ELSEVIER

Journal of Photochemistry and Photobiology
A: Chemistry 86 (1995) 209–217

Journal of PHOTOCHEMISTRY AND PHOTOBIOLOGY A:CHEMISTRY

Chiral discrimination in the fluorescence quenching of pyrene complexed to β-cyclodextrin

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Received 3 March 1994; accepted 2 August 1994

Abstract

Chiral discrimination was observed for the quenching by D- and L-tryptophan of excited singlet pyrene when complexed to β-CD in the presence of alcohols or short chain alkyl sulfates. Alcohols or alkyl sulfates were added to increase the association constant of pyrene with β-cyclodextrin. In all cases the quenching by D-tryptophan is more efficient than for L-tryptophan. The largest discrimination was observed in the presence of tert-butanol ($K_{sv}(D)/K_{sv}(L)) = 3.6$) owing to a marked decrease of the quenching efficiency by L-tryptophan. Quenching occurs through a static mechanism indicating that pyrene and tryptophan are in close proximity within the cyclodextrin complex.

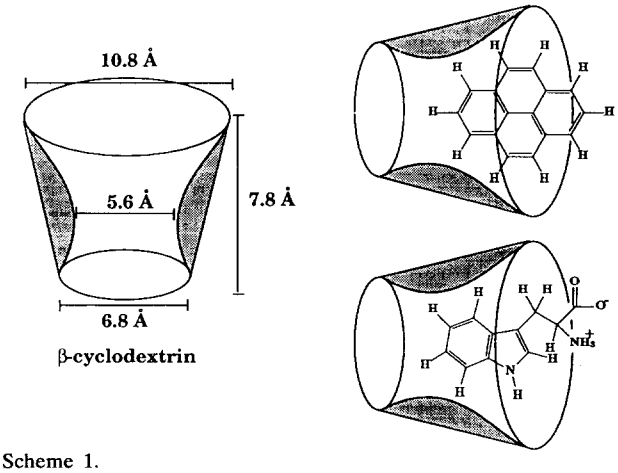
Keywords: Chiral discrimination; Fluorescence quenching; Pyrene; β-Cyclodextrin

1. Introduction

Structured microheterogeneous environments can significantly alter the chemical reactivity of a variety of processes. Some general mechanisms, such as the increase in local concentrations of reactants at interfaces, or higher reactivity through spatial confinement of the reaction have been firmly established [1,2]. However, the importance of specific interactions between reactants and structured environments has received much less attention. We chose to investigate specific interactions by selecting a molecular property, chirality, that requires a strict spatial arrangement between the molecules involved in the chemical process. A chiral host provides the chiral environment that influences the reaction between the complexed achiral guest and a chiral quencher molecule.

We chose β-cyclodextrin (β-CD) as the host molecule. CDs are cyclic oligosaccharides with 6(α), 7(β) or 8(γ) D-glucose units which have a doughnut shaped hydrophobic cavity. They have been popular host molecules and their complexation with a variety of compounds has been described over the last decades. The important feature for our experiments is that CDs provide a chiral environment. Complexation of guest molecules occurs within the hydrophobic cavity while the glucose hydroxyl groups at both entrances of the CD cavity can further stabilize complex formation through hydrogen bonds. A comparison of the size of β-CD with respect to pyrene and tryptophan, the molecules employed in our study, is shown in Scheme 1. The positioning of pyrene and tryptophan within the cavity is arbitrary and is included to emphasize the size relationship.

Photochemistry and photophysics have played an important role in investigating phenomena in constrained media as a result of the wide range of accessible time-domains (picosecond to millisecond) [1,2]. Chiral discrimination for quenching processes in homogeneous



Scheme 1.

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solution was described for different systems. In nonpolar solvents chiral discrimination was observed for the quenching of (R)-1, 1′-binaphthyl by different chiral amines [3]. Exciplex formation was suggested to be critical for this discrimination to occur. Weak chiral discrimination in pyrene excimer and exciplex formation was observed and it was enhanced in chiral solvents [4–6]. Steady-state and time-resolved (millisecond time-domain) circularly polarized luminescence were used to investigate chiral discrimination in the quenching of excited metal centered complexes with configurational chirality [7–11]. Chiral crown ethers with L- tryptophan moieties efficiently complexed cations, such as terbium (III). Once bound, Tb^{3+} quenched the tryptophan emission. The quenching efficiency of the L-crown was twice than that of the D-enantiomer [12]. In a further example, the dynamic quenching of an alcohol dehydrogenase cofactor (ϵNAD^+) by methionine showed a ratio of about 3 for the quenching efficiencies by L- and D-methionine [13]. These studies in organized systems show the potentiality of chiral environments to lead to chiral discrimination.

The photochemical reaction that we studied to assess the possibility of chiral discrimination was between an achiral excited state (pyrene) and a chiral quencher molecule (D- and L-tryptophan). Thus, no chiral discrimination can occur in the homogenous solvent (water). When pyrene is complexed to β-CD any chiral discrimination in the quenching process will be a result of the interaction of the host (β-CD) with tryptophan. Pyrene was chosen as the probe molecule because of its long singlet lifetime and the dependence of the fluorescence intensities of the different vibrational bands on solvent polarity [14,15]. To simplify the interpretation of the quenching data, pyrene should remain bound to β-CD during its excited state lifetime. Dissociation rate constants of aromatic molecules from cyclodextrin cavities vary considerably from 1×10^7 s^{-1} for excited triplet xanthone [16] to 5×10^4 s^{-1} and 1×10^5 s^{-1} for pyrene and naphthalene, respectively [17]. Thus, in the case of pyrene the excited state lifetime (<500 ns, $k>2\times10^6$ s^{-1}) is short enough to assure that no relocation will occur for the excited singlet state. As quencher molecules we chose D- and L-tryptophan as the quenching of excited singlet pyrene by these amino acids had been previously reported [18].

2. Experimental details

β-CD, a generous gift from American Maize Products was used as received. Pyrene was purchased from either Polysciences Inc. (high purity; used as received) or Aldrich (99%, recrystallized at least twice from ethanol). No impurities were detected by gas chromatography and the pyrene fluorescence decay in water was monoexponential ($t\geqslant193$ ns). 2-Butanol (2-BuOH) (99% Aldrich), tert-butanol (*t*-BuOH) (98% BDH), 1-butanol (1-BuOH) (>99.4% Anachemia), sodium 1-butylsulfate (C_4–SO_4) (Lancaster) and sodium 1-hexylsulfate (C_6–SO_4) (Lancaster) were used as received. D-Tryptophan (99+% Aldrich, 99% ICN), L-tryptophan (99% Aldrich, 99+% ICN, BDH) and DL-tryptophan (99+% Aldrich) were used without purification. A weak emission different from tryptophan fluorescence (excitation at 280 nm) was observed in the same spectral region as pyrene fluorescence when tryptophan was excited between 330 and 340 nm. The magnitude of this emission was small when compared to pyrene in water but was significant when compared to the small intensity differences in the quenching studies. The emission intensity was dependent on the source of tryptophan and we attributed it to an impurity. This impurity could not be detected by reversed-phase HPLC using UV detection (280, 300, 320 or 340 nm) on a Varian 5000 liquid chromatograph with a 2 mm 25×0.46 cm CSC-S ODS2 column (Eluents at pH 2.51; Gradients from 50% to 30% of B. A: 25 mM sodium hexasulfonate/25 mM triethylamine hydrochloride/water; and B: 25 mM sodium hexasulfonate/25 mM triethylamine hydrochloride/water/ethanol (1:9 v/v)). TLC plates (0.25 mm silica gel Polygram Sil G/UV_{254} plate) run with solvent mixtures suggested in the literature [19] for simple indole derivatives showed one spot. Recrystallization from ethanol/water did not decrease the impurity emission. The impurity emission was measured in blank experiments and subtracted from the pyrene fluorescence (vide infra).

Pyrene-β-CD complexes were prepared by addition of pyrene methanolic stock solutions (2–4 mM) to water ([pyrene]=0.5 μM) and addition of the appropriately weighed amount of CD. To ensure a constant pyrene concentration, tryptophan solutions were prepared by dissolving the solid in the same pyrene/CD solution which was employed to measure the fluorescence intensity in the absence of quencher. Tryptophan blank solutions were prepared by solubilization in CD/water solutions with CD concentrations equal to those in the pyrene solutions. For several experiments the tryptophan concentration was checked by measuring the value for ϵ at 279 nm. The standard deviation for the average ϵ at this wavelength (5600 M^{-1} cm^{-1}, 15 determinations) was below 2%. All solutions for the pyrene/β-CD/alcohol(or alkyl sulfate) system were prepared from the same aqueous stock solutions containing β-CD and alcohols or alkyl sulfates (third components). The following solutions were prepared: (i) Solution A was prepared by injecting the appropriate volume of a pyrene methanolic stock solution into the CD/third component solution ([pyrene]final=0.97 μM). This solution was employed to measure the emission intensity in the absence of quencher. (ii) A solution at the highest

tryptophan concentration employed was prepared by dissolving the solid with solution A. Several solutions at lower tryptophan concentrations were prepared by dilution. (iii) Blank solutions containing all components but pyrene were prepared by dissolving tryptophan into the β-CD/third component stock solution. All samples were left to equilibrate for at least 2 hours in the dark.

Two different quenching methods were used. (i) In the addition method small aliquots of a concentrated tryptophan solution were added to a pyrene-CD solution. The decrease of the fluorescence intensity (integrated area between 365 and 400 nm or intensity value at 400 nm) was measured for each tryptophan concentration. (ii) For high tryptophan concentrations the dilution method was employed as tryptophan is not very soluble in water. The pyrene emission was measured at different tryptophan concentrations prepared by dilution. The emission for the blank containing tryptophan and all other components but pyrene was measured at each tryptophan concentration and was subtracted from the integrated pyrene emission intensity (365–400 nm). The tryptophan emission was always smaller than 15% of the total pyrene emission. The quenching experiments for pyrene in water and pyrene–CD complexes were performed in solutions deaerated by bubbling nitrogen for 15 minutes, whereas in the presence of third components, aerated solutions were employed. A standard was used to correct for drift in the excitation intensity of the fluorimeter.

The absorption of pyrene at the excitation wavelength (340 nm) was less than 0.06. Tryptophan absorbed at the same wavelength and at high tryptophan concentrations the amount of light absorbed by pyrene decreased leading to a decrease of the pyrene emission intensity that is not related to quenching. This inner filter effect was corrected for by calculating the decrease of the excitation light intensity absorbed by pyrene in the presence of tryptophan. The excitation and detection monochromators in the fluorimeter are at a 90° angle and the sample is irradiated at the center of the cell. With an excitation slit of 2.0 nm the width of the irradiated area on the cell was 0.2 cm. For each experiment the fraction of absorbed light ($1-10^{-A}$, where A is the absorbance) between 0.4 cm and 0.6 cm was calculated for pyrene in the absence (f_0^{abs}) and presence (f^{abs}) of the highest tryptophan concentration employed. The fractional decrease ($\Delta f = (f_0^{abs} - f^{abs})/f^{abs}$) due to the inner filter effect normalized for the tryptophan concentration (Δf/[tryptophan]) corresponds to the contribution of this artifact to the measured slope in the quenching plot. In order to obtain the values for the Stern-Volmer constants the value for Δf/[tryptophan] was subtracted from the slope of the quenching plot. We simulated the inner filter effect by placing a second cell containing tryptophan between the excitation beam and the cell containing pyrene. The decrease of the pyrene emission intensity observed was within 10% of the calculated value. The contribution of Δf/[tryptophan] to the measured slope was more prominent for low quenching efficiencies and was generally smaller than 30%.

Steady-state fluorescence spectra were acquired with a Perkin Elmer MPF 66 fluorimeter. The samples were kept at 20.0±0.5 °C (Haake F 3 circulating bath). The excitation and emission slits were set at 2.0 nm. When necessary, the fluorescence intensity was decreased by attenuating the excitation beam with neutral density filters. The intensity ratio of the pyrene fluorescence for vibrational bands (I/III ratio) has been shown to be sensitive to solvent polarity [14,15]. Slightly different values were reported for the I/III ratio in different solvents. For this reason we determined I/III ratios for pyrene (0.5 μM) in different solvents under our experimental conditions (representative values are 0.62 in cyclohexane, 1.04 in tert-butanol, 1.28 in ethanol, 1.94 in water).

Lifetime measurements were performed on a PTI LS-1 time-correlated single photon counter (20±4 °C, for experiments with pyrene in water and complexed to β-CD and 20.0±0.5 °C (RM6 Lauda circulator) for complexes with β-CD/alcohols or alkylsulfates). Water containing silica gel or small amounts of milk was employed as the scattering solution to determine the instrument response function. All decays were recorded for a duration of at least 5 lifetimes. Visual analysis of residuals, the autocorrelation, χ^2 values and DW (Durbin-Watson) parameters were used as criteria for fitting and to differentiate between mono- and biexponential decays. Typical values of χ^2 are between 0.9 and 1.3 and DW>1.70 and >1.75 for mono- and biexponential fits, respectively [20]. UV–Vis spectra were recorded on a Varian Cary 5, a Cary 1 or a Philips PU8740 spectrophotometer at room temperature. The Cary 5 was employed for difference spectra.

3. Results

3.1. Characterization of pyrene–cyclodextrin complexes

Complexation of pyrene with β-CD has been extensively studied by several groups but some controversy still existed as to the stoichiometry of the complex. Several different spectroscopic techniques, such as absorption, steady-state and time-resolved fluorescence were employed to determine the values for the equilibrium constants between pyrene and β-CD. The reported values for a 1:1 pyrene/β-CD complex range from 7.6 M^{-1} to 277 M^{-1} [17,21–23]. A recent study demonstrated the sequential formation of pyrene/β-CD complexes with 1:1 and 1:2 stoichiometries. The estimated range for the equilibrium constant of the 1:1

complex was 120–260 M^{-1} and the ratio between the equilibrium constants for the 1:2 and 1:1 complexes was determined to be between 0.5 and 2 [24]. These values are similar to those determined in an earlier less extensive study [25] that also considered the sequential formation of pyrene/β-CD complexes. The association constant of pyrene to β-CD can be enhanced in the presence of alcohols or short-chain alkyl sulfates that co-include with pyrene into the CD cavity [17,21,26]. A 1:1:1 stoichiometry was suggested for the complex in the presence of alkylsulfates and the equilibrium constants for pyrene and β-CD/alkylsulfates were 3000 and 1400 M^{-1} for 1-butylsulfate and 1-hexylsulfate, respectively [17]. Two different stoichiometries (1:1:1 and 1:2:2 pyrene:β-CD:alcohol) were proposed for the complex in the presence of alcohols [21,26]. It is important for the interpretation of our quenching data to establish the ratio of pyrene complexed to CD. Owing to the discrepancy of the equilibrium constant values for the pyrene β-CD complexation we determined the degree of binding under our experimental conditions. The experimental methodology employed is similar to that reported previously [17,21,27] and for this reason only the pertinent results for our studies will be mentioned.

In the presence of β-CD a new peak at longer wavelengths was observed in the UV–Vis absorption and fluorescence excitation spectra. Fluorescence spectra and decay traces were obtained by excitation at 337 nm, the isosbestic point between free and complexed pyrene. The fluorescence decay of pyrene in the presence of 12.5 mM β-CD was fitted to the sum of two exponentials ($A_1 = 0.31$, $\tau_1 = 128$ ns, $A_2 = 0.69$, $\tau_2 = 288$ ns). The short lived component corresponds to free pyrene and pyrene–β-CD complex with a 1:1 stoichiometry whereas the long lived component corresponds to pyrene–β-CD with a 1:2 stoichiometry [24]. The degree of binding of pyrene to β-CD as a 1:2 complex was calculated from the pre-exponential factors assuming that the ratio of radiative rate constants were inversely proportional to the ratio of lifetimes in the absence of oxygen:

$$\frac{[\text{pyrene-}(\beta\text{-CD})_2]}{[\text{pyrene}] + [\text{pyrene-}\beta\text{-CD}]} = \frac{A_2}{A_1}\frac{\epsilon_1}{\epsilon_2}\frac{\tau_2}{\tau_1} \quad (1)$$

The ratio of absorption coefficients is unity as pyrene was excited at the isosbestic point. 78% of the pyrene was complexed to β-CD with a 1:2 stoichiometry whereas 22% of the pyrene was free in solution or bound as a 1:1 complex. These values are within the range of the recently published equilibrium constants [24].

To enhance the pyrene association to β-CD we added alcohols or alkylsulfates as ternary complexation agents [17,21,26]. Pronounced changes in the absorption and fluorescence excitation spectra were observed when pyrene was complexed to β-CD in the presence of alcohols (1-BuOH, 2-BuOH and *t*-BuOH) or alkylsulfates (C_4–SO_4 and C_6–SO_4). The pyrene absorption maximum shifted from 335 nm in water to 339 nm. This shift was similar to that observed for pyrene/β-CD in the presence of pentanol [21]. It was crucial for our quenching experiments (vide infra) to establish the degree of complexed pyrene. Based on the published values for the equilibrium constants [17,21,26] more than 90% of the pyrene should be complexed under our experimental conditions. To verify that this was indeed the case, we determined the pyrene fluorescence lifetimes for different experimental conditions (Table 1). The pyrene (< 0.5 μM) fluorescence decay in water was monoexponential and the lifetime observed (Table 1) in the absence of oxygen was comparable to that in the literature [17]. In the presence of 10 mM β-CD the pyrene emission decay was fitted to the sum of two exponentials (vide infra). In the presence of 20 mM of any alcohol or alkyl sulfate the fluorescence decay was monoexponential and the lifetime increased to values in excess of 450 ns (Table 1). Decay traces were fitted to both, a single exponential decay and to the sum of two exponentials. No improvement was observed in the latter case. This result indicates that all pyrene is complexed. To increase the signal to noise ratio experiments with CD and alcohols or alkyl sulfates were performed with 0.97 μM pyrene. In all cases the fluorescence decay was monoexponential and no excimer emission was observed at 480 nm. Although previously observed [17,28,29], it is important to mention the remarkable protective effect that β-CD has on the excited states of included molecules. Whereas in homogeneous solution oxygen decreases the pyrene lifetime by a factor of 1.5, the decrease is much smaller (< 1.1) when pyrene is included in β-CD and pyrene is completely protected from oxygen quenching when complexed to β-CD in the presence of alcohols and alkyl sulfates.

The fluorescence intensity ratio between the first and third vibrational bands (I/III ratios) of pyrene monomer emission is very sensitive to the polarity of the pyrene environment [14,15]. This parameter was employed in our studies to check the average polarity sensed by pyrene molecules at different quencher concentrations (vide infra). I/III ratios for pyrene in solution and complexed to CD are shown in Table 1. The value for pyrene in the presence of only β-CD does correspond to a combined value for all pyrene species, i.e. free in water and for β-CD complexes with 1:1 and 1:2 stoichiometries. The presence of pyrene in more than one environment also explains the dependence of the I/III ratio on the excitation wavelength and β-CD concentration. In the presence of alcohols or alkyl sulfates the free pyrene concentration is negligible and the I/III ratio corresponds to the polarity sensed by pyrene in the ternary complex. The I/III ratios for complexed

Table 1
Excited singlet pyrene lifetimes and I/III emission intensity ratios in water and when complexed to β-CD in the absence and presence of alcohols and alkyl sulfates

	I/III	I/III-literature values	τ(ns)-under N_2	τ(ns)-air
Water [a]	1.94	1.87 (ref. 14)	193	128
β-CD [a–c]	1.01	1.17 (ref. 26)	311 [c]	288 [c]
		1.03 (ref. 17)		
β-CD–1−BuOH [d]	0.63	0.56 (ref. 26)	456	448
β-CD–2BuOH [d]	0.72		452	447
β-CD–t-BuOH [d]	0.81	0.70 (ref. 26)	453	447
β-CD–C_4-SO_4 [d]	0.72	0.71 (ref. 17)	457	447
β-CD–C_6SO_4 [d]	0.59	0.59 (ref. 17)	468	457

[a] [pyrene] ≈ 0.5 μM.
[b] [β-CD] = 12.5 mM.
[c] Double exponential decay; the short lifetime corresponds to pyrene in water (fixed at 193 and 128, in the presence of nitrogen and air, respectively); the lifetime shown corresponds to pyrene complexed to β-CD.
[d] [β-CD] = 13 mM, [pyrene] = 0.97 μM and [alcohol or alkyl sulfate] = 20 mM.

pyrene correspond to polarities in non-polar environments, e.g. 0.59 (C_6–SO_4) corresponds to the polarity of cyclohexane. This suggests the exclusion of water from the surroundings of pyrene. Our values for the I/III ratios are in good agreement with the literature values for complexes with alkyl sulfates [17] and somewhat higher than the values published in the presence of alcohols [26].

3.2. Quenching of excited singlet pyrene in water and when complexed to β-CD

Tryptophan efficiently quenches pyrene fluorescence ($2.2\times10\ M^{-1}\ s^{-1}$, pH independent) probably through a charge-transfer mechanism [18]. This suggests that a close contact between pyrene and tryptophan is necessary for deactivation of the excited state. It was important for us to choose a quenching process that does not involve any long-range deactivation mechanism as no chiral discrimination was expected for long-range processes.

Two quenching mechanisms, dynamic and static quenching, should be considered. Dynamic quenching is due to the diffusion of the quencher to the excited state within the CD cavity and this process yields information on the dynamics (mobility) of the quencher through the chiral matrix (β-CD). The second mechanism (static quenching) involves a complex containing pyrene, β-CD and tryptophan prior to excitation of the probe and no dynamic information can be obtained. However, static quenching provides information on the relative positioning of probe and quencher within the chiral host molecule.

Quenching efficiencies were determined by measuring the decrease of steady-state fluorescence intensities and fluorescence lifetimes with increasing quencher concentrations. For dynamic quenching the data were treated according to the Stern-Volmer formalism (Eq. 2):

$$\frac{I_0}{I} = \frac{\tau_0}{\tau} = 1 + K_{sv}[\mathrm{Trp}] \quad (2)$$

where I_0 and I are the fluorescence intensities in the absence and presence of quencher, τ_0 and τ are the pyrene fluorescence lifetimes in the absence and presence of quencher, and K_{sv} is the Stern-Volmer constant which is equal to the product of the quenching rate constant and the lifetime in the absence of quencher.

In a process involving dynamic quenching, equal values of I_0/I and τ_0/τ are obtained at each quencher concentration. In the static mechanism the quenching process does not require the diffusion of the reaction partners. A fraction of the excited states is immediately removed upon excitation leading to a decrease of the observed steady-state fluorescence intensity. However, the fluorescence lifetime is constant as excited states not complexed to quencher molecules are not being quenched. Thus, τ_0/τ is equal to unity and K_{sv} in Eq. (2) corresponds to an effective association constant of the complex [30].

No complexation between pyrene and tryptophan was observed in aqueous solution [18]. We measured the decrease of the pyrene fluorescence in water in the presence of tryptophan (Fig. 1) and the rate constants for quenching were determined by employing Eq. (2). Equal I_0/I and τ_0/τ values were obtained. The fluorescence decay was monitored at 400 nm. In the presence of tryptophan a very fast decay due to tryptophan emission was observed. This fast decay was not included when curve fitting the data. As expected the same quenching efficiency was observed for both enantiomers of tryptophan. Average k_q values obtained were $(4.9\pm0.4)\times10^9\ M^{-1}\ s^{-1}$ (13 determinations), $(4.9\pm0.6)\times10^9\ M^{-1}\ s^{-1}$ (5 determinations) and $(5.1\pm0.5)\times10^9\ M^{-1}\ s^{-1}$ (3 determinations) for L-, D- and D/L-tryptophan, respectively. The I/III ratio for pyrene emission was constant throughout the experi-

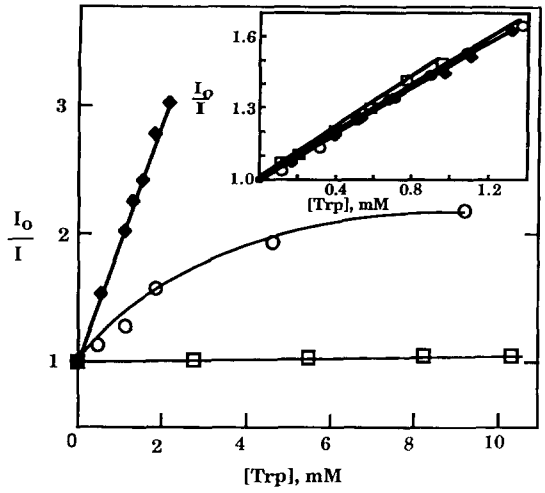

Fig. 1. Quenching plots for excited singlet pyrene by tryptophan in water (♦), in the presence of 10 mM β-CD (○) and in the presence of 13 mM β-CD and 20 mM tert-butanol (□). The inset shows the quenching plot for pyrene in the presence of 10 mM β-CD by (□)L-, (○)D- and (♦)D/L-tryptophan.

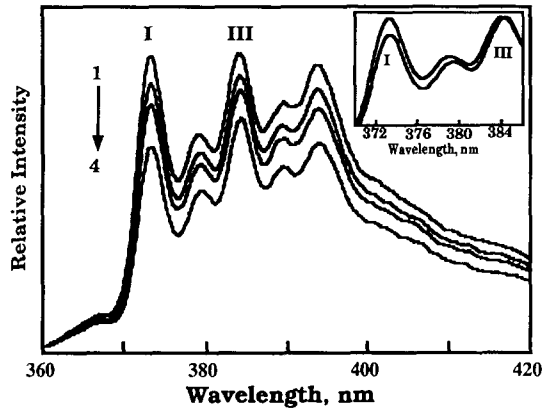

Fig. 2. Fluorescence spectra of pyrene (0.5 μM)/β-CD(10 mM) with increasing L-tryptophan concentrations: **1**, 0 M; **2** 0.3 mM; **3** 0.5 mM and **4** 1.0 mM. The inset shows the change in the I/III intensity ratio when 1.0 mM of tryptophan was added.

ment. Our k_q values were higher by a factor of 2 when compared to those previously published [18]. We obtained the same quenching rate constant for several sources of tryptophan including that employed in the previous report and we do not have a rationale for the observed difference.

Pyrene in the presence of β-CD was excited at the isosbestic point (337 nm) to ensure equal excitation efficiencies for free and CD-complexed pyrene. The decrease of the fluorescence emission intensity is shown in Fig. 2. The Stern-Volmer plot for pyrene quenching by tryptophan in the presence of 10 mM β-CD (Fig. 1) was initially linear but leveled off at higher quencher concentrations. This behavior suggests that pyrene in at least two different environments was being quenched. The decrease of the quenching efficiency at high quencher concentrations indicates that complexation of pyrene to CD protects the excited state from deactivation by tryptophan. We assigned the process at high tryptophan concentrations to the quenching of pyrene–β-CD complexes with a 1:2 stoichiometry. At low tryptophan concentrations pyrene in water and probably pyrene complexed to β-CD with a 1:1 stoichiometry were quenched. No chiral discrimination was observed for the quenching processes at low tryptophan concentrations (inset Fig. 1).

A careful analysis of the I/III intensity ratios in the presence of tryptophan substantiates the qualitative picture drawn above. We mentioned earlier that the I/III ratio measured for pyrene in the presence of β-CD corresponds to the combination of the values for pyrene in solution and complexed to β-CD. At low tryptophan concentrations we primarily quenched those pyrene molecules that were involved in processes with high quenching efficiency. Thus, with increasing tryptophan concentration the relative contribution of the pyrene complexed to β-CD with a 1:2 stoichiometry which has a lower value for the I/III ratio increased. For this reason a decrease of the I/III ratio was observed when the tryptophan concentration increased (Fig. 3). At high tryptophan concentrations (>6 mM) the I/III ratio levels off suggesting that only one pyrene species remains unquenched.

In the presence of β-CD three pyrene species were present and most of the pyrene quenching was due to pyrene in water and possibly for the pyrene–β-CD as a 1:1 complex. In addition, the pyrene emission intensity increased at low tryptophan concentrations when pyrene was excited at 340 nm, a wavelength where the complexed pyrene had a much higher absorption coefficient than the aqueous pyrene. This indicates that the equilibrium constant between pyrene and β-CD increased in the presence of tryptophan. Indeed, tryptophan and other amino acids have been shown to act as zwitterionic ternary complexation agents [31] in a manner similar

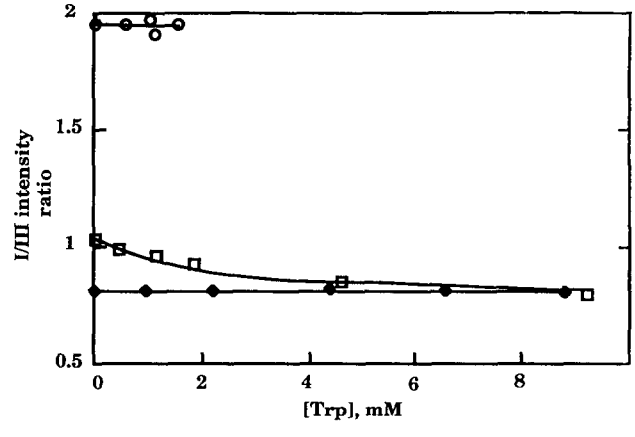

Fig. 3. Dependence of the pyrene I/III emission intensity ratio with increasing concentrations of tryptophan in the absence (○) and presence of (□) 10 mM β-CD or (♦) 13 mM β-CD/20 mM *t*-BuOH.

to alcohols and alkyl sulfates. This extra complexity makes it extremely difficult to analyze quantitatively the quenching of complexed pyrene and to determine if chiral discrimination occurs.

3.3. Quenching of excited singlet pyrene β-CD complexes in the presence of alcohols or alkyl sulfates

Conditions where most of the pyrene was complexed (vide supra) were chosen to avoid the complexity of having to analyze for quenching of several excited pyrene species. The tryptophan quenching efficiency for β-CD complexed pyrene in the presence of alcohols or alkyl sulfates decreased dramatically when compared to quenching in the presence of only β-CD (Fig. 1). The low quenching efficiency required the use of high tryptophan concentrations and corrections for tryptophan impurity emission and inner filter effects were performed (vide supra).

Typical Stern-Volmer plots are shown in Fig. 4 and the corrected K_{sv} values are given in Table 2. The chiral discrimination was fairly small in the presence of linear alcohols or alkyl sulfates but was significantly larger for a bulky molecule such as tert-butanol. The magnitude

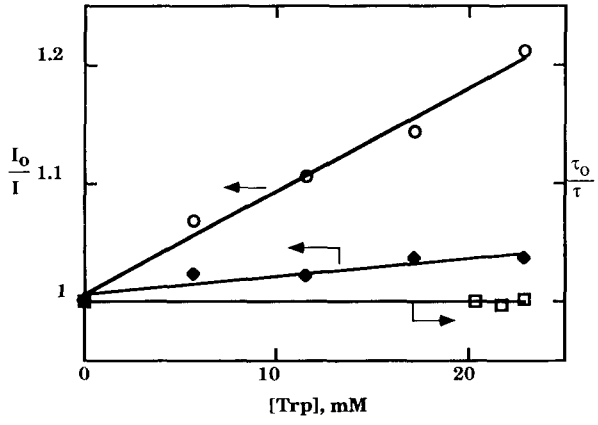

Fig. 4. Quenching plot for the corrected steady-state emission intensity decrease of singlet pyrene/β-CD/t-BuOH by (○) D- and (◆) L-tryptophan. (□) represents the lifetime ratio in the absence and presence of L-tryptophan.

Table 2
Stern-Volmer constants (M^{-1}) and ratio of constants for the quenching of pyrene (0.97 μM) complexed to β-CD (13 mM) in the presence of alcohols or alkyl sulfates (third component, 20 mM) by D- and L-tryptophan (Trp). The number in parenthesis indicates the number of independent experiments performed

Third component	D-Trp	L-Trp	$\frac{K_{sv}\text{ (D-Trp)}}{K_{sv}\text{ (L-Trp)}}$
t-BuOH	5±1(6)	1.4±0.7(4)	3.6
2-BuOH	4.0±0.4(3)	2.3±0.3(3)	1.7
1-BuOH	5±1(3)	2.6±0.1(3)	1.9
C_4–SO_4	5±2(4)	4±1(7)	1.3
C_6–SO_4	3.2±0.5(3)	1.6±0.3(3)	2.0

of the chiral discrimination observed was similar to quenching studies involving crown ethers [12] or enzymes [13]. Our relatively large errors reflect the small intensity decreases being measured. The smallest I_0/I value that we could measure after corrections was 1.02. The Stern-Volmer constant for the quenching in the presence of t-BuOH and C_6–SO_4 by L-tryptophan were 1.4 M^{-1} and 1.6 M^{-1}, respectively. These values were very close to our detection limit as they correspond to I_0/I values at the highest tryptophan concentration (30 mM) employed of 1.04 and 1.05, respectively. Most of our quenching experiments were performed in pairs with samples for D- and L-tryptophan being prepared in parallel from the same stock solutions. In all instances we observed a higher quenching efficiency for D-tryptophan than for L-tryptophan, although the absolute values for K_{sv} varied between experiments. This fact gives us confidence that even in cases (pyrene/β-CD/C_4–SO_4) where the K_{sv} values for both enantiomers are the same within the experimental error chiral discrimination did occur. Quenching of pyrene/β-CD/t-BuOH by D/L-tryptophan was also studied but this experiment has intrinsically much higher errors because of the lower solubility of the racemate. For this reason we cannot determine if the value obtained (4.5 M^{-1}) is higher than the expected average (3.2 M^{-1}) for the quenching by D- and L-tryptophan.

Contrary to the experiments in presence of only β-CD the I/III ratio stayed constant with the addition of tryptophan (Fig. 3), suggesting that pyrene inside the CD cavity was quenched. The constant value of the I/III ratio also indicated that proper corrections for the emission of tryptophan were performed. A systematic error in the correction would lead to systematic changes in the I/III ratio due to the contribution of the broad tryptophan emission to the well resolved pyrene fluorescence.

The same lifetime for pyrene in the absence and presence of D- or L-tryptophan ($\tau_0/\tau = 1$, Fig. 4) was obtained, indicating that only static quenching occurred. This result eliminates any dynamic quenching involving the tryptophan from the aqueous phase. Thus, pyrene and tryptophan have to form a complex within the cyclodextrin cavity prior to excitation of pyrene. However, we cannot rule out the relocation of tryptophan within the CD cavity as this phenomenon is expected to occur in the picosecond time-domain and cannot be measured with our time-resolved single photon counter (time resolution of ca. 1 ns).

4. Discussion

The mode of pyrene and tryptophan inclusion inside the CD cavity and the mechanism for interaction are important in order to understand the chiral discrimi-

nation observed for the quenching process. CPK models [32] and molecular modeling [24] of CD-pyrene complexes showed that pyrene does not completely fit within the β-CD cavity. This leads to the formation of a pyrene β-CD complex with 1:2 stoichiometry at high CD concentrations. In the absence of alcohols or alkyl sulfates three pyrene species are present in solution, i.e. free pyrene and pyrene/β-CD complexes with 1:1 and 1:2 stoichiometries. The quenching plot is initially linear and than levels off at higher tryptophan concentrations. The linear region corresponds to the quenching of free pyrene and complexed pyrene with a 1:1 stoichiometry. No chiral discrimination was observed for this linear region suggesting that no chiral discrimination occurs for the quenching of pyrene in a 1:1 complex with β-CD. This can be explained by the fact that part of the pyrene molecule protrudes from the CD cavity and tryptophan will not have to interact strongly with the CD cavity for quenching to occur. At high tryptophan concentrations, when a plateau region is observed in the quenching plot, all free pyrene and pyrene complexed to CD with a 1:1 stoichiometry have been quenched and further quenching is due to pyrene/β-CD complexes with a 1:2 stoichiometry. In principle we should have observed the same chiral discrimination determined in the presence of alcohols or alkyl sulfates. However, we observed that tryptophan also enhances the equilibrium constant between pyrene and β-CD. This observation suggests that the quenching in the plateau region probably occurs through a static mechanism as for the tryptophan quenching in the presence of alcohols or alkyl sulfates. Unfortunately this enhancement of the pyrene β-CD complexation with addition of tryptophan precludes any quantitative analysis of the quenching in the plateau region. For this reason the assessment of chiral discrimination was investigated in the presence of alcohols or alkyl sulfates when all the pyrene is bound to β-CD before the addition of any tryptophan.

One possibility for the quenching of complexed pyrene by tryptophan is a complex in which the tryptophan is included inside the CD cavity and pyrene is capping one of the entrances. This mode of interaction was suggested for the static quenching of pyrene/β-CD by amines [33]. We exclude this possibility as the low I/III ratios for the complexes in the presence of alcohols or alkyl sulfates and the protection from oxygen quenching indicate that pyrene is included within the CD cavity and the third component is protecting the excited state from interaction with molecules residing in the homogeneous solvent. Shielding effects by alcohols were previously observed for the quenching of triplet 1-bromonaphthalene by oxygen when included in glucosyl-β-CD [34] and the iodide quenching of 1-cyanonaphthalene in β-CD [35]. For static quenching to occur tryptophan has to be located in the vicinity of pyrene within the same CD complex. Due to the dimensions of pyrene and tryptophan the complex probably involves two CDs. Indeed, preliminary studies on the enhancement of the association constant between pyrene and β-CD by amino acids suggests a 1:2 stoichiometry for pyrene and CD [31]. The important point to emphasize is that regardless of the stoichiometry of the complex the chiral discrimination observed is due to the different complexation pattern of D- and L-tryptophan with β-CD.

A detailed NMR study complemented with molecular modeling calculations described the enantioselective binding of tryptophan to α-CD [36]. This study suggested that the inclusion mode of both enantiomers is similar (indole moiety partially included inside the cavity and hydrogen bonding to hydroxyl groups) but there is a much higher number of hydrogen bonds being formed with the R-enantiomer (D-tryptophan). This is reflected in the stronger binding of D-tryptophan when compared to its enantiomer. In addition, the orientation of the indole moiety within the CD cavity is slightly different for both enantiomers. No detailed analysis has been performed for β-CD but the equilibrium constants of both enantiomers of tryptophan with β-CD have been estimated at pH 8.9 and 12 °C. The values are (8 ± 1) and (9 ± 1) M^{-1} for L- and D-tryptophan, respectively [37]. One possibility for the chiral discrimination observed in our quenching experiments is that the binding constant for both enantiomers is different. Based on the values above differential binding could account for a ratio of 1.1 between the K_{sv} values for D- and L-tryptophan. This value is much lower than the discrimination observed (Table 2). We do not believe that achiral molecules, such as alcohols and alkyl sulfates can affect the differential binding of the tryptophan enantiomers to β-CD to such an extent as to lead to quenching efficiency ratios (K_{sv}(D)/K_{sv}(L)) higher than 2. Thus, more subtle differences in the relative positioning of tryptophan with respect to pyrene could account for the discrimination observed.

The quenching mechanism of singlet pyrene by tryptophan has not been firmly established, but charge transfer interactions were proposed [18]. The indole moiety of tryptophan is responsible for the quenching as the quenching behavior of tryptophan is very similar to that of indole [18,38] and other amino acids do not quench singlet pyrene. A charge transfer mechanism is also supported by the observation of exciplex emission for the interaction of pyrene derivatives with 1, 2-dimethylindole [39]. For static quenching to occur through a charge transfer mechanism the pyrene and indole moieties have to be in close proximity. In all cases, we observe quenching by both enantiomers indicating that the indole group is included in the CD cavity. Chiral discrimination could be due to the different relative location of the tryptophan indole moiety with

respect to the pyrene π system. Evidence for different location of the indole ring within CD cavities has been described for the complexation of this amino acid with α-CD [36].

For all alcohols or alkyl sulfates but C_6–SO_4 the quenching efficiency by D-tryptophan is the same within experimental error (Table 2). However, the quenching efficiency by L-tryptophan is more sensitive to the structure of the alcohol or alkyl sulfate. This suggests that the relative positioning between pyrene and the indole is changed more easily for the enantiomer (L-tryptophan) that has a less favorable interaction with the CD cavity.

In summary, the chiral discrimination in the quenching by D- or L-tryptophan of excited singlet pyrene complexed to β-CD/alcohols or alkyl sulfates occurs through a static mechanism which requires tryptophan to be in close proximity to pyrene. The major effect leading to chiral discrimination is probably the different relative positioning of the indole ring with respect to pyrene although differential binding efficiencies of tryptophan to CD may also play a minor role. The structure of the added alcohols or alkyl sulfates has a marked effect on the quenching efficiency of L-tryptophan; the enantiomer that has a weaker complexation to the β-CD cavity. These results confirm that chiral environments can induce chiral discrimination for a reaction where only one of the reactants is chiral. We are currently searching for other chiral quenchers with higher quenching efficiencies so that detailed issues such as stoichiometry of complexation and the effect of the structure of added alcohols and alkyl sulfates on the chiral discrimination can be investigated.

Acknowledgments

This research was supported by the Natural Sciences and Engineering Research Council of Canada. The University of Victoria is also gratefully acknowledged for providing set up funds.

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