buffer pH 9.6 was used as a reference[26] (Φ_F = 0.85)[27] and both sample and reference were excited at the same wavelength. To avoid inner filter effects, the absorbance of the solutions at the excitation wavelength was kept below 0.10.

Time-resolved experiments. NanoLED sources (maxima at 341 and 461 nm) were used for excitation. The emitted photons, after passing through the iHR320 monochromator, were detected by a TBX-04 detector connected to a TBX-PS power supply and counted by a FluoroHub-B module, controlled using the DataStation measurement control software application. The selected counting time window for the measurements reported in this study was 0–200 ns.

For a given solution, the decay curve was measured at multiple emission wavelengths to construct time-resolved emission spectra (TRES), 3D data set of counts *versus* time and *versus* wavelength. This 3D data set was then sliced orthogonally to the time axis to produce 2D spectra of counts *versus* wavelength in order to visualize how the emission spectrum evolves during the fluorescence lifetime. The Global Analysis of TRES, a fit calculation (up to 5 exponentials) performed globally on up to 100 separate decay curves, was carried out using the DAS6 Fluorescence Decay Analysis software.

Results and discussion

6-Formyldihydropterin and sepiapterin

In contrast to most aromatic and reduced pterin derivatives, 6-formyldihydropterin (H_2Fop) and sepiapterin (Sep) present an intense and broad absorption band in the spectral range 350–500 nm (Fig. 2). This particular spectral feature results from a charge transfer across the conjugated system of the pterin moiety between the amino group in position 2 and the carbonyl group of the 6-substituent.[28] Thus, the fluorescence of these compounds may be studied without the interference of the emission of oxidized pterins. In fact, the latter derivatives do not absorb above 410 nm (Fig. 2),[17,18] therefore the dihydropterins may be excited selectively by irradiation in the range 420–470 nm, even in the presence of a significant concentration of oxidized derivatives. Therefore, H_2Fop and Sep are suitable compounds for a first approach to the study of the emission properties of dihydropterins.

The fluorescence spectra of the two compounds in air-equilibrated aqueous solutions, obtained by excitation at 425 nm, are similar and show a broad emission band centered at 525 nm (Fig. 3, Table 1). The fluorescence quantum yields

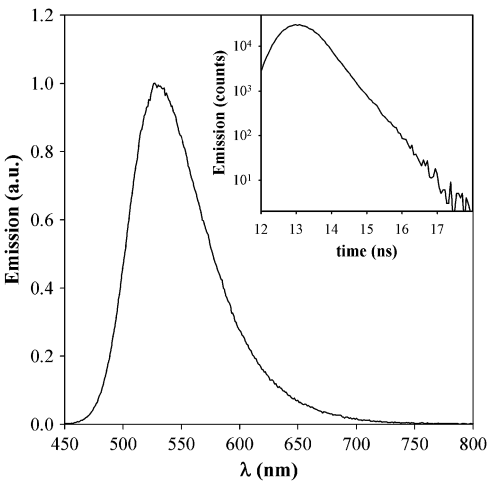

Fig. 3 Corrected and normalized fluorescence spectra of an air-equilibrated aqueous solution (pH 6.5) of H_2Fop obtained by excitation at 425 nm. Inset: decay of the H_2Fop emission (17 μM, pH = 6.0, λ_{exc} = 461 nm, λ_{an} = 530 nm).

(Φ_F) were determined from the steady-state measurements using eqn (1) (Experimental Section) and values of 8.7 ($\pm$0.3) $\times$ 10^{-3} and 7.0 ($\pm$0.6) $\times$ 10^{-3} were obtained for H_2Fop and Sep, respectively. These values are much lower than those previously reported for unconjugated oxidized pterins (Table 1).[17–19] For both compounds, Φ_F was determined in Ar-, air- and O_2-saturated solutions and the same value was obtained, within experimental error.

Time-resolved studies of the fluorescence were performed using excitation at 461 nm and fluorescence decays were analyzed in the 500–550 nm wavelength range. A first-order rate law was observed for all the decays. A typical trace recorded for H_2Fop is shown in Fig. 3. Fluorescence lifetimes (τ_F) were determined to be 0.34 ($\pm$0.06) ns and 0.28 ($\pm$0.05) ns for H_2Fop and Sep, respectively (Table 1). These values are much lower than those reported for unconjugated oxidized pterins.[17–19] In particular, τ_F for 6-formylpterin (Fop) has been reported to be 7.9 ($\pm$0.4) ns (to the best of our knowledge, no results have been published for the oxidized analogue of Sep).

HPLC analysis of dihydropterins solutions

As mentioned in the Introduction, the solutions of dihydropterins are inevitably contaminated with oxidized pterins. Except for the special cases discussed in the previous section, those contaminations, although in very low amounts, can significantly interfere in the study of the emission properties of dihydropterins due to: (i) the partial superimposition of the absorption spectra of both classes of pterins derivatives and the very large differences in the Φ_F values ($\Phi_{F(H_2Pt)} \ll \Phi_{F(Pt)}$). Therefore, to assess the chemical nature and the magnitude of the contamination, the solutions of dihydrobiopterin (H_2Bip), dihydroneopterin (H_2Nep), 6-hydroxymethyldihydropterin (H_2Hmp) and 6-methyldihydropterin (H_2Mep) were analyzed by HPLC.

It was found that in all cases, the solution of a given dihydropterin derivative was contaminated with its oxidized analogue. In addition, very small amounts of dihydroxanthopterin (H_2Xap) were also detected in the cases of H_2Bip, H_2Nep and H_2Hmp. However, a quick evaluation of the absorption and emission spectra of H_2Xap indicated that it does not cause a significant interference because: (i) its molar absorption coefficient is very weak above 330 nm and (ii) its Φ_F value is of the same order of magnitude as those of other dihydropterins. For the dihydropterins mentioned in the previous paragraph (H_2Bip, H_2Nep, H_2Hmp, H_2Mep), the molar percentages of the corresponding oxidized analogue and H_2Xap were calculated from the HPLC chromatograms using commercial standards of the impurities and the results are listed in Table 1.

Dihydrobiopterin, dihydroneopterin and 6-hydroxymethyldihydropterin

H_2Bip, H_2Nep and H_2Hmp undergo autooxidation (reaction with dissolved O_2), but at low rate, their half-life ($t_{1/2}$) in air-equilibrated aqueous solutions at 25 °C being longer than 48 h.[24] For the three compounds, the main product of the autooxidation is H_2Xap (the fraction of consumed reactant converted into H_2Xap is more than 90%). Therefore, taking into account the low reaction rate and the properties discussed for H_2Xap, the oxidation of H_2Bip, H_2Nep and H_2Hmp by dissolved O_2 cannot cause an additional interference in the study of their emission properties.

In steady-state measurements, a dependence of the emission spectra with the excitation wavelength was observed for the three compounds, thus suggesting the presence of more than one fluorescent component. Spectra obtained by excitation above 380 nm, where dihydropterins do not absorb significantly, showed a red shift in the emission maxima, which is consistent with a significant contribution of the corresponding oxidized pterin derivative.

Time-resolved experiments were performed by excitation at 341 nm (see Experimental Section), *i.e.* both dihydro- and oxidized pterins absorb at this wavelength. The emission decays were clearly biexponential with a short-lived component (τ_F < 1 ns) and a longer-lived component (1 ns < τ_F < 5 ns). The shorter τ_F values were of the same order of magnitude as those determined for H_2Fop and Sep in this work (*vide supra*), whereas the longer τ_F values were of the same order of magnitude as those previously reported for oxidized pterins.[17–19] The τ_F values of both components were constant independently of the wavelength of analysis. However, the comparative assessment of the pre-exponential factor as a function of emission wavelength showed a contribution of the longer-lived component increasing with the analysis wavelength (Fig. 4).

In agreement with these results, the global analysis of time-resolved emission spectra (TRES) also revealed the presence of two components. The corresponding spectra were obtained from the corrected plots of the pre-exponential factors as a function of the wavelength. For each dihydropterin solution analyzed, the spectrum and the τ_F value of the long-lived component matched very well those previously published for the corresponding oxidized pterin derivative (Table 2). In addition, data obtained from global analysis for the longer-lived components were equal, within experimental error, to those obtained in independent experiments, carried out with our experimental set-up, using standard solutions of the

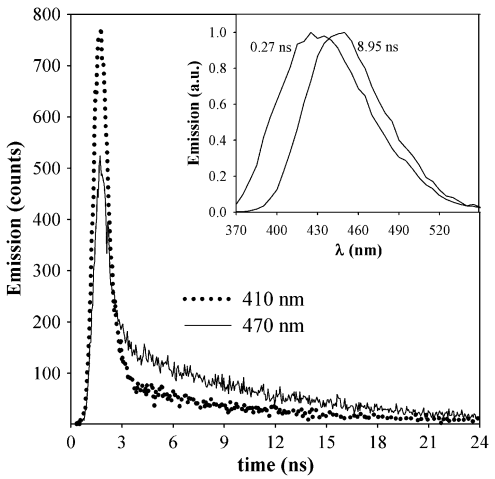

Fig. 4 Decays of the H_2Bip emission in aqueous solution (17 μM, pH = 7.0, λ_{exc} = 341 nm) registered at two different wavelengths. Inset: Fluorescence spectra registered at different times after the excitation.

Table 2 Comparison of wavelengths of fluorescence maxima (λ_F) and fluorescence lifetimes (τ_F) of the long-lived component in global analysis of dihydropterin solutions with data obtained from analysis of solutions of oxidized pterins. All the values listed were obtained by excitation into the lowest-energy band of each compound

Long-lived component in global analysis of dihydropterin solutions			Analysis of oxidized pterin solutions				
			Previously reported data[a]		This work (global analysis)		
Compound	λ_F (nm) (±5)	τ_F (ns) (±0.4)	Compound	λ_F (nm) (±3)	τ_F (ns) (±0.4)	λ_F (nm) (±4)	τ_F (ns) (±0.4)
H_2Bip	444	9.4	Bip	441	9.1	445	9.7
H_2Nep	443	9.2	Nep	440	8.9	445	9.6
H_2Hmp	449	10.8	Hmp	449	11.0	450	10.8
H_2Mep	448	13.1	Mep	448	13.3	445	13.6

[a] Data from ref. 18 and 19.

corresponding oxidized pterins (Table 2). The comparison between the spectrum obtained by global analysis for the longer-lived component in solutions of H_2Bip and the unique species detected in global analysis of Bip solutions is shown in the inset of Fig. 5. Similar results were observed for H_2Nep and H_2Hmp (Fig. S1).†

Therefore, for a given dihydropterin derivative, the longer-lived component was assigned to the corresponding oxidized analogue, present in the solution as a contaminant. Consequently, the short-lived components were assigned to the dihydropterins. The spectra of these compounds are shown in Fig. 5 and Fig. S1 (ESI), and the corresponding wavelengths of fluorescence maxima (λ_F) and τ_F values are listed in Table 1. These τ_F values are very similar to those determined for H_2Fop and Sep (*vide supra*).

The Φ_F values of the compounds analyzed in this section cannot be estimated directly from steady-sate measurements using eqn (1), as in the case of H_2Fop and Sep, because the integrated intensities have a significant contribution of the corresponding oxidized derivatives. Taking advantage that the Φ_F values of the oxidized pterins are known,[18,19] we calculated the Φ_F values of dihydropterins from global analysis data, using the following equation:

$$\frac{\Phi_F^{H_2Pt}}{\Phi_F^{Pt}} = \frac{\tau_F^{H_2Pt} I^{H_2Pt} A^{Pt}}{\tau_F^{Pt} I^{Pt} A^{H_2Pt}} \quad (2)$$

where the superscripts H_2Pt and Pt refer to a given dihydropterin (short-lived component) and the corresponding oxidized derivative (longer-lived component), respectively. In this case, the integrated intensity (I) was calculated from the wavelength integrated pre-exponential factors and A is the absorbance at 341 nm (emission maximum of the NanoLED used for excitation). A^{H_2Pt} and A^{Pt} were calculated using the Lambert-Beer equation:

$$A = \varepsilon l c \quad (3)$$

where ε is the known molar absorption coefficient at 341 nm, l is the optical pathlength of the fluorescence cell (1 cm) and c is the molar concentration in the sample, determined by HPLC. The results obtained (Table 1) are of the same order of magnitude as those for H_2Fop and Sep (*vide supra*) and, consequently, much lower than those of the corresponding oxidized derivatives ($\Phi_F^{Bip} = 0.36$, $\Phi_F^{Nep} = 0.38$, $\Phi_F^{Hmp} = 0.53$).[18,19]

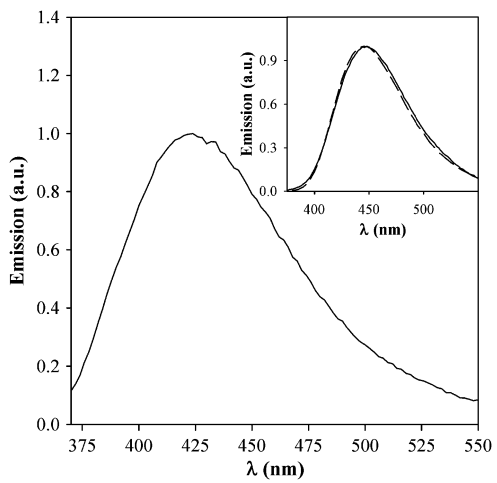

Fig. 5 Spectrum obtained by global analysis of TRES for the short-lived component in solutions of H_2Bip (17 μM, pH = 7.0, λ_{exc} = 341 nm). Inset: comparison between the spectrum obtained by global analysis of TRES for the longer-lived component in solutions of H_2Bip (solid line) and the unique species detected in global analysis of TRES of Bip solutions (dashed line).

6-Methyldihydropterin

In contrast to the compounds analyzed in the previous section, autooxidation of H_2Mep is rather fast ($t_{1/2}$ in air-equilibrated aqueous solutions being 6.5 h at 25 °C) and yields different products. In fact, the chemical reaction between dissolved O_2 and H_2Mep leads to oxidation through two reaction pathways: (a) oxidation of the dihydropyrazine ring yielding the aromatic pyrazine moiety (Mep) (~50%) and (b) oxidation and cleavage of the dihydropterin molecule to yield non-pterinic substances (~50%), whose spectra lack the characteristic absorption bands of pterins and dihydropterins in the UV-A region.[24] Therefore, a variation of the emission properties of the air-equilibrated solutions of H_2Mep with time was expected.

In fact, in steady-state experiments a significant increase in the emission of air-equilibrated solutions of H_2Mep was observed as a function of time. In contrast, such an increase was not observed when the H_2Mep solutions were bubbled with Ar immediately after preparation. This behavior can be interpreted as follows: in the presence of O_2 the oxidation of H_2Mep (poorly fluorescent) into Mep (highly fluorescent)

results in an increase in the total emission of the solution; on the other hand, in the absence of O_2, the chemical conversion does not take place and, consequently, the emission of the solutions does not change significantly with time.

A set of time-resolved experiments, similar to that described in the previous Section, was carried in O_2-free solutions of H_2Mep. The emission pattern observed was similar to that corresponding to H_2Bip, H_2Nep and H_2Hmp, *i.e.* two fluorescent components, the slower one being compatible with oxidized pterins. Global analysis of TRES allowed to unequivocally assign the longer-lived component to Mep and to obtain the emission spectrum (Fig. S2, ESI) and the τ_F value of H_2Mep (Table 1), which are, as expected, similar to those found for the other dihydropterins analyzed in the previous section. Likewise, Φ_F was calculated from eqn (2) and a value of $3(\pm 1) \times 10^{-3}$ was obtained.

In another set of time-resolved experiments, air-equilibrated H_2Mep solutions were analyzed at different times after preparation, under otherwise identical conditions. Global analysis of the results revealed that the concentration of the short-lived component decreased, whereas the concentration of the longer-lived component increased, as a function of irradiation time. This behavior was expected considering the conversion of H_2Mep into Mep and confirmed that the short-lived component corresponds to H_2Mep.

Conclusions

The fluorescence properties of six compounds of the family of dihydropterins (H_2Fop, Sep, H_2Bip, H_2Nep, H_2Hmp, and H_2Mep, Fig. 1) have been studied in aqueous solution at pH 6–7, using the single-photon-counting technique. A difficulty arose for these investigations as dihydropterins are more or less unstable in the presence of O_2 and their solutions are always contaminated with oxidized pterins, which have much higher fluorescence quantum yields (Φ_F). Fluorescence measurements were combined with HPLC analysis of the solutions to determine the degree of contamination.

Only in the case of H_2Fop and Sep that present particular spectral features (intense and broad absorption bands centered at about 425 nm, where oxidized pterins do not absorb), interference of the emission of oxidized pterins could be avoided. The fluorescence quantum yields (Φ_F), determined from the steady-state measurements, as well as the fluorescence lifetimes (τ_F) (Table 1), are much lower than those previously reported for unconjugated oxidized pterins.

On the other hand, the absorption spectra of H_2Bip, H_2Nep, H_2Hmp, and H_2Mep are partially superimposed to those of the corresponding oxidized derivatives. The emission decays were clearly biexponential with a short-lived component ($\tau_F < 1$ ns) and a longer-lived component (1 ns $< \tau_F <$ 5 ns). By means of global analysis of time-resolved emission spectra, and, taking into account previous and new results on oxidized pterins, the longer-lived component was assigned to the corresponding oxidized analogue, present in the solution as a contaminant. On the other hand, the short-lived components were assigned to the dihydropterins and their τ_F values were calculated. Taking advantage that the Φ_F values of the oxidized pterins are known, the Φ_F values of dihydropterins were obtained from global analysis data and values in the range $3–9 \times 10^{-3}$ were obtained.

The small Φ_F values and the short τ_F measured in our experiments reveal a fast non-radiative deactivation of the singlet excited states of dihydropterins. We have shown previously that a reaction (tautomerization, dimerization) may also occur from the S_1 state of some dihydropterins derivatives, but with low quantum yields of substrate consumption ($\Phi_{-H2Pt} = 5.3 \times 10^{-2}$, 3.8×10^{-2} and 10^{-2} for H_2Bip, H_2Nep and H_2Fop respectively).[29–31] Quantum yields of intersystem crossing (Φ_{ISC}) are most probably small, as oxygen does not affect significantly product formation. Therefore, we conclude that non-radiative decay by internal conversion most probably dominates deactivation of the S_1 state of dihydropterins.

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