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Spectral Properties, Cation Selectivity, and Dynamic Efficiency of Fluorescent Alkali Ion Indicators in Aqueous Solution Around Neutral pH

Pierre Buet,[1] Bernd Gersch,[1] and Ernst Grell[1,2]

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The spectral properties of two fluorescent alkali ion indicators, the commercially available cryptand CD222 and a new bipyridyl-type cryptand, F[bpy.bpy.2], bearing the trifluorocoumarino residue are investigated in aqueous solution as a function of pH as well as around neutral pH in the presence of alkali and alkaline earth cations. From the values of the acidity constants it is concluded that bridgehead nitrogen deprotonation occurs at a much lower pH for CD222 (pK_a below 5.5) than for F[bpy.bpy.2]. Spectrofluorometric titrations with salts of NH_4^+, TI^+, and alkali as well as alkaline earth cations indicate that both indicators are K^+ selective. F[bpy.bpy.2] shows the higher K^+/Na^+ selectivity and larger fluorescence intensity changes but the slower dynamic response. Under suitable conditions, alkali ion binding by CD222 can occur in less than 1 ms.

KEY WORDS: Alkali ion indicator; CD222; fluorescent cryptands; stability constants; dynamics of cation binding.

INTRODUCTION

Suitable alkali ion indicators are demanded for the fast, time-resolved, and selective recording of alkali ion fluxes in cellular systems which are not accessible to ion-selective microelectrodes or sensor systems [1]. Fluorescent ligands consisting of a fluorophore attached to a cation coordinating molecular unit, for example, for alkaline earth ions, have proven to be applicable as indispensable indicator systems [2]. To be applicable as a biological indicator, the probe molecule is expected to exhibit, besides sufficient water solubility, (i) a high affinity at neutral pH to permit detection of low cation concentrations, (ii) a high selectivity to enable detection among other cations, (iii) a high quantum yield and an excitation wavelength above 300 nm, (iv) a marked fluorescence change upon cation binding, and (v) fast binding kinetics to allow time-resolved studies, also in small volumes.

Concerning alkali ions, different structure modifications of the parent cryptands [2.2.1] and [2.2.2] [3], known for their Na^+ and K^+ selectivity, respectively, as well as on the basis of crown ethers [4], have been undertaken to develop fluorescent ligands of a high affinity for alkali ions at neutral pH. A typical example is the commercially available indicator CD222, a cryptand originally synthesized by Crossley *et al.* [5]. Its structure consists of two carboxycoumarino residues in one bridge of the cryptand (Fig. 1), but its properties have not yet been characterized in great detail. The aromatic residue next to a bridgehead nitrogen will reduce its basicity as well as the bridge flexibility. Besides CD222, the new fluorescent cryptand F[bpy.bpy.2] (Fig. 1), a modified ligand of the recently described cryptand F222 [6,7], is introduced here. It is hoped that the bipyridyl bridges will provide an increased rigidity and a higher degree of preorganization, compared to [2.2.2] as well as CD222,

[1] Max-Planck-Institute of Biophysics, Kennedy Allee 70, D-60596 Frankfurt, Germany.
[2] To whom correspondence should be addressed. Fax: +4969 6303346. E-mail: ernst.grell@mpibp-frankfurt.mpg.de.

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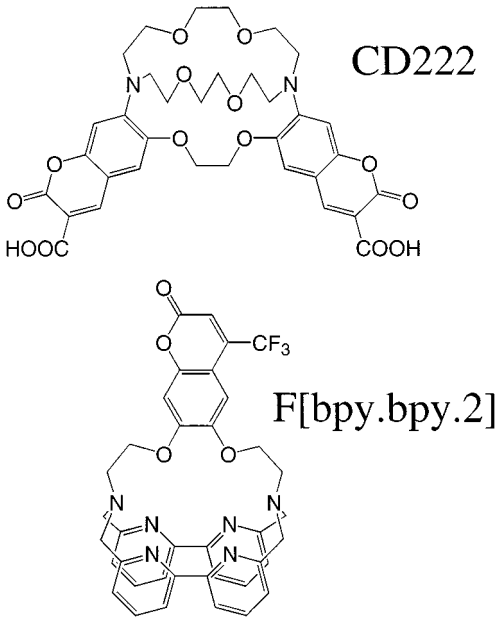

Fig. 1. Chemical structure of the fluorescent cryptands CD222 and F[bpy.bpy.2].

and will thus lead to a higher alkali ion affinity. In this contribution we report the spectral properties and the alkali as well as alkaline earth ion binding affinities of these two ligands at different pH values together with dynamic aspects of cation binding [8].

EXPERIMENTAL

Materials

Chemicals. General chemicals were of analytical grade, supplied by *Fluka* or *Merck*. Choline chloride (ChCl), BisTris, imidazole, and Tris, all of grade *Biochemica* MicroSelect, and *N*-ethylmorpholine (Nem), of grade *BioChemica*, were purchased from *Fluka*. 5,5-Diethylbarbituric acid (Veronal; *Merck*) was recrystallized from water; tetramethylammonium hydroxide (TMAOH; *Fluka*), from ethanol/diethyl ether. Aqueous solutions were prepared with Milli-Q (*Millipore*) filtered water (>17 MΩ/cm) in LDPE (low-density polyethylene) flasks, and the pH adjustment was done without contacting the buffer solution used for the measurements with the combined electrode to minimize trace contents of potassium.

CD222. 19^2,24^2-Dioxo-4,7,13,16,20,23,-hexaoxa-1,10-diaza-19(7,6),24(6,7)-di(2H-2-benzopyrana)bicyclo[8.8.6]tetracosaphane-19^3,24^3-dicarboxylic acid (CD222; *Molecular Probes*), was used without further purification. A test realized on a silica gel $60WF_{254}$s-precoated TLC sheet (*Merck*) using the eluent *n*-butanol/H_2O/pyridine/acetic acid (50/20/15/15, v/v/v/v) provided evidence for a main component (at least about 90% according to the analytical characterization by *Molecular Probes*) with an R_f value of 0.32 and a minor contamination with an R_f of 0.19. Stock solutions (about 3 m*M*) were prepared by dissolving the ≅1.0 mg of the ligand in 500 μl dimethylsulfoxide (DMSO) (stored at 10°C); determination of concentration in 50 m*M* histidine/HCl, pH 7.0, was based on the published extinction coefficient of 31,000 M^{-1} cm^{-1} at 390 nm [5]. Aqueous solutions prepared with this stock solution had a DMSO content of less than 1% (v/v).

F[bpy.bpy.2]. *N*,*N*′,*N*,*N*′-[Bis(2,2′-bipyridine-6,6′-dimethylene)]-5,6-bis(2-aminoethoxy)-4-trifluoromethylcoumarine (F[bpy.bpy.2]) was synthesized, purified, and analytically characterized in the form of its Na^+ complex [9,10]. For preparation of the uncomplexed, pure cryptand, 14 mg of the isolated Na^+ cryptate dissolved in 0.7 ml of methanol was chromatographed on Sephadex LH 20 (*Pharmacia*) with the eluent methanol/H_2O/formic acid (50/40/10, v/v/v), and the fraction characterized by TLC with methanol/dichloromethane (1/12.5, v/v) (Na^+ cryptate, $R_f = 0.35$; free cryptand, $R_f = 0$). The $R_f = 0$ fractions were collected and evaporated at 35°C under low pressure using only quartz glass equipment. The residue was rechromatographed on Sephadex LH 20 using MeOH. After evaporation and drying over P_2O_5, 8 mg of a yellowish, chromatographically pure oil (70%) was obtained. TLC plates (as above) showed a single spot with $R_f = 0$ and treatment with NaCl allowed us to detect again a single spot, but with $R_f = 0.35$. About 0.5 m*M* stock solutions of F[bpy.bpy.2] were prepared in methanol Uvasol (*Merck*) and the concentration was calculated on the basis of the extinction coefficient of 10,800 M^{-1} cm^{-1} at 365 nm after dilution with an aqueous buffer solution of pH 8.0. Aqueous solutions prepared from stock solutions always contained less than 1.5% (v/v) methanol.

F221 and F222. Compounds and solutions were as specified in Ref. 6.

Spectra and Titrations

Absorption Spectra. Absorption spectra were recorded using an Uvikon 810 (*Kontron*), an HP 8450A (*Hewlett Packard*), or a U-3000 (*Hitachi*) spectrophotometer (double-beam technology, thermostated cuvette holders) coupled to a computer.

Fluorescence Spectra. Uncorrected emission and excitation spectra of the fluorescent compounds were obtained with a *Spex* Fluorolog 212 instrument (thermo-

stated cuvette holders, excitation and emission slit widths of about 2 mm, typical concentrations of fluorescent compounds of about 1.0×10^{-5} *M*), interfaced to a personal computer. CD222 was excited at 398 nm, unless specified differently. Corrected spectra of F[bpy.bpy.2] were obtained with an Aminco Bowman Series 2 luminescence spectrometer using a calibrated light source [11]. The excitation wavelength for F[bpy.bpy.2] was 366 nm.

Spectrofluorimetric and Spectrophotometric pH Titrations. Spectrophotometric pH titrations with CD222 were carried out under stirring directly in a 2-cm cuvette. For the pH measurement in the cuvette, a specially designed, combined microelectrode (Möller, Zürich) containing 500 m*M* tetramethylammonium chloride in the reference compartment (omitting K^+ leakage) was employed. Starting with a solution of 15 μ*M* CD222 in 20 m*M* Veronal/TMAOH, pH 8.1, two pH titrations were carried out: the first titration by stepwise addition of small volumes (*Eppendorf* micropipettes) of 0.1 and 1 *M* TMAOH up to about pH 12 and the second by adding 0.01, 0.1, and 1 *M* HCl down to about pH 2. After correction of dilutions, the absorption values of 17 selected wavelengths between 300 and 460 nm, obtained from the spectra at 28 pH values between pH 2.1 and pH 11.5, were analyzed with the nonlinear fitting program LETAGROP [12–14] for three protolytic equilibria, leading to the corresponding dissociation constants and spectral parameters of all protolytic states considered. The spectrofluorometric titrations of CD222 were carried out accordingly from pH 3.35 to pH 11.6, starting with a 10 μ*M* ligand solution in 20 m*M* Veronal/TMAOH, pH 8.2. The emission spectra at 21 pH values were recorded between 410 and 660 nm (excitation at 398 nm). Again, the evaluation was done with LETAGROP for two protolytic equilibria.

The spectrophotometric pH titration of F[bpy.bpy.2] was carried out as indicated above with a 4 μ*M* solution in 10 m*M* 3-amonipropionic acid/TMAOH, pH 8.46, by adding small volumes of 0.01, 0.1, 1, and 10 *M* HCl. After having performed the necessary corrections, the intensities of 25 selected wavelengths between 380 and 640 nm, obtained from the spectra measured at 32 pH values between 8.46 and 0.65, were analyzed using the program LETAGROP for two protolytic equilibria. The spectrofluorometric pH titration was done accordingly (excitation at 360 nm).

Spectrofluorimetric Cation Titrations. The stability constants of the alkali or alkaline earth cation complexes of 10 μ*M* CD222 and 4 μ*M* F[bpy.bpy.2] were determined by spectrofluorometric titrations (excitation of CD222 as given above and of F[bpy.bpy.2] at 366 nm) at a constant ionic strength ($I = 0.186$ *M*, adjusted with choline chloride) at 25°C in 100 m*M* Tris/HCl, pH 8.0, 9.0, and 10.0, by adding small volumes of ionic strength-adjusted stock solutions, provided that the concentration of the salt of the monovalent cation was not 129 m*M* or higher and that of the divalent cation was not 43 m*M* or higher. In the case of weak cation binding, total ionic strength values higher than 0.186 *M* could not be omitted at the end of the titration experiments. The signal was recorded at 486 nm for CD222 (excitation at 398 nm) and at 480 nm for F[bpy.bpy.2] (excitation at 366 nm) after equilibration had been reached, which lasted 5 to 30 min, depending on the system under investigation. For the purpose of comparison of cation titration amplitudes (e.g., fluorescence changes in Table II), the same excitation (high-wavelength main band) and emission wavelengths were applied for each of the ligands. This, however, does not exclude the existence of different conditions providing even larger fluorescence intensity changes. In the case of CD222, the fluorescence change induced by K^+ binding was checked for different lots. Apparently, it depended on the sample purity and was also reduced upon sample aging, but the determined affinities were the same.

The dependence of the fluorescence intensity (F_{obs}) as a function of the total concentration of added cation ($[M^{n+}]_{tot}$) was analyzed on the basis of a 1:1 stoichiometry,

$$\mathrm{L} + \mathrm{M}^{n+} \overset{K}{\rightleftharpoons} \mathrm{LM}^{n+}$$

with the nonlinear fitting programs KOD11 [15], LETAGROP, and Origin [16] according to the equation

$$F_{obs} = \Phi_L[L] + \Phi_{LM^{n+}}([L]_{tot} - [L]) \qquad (1)$$

where the concentration of unbound ligand [L] is given by

$$[L] = \frac{K([L]_{tot} - [M^{n+}]_{tot}) - 1 + \sqrt{(K([M^{n+}]_{tot} - [L]_{tot}) + 1)^2 + 4K[L]_{tot}}}{2K} \qquad (2)$$

with *K* corresponding to the stability constant of the complex LM^{n+} characterized by the fluorescent quantum yield $\Phi_{LM^{n+}}$ and $[L]_{tot}$ to the total concentration of ligand L characterized by the fluorescence quantum yield Φ_L.

Kinetic Studies

Fluorescence stopped-flow studies were carried out with a SF17MV instrument (Applied Photophysics), controlled by an on-line PC and equipped with a thermostat. The dead time of the setup was 1.6 ms [17]. Before mixing, the concentration of CD222 was 30 μ*M*, that of F[bpy.bpy.2] 12 μ*M*. The applied media were 100 m*M* Nem/HCl, pH 7.75, and 100 m*M* imidazole/HCl, pH 7.5.

Excitation was at 360 nm, excepting the 400 nm for CD222, and the emission intensity above 475 nm was followed for all fluorescent crytands as a function of time. All evaluations, based on single exponentials, were done by employing the manufacturer's software.

Temperature

All measurement studies were carried out at 25°C.

RESULTS

Special Properties and pH Dependence of Ligands

Between pH 7.5 and pH 12, the absorption spectrum of CD222 was fairly insensitive to the variation of pH and characterized by a single major band above 280 nm, with a maximum at 393 nm, and a weaker band centered at 254 nm (cf. Fig. 2 and Table I). The gradual decrease in pH led to an intensity decrease and a bathochromic shift of the more intense band. At pH 2 a new maximum was observed at 460 nm, with an extinction coefficient of $1.1 \times 10^4\ M^{-1}\ cm^{-1}$ (cf. Fig. 2). The pH dependence of the absorption between pH 2 and pH 12 was incompatible with protolysis of a mono- or diprotic acid. However, if a triprotic acid model was assumed, a quantitative description of the pH dependence could be achieved. The absorption values of 17 representative wavelengths, chosen between 300 and 460 nm, were analyzed using the nonlinear fitting program LETAGROP. This provided the three acid dissociation constants, expressed as pK_a values of 3.0 ± 0.1, 4.1 ± 0.1, and 6.3 ± 0.1, together with the absorption spectra of the four protolytic states involved (Fig. 2). In contrast to this behavior, the fluorescence emission spectrum was less sensitive to pH. Within the same pH range, the fluorescence maximum at 480 nm (excitation at 398 nm) remained but its intensity was dependent on pH, as shown in Fig. 2. The simplest model consistent with the experimental data corresponds to that of a diprotic acid. Again employing LETAGROP, the two pK_a values, 4.20 ± 0.05 and 7.5 ± 0.1, could be determined. The lower pK_a value corresponds to the second one obtained from the pH dependence of the absorption spectra, given above. On the other hand, the dissociation of the second proton ($pK_a = 7.5$) does not affect the absorption spectra markedly. Thus, the four mean pK_a values ($pK_{a1} = 3.0$, $pK_{a2} = 4.15$, $pK_{a3} = 6.3$, and $pK_{a4} = 7.5$) characterize the acidity of the two carboxylic acid groups and the two bridgehead nitrogens of CD222.

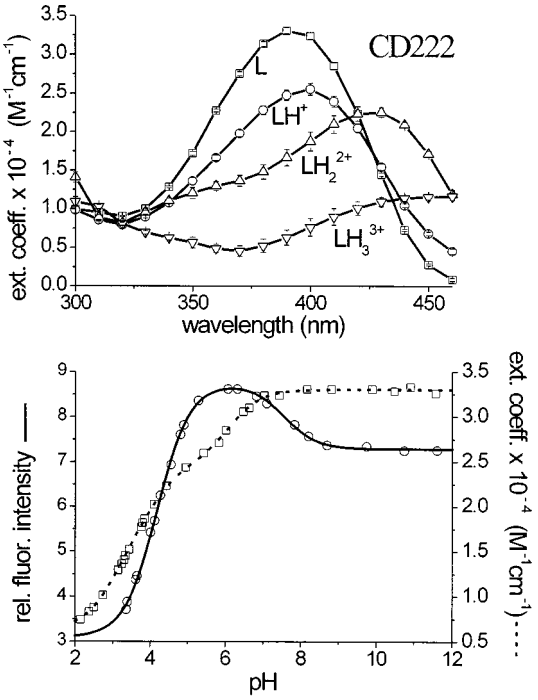

Fig. 2. pH dependence of spectral properties of CD222 in the absence of coordinating cations. Top: Absorption spectra of the four protolytic states of CD222 in 20 m*M* Veronal buffer, resulting from the evaluation of the pH titration employing the program LETAGROP. Bottom: Plot of the pH dependence of the absorption (extinction coefficient at 390 nm; dashed line) and of the fluorescence intensity (excitation at 398 nm, emission at 486 nm; solid line), determined in the same medium. Details are given under Experimental. The pK_a related to the theoretival curves are given in the text.

With regard to F[bpy.bpy.2], its absorption spectrum (cf. Fig. 3) at pH 3.5 exhibited a principal maximum at 295 nm ($\varepsilon = 30{,}200\ M^{-1}\ cm^{-1}$), attributed mainly to the chelating bipyridyl part of the ligand, and a second band at 355 nm ($\varepsilon = 12{,}900\ M^{-1}\ cm^{-1}$), assigned preferentially to the trifluorocoumarine residue. The absorption of F[bpy.bpy.2] was very sensitive to pH: both indicated bands showed a small bathochromic shift and a small intensity decrease upon an increase in pH up to 8.5. At a pH of about 0, only one major absorption band, with a maximum at 316 nm ($\varepsilon = 44{,}000\ M^{-1}\ cm^{-1}$), was detectable (Fig. 3). Intact F[bpy.bpy.2] can be investigated only up to about pH 8.5, because the lactone ring of the trifluorocoumarino moiety is opened at a high pH due to hydrolysis, as discussed previously for similar compounds [6]. At pH 3.5, the fluorescence excitation spectrum (corresponding to the absorption spectrum) also showed two maxima (302 and 365 nm) and the emission spectrum showed an intense emission band with a maximum at 470 nm (Fig. 3, Table I). An increase in pH to 8.5 was accompanied by an emission intensity decrease

Table I. Spectral Parameters of Absorption (Extinction Coefficient ε and λ_{max}) and Fluorescence (λ_{max} of emission) Spectra of the Cation Complexes of CD222 and F[bpy.bpy.2] at pH 8.0 (Total Ionic Strength, 0.186 *M*) and 25°C

	CD222			F[bpy.bpy.2]		
Cation	Absorption, ε (M^{-1} cm^{-1})/λ_{max} (nm)		Fluorescence, λ^{em}_{max} (nm)[a]	Absorption, ε (M^{-1} cm^{-1})/λ_{max} (nm)		Fluorescence, λ^{em}_{max} (nm)[a]
—[b]	19,800/254	33,000/393	480	28,900/299	10,800/365	470
Na^{+}[c]	12,000/301	24,000/364	478	27,600/299	9,500/362	466
K^{+}[c]	12,500/299	20,700/370	468	28,700/297	9,700/362	466
NH_4^+	11,900/301[e]	19,200/370[e]	480[e]	28,700/299[d]	10,200/366[d]	470[d]
Tl^{+}[g]	14,600/299	19,200/361	471	27,600/301	10,500/363	472
Rb^{+}	11,300/300[e]	20,800/370[e]	476[e]	28,100/299[d]	10,000/363[d]	468[d]
Mg^{2+}[d]	18,000/260	33,700/412	491			
Ca^{2+}	16,500/294[d]	16,600/359[d]	474[d]	37,000/300[f]	11,000/362[f]	468[f]
Ba^{2+}[f]	20,200/298	20,500/352	475	35,000/300	9,800/354	468

[a] Determined from uncorrected fluorescence emission spectra (excitation: CD222 at 398 nm and F[bpy.bpy.2] at 366 nm).
[b] 10 μ*M* CD222 or 4 μ*M* F[bpy.bpy.2] in 100 m*M* Tris/HCl containing different concentrations of salts: 129 m*M* ChCl.
[c] 129 m*M*.
[d] 250 m*M*, or
[e] 1 *M* of the chloride salt of the considered monovalent cation and
[f] 43 m*M* of the divalent cation salt.
[g] 10 μM CD222 or 4 μ*M* F[bpy.bpy.2] in 100 m*M* Tris/HNO_3 containing 129 m*M* $TlNO_3$.

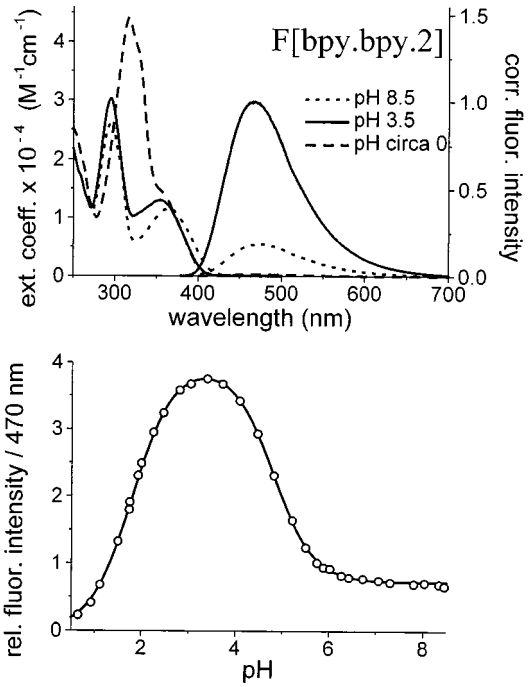

Fig. 3. pH dependence of spectral properties of F[bpy.bpy.2]. Top: Absorption spectra between 250 and 420 nm and relative, corrected fluorescence emission spectra between 380 and 700 nm (excitation, 360 nm) of F[bpy.bpy.2] (4 μ*M*) in 10 m*M* 3-aminopropionic acid/TMAOH, pH 3.5 (——), 10 m*M* Veronal/TMAOH, pH 8.5 (-----), and 1 *M* HCl_l ~ pH 0 (— — —). Bottom: Spectrofluorimetric pH titration of 4 μ*M* F[bpy.bpy.2] in 10 m*M* 3-aminopropionic acid/TMAOH at pH 8.46 with HCl. The pK_a values are given in the text (further details under Experimental). The emission intensities are dilution corrected.

and a small bathochromic shift. At a pH even lower than 3.5, the fluorescence intensity was decreased due to a quenching process (Fig. 3). This is the expression of a strong interaction between the fluorophore and the protonated bipyridyl moieties, because very similar molecules, F221 and F222, exhibited maximum fluorescence under these conditions [6]. The evaluation of the spectrofluorimetric pH titration of F[bpy.bpy.2] according to a diprotic acid provided pK_{a1}, and pK_{a2} values of 1.8 ± 0.1 and 4.8 ± 0.1, respectively (cf. Fig. 3).

Spectral Properties of Cation Complexes

After the determination of the stability constants of several cation complexes at pH 8.0 (cf. next paragraph), it was possible to characterize also the corresponding absorption and fluorescence spectra. If the affinity was not too weak, the measurements were done under the condition of a constant total ionic strength of 0.186 *M*. The results together with data obtained in the absence of bindable cations (ionic strength adjusted with choline chloride, which is not capable to bind within the cavity) are summarized in Table I and, to some extent, in Table II. Above 280 nm, the cation complexes of CD222 showed two bands in the absorption spectrum (except for Mg^{2+}), with maxima around 300 and 370 nm (Table I). For the alkali ion cryptates, the 370-nm band is more intense than the 300-nm one, whereas both bands are of about equal intensity for the complex with alkaline earth cations. The binding of the very small Mg^{2+} showed

Table II. Cation Binding to CD222 and F[bpy.bpy.2] at pH 8.0 (Total Ionic Strength, 0.186 *M*, Adjusted with Choline Chloride) and 25°C: Stability Constant *K*, Relative Cation Selectivity, and Relative Fluorescence Change (ΔF) Under the Conditions Chosen for the Spectrofluorometric Titrations (Further Details Given Under "Experimental")

	CD222			F[bpy.bpy.2]		
Cation	log K^a	ΔF(%)^b	Selectivity, K_i/K_{K^+}	logK^a	ΔF (%)^c	Selectivity, K_i/K_{K^+}
Li^+	<1	—		<1.5	—	
Na^+	2.4	−45	0.29	3.1	+55	0.29
K^+	3.0	+100	1	3.6	+205	1
NH_4^+	0.9	+30	0.009	2.2	+25	0.039
Tl^+	2.7	−60	0.6	5.2	−20	40
Rb^+	<0.5	>0		<0.5	<0	
Mg^{2+}	1.5	−40	0.037	<1		
Ca^{2+}	1.9	−35	0.093	5.9	+320	200
Ba^{2+}	4.3	−55	26	5.2	+610	40

[a] Estimated error, ± 0.1.
[b] Determined from uncorrected fluorescence emission spectra at 486 nm (excitation at 398 nm) and
[c] at 480 nm (excitation 366 nm).

rather unusual band positions in the absorption spectrum. Concerning fluorescence, except for the Tl^+ and, again, for the Mg^{2+} complex, the position of the maximum of the main fluorescence emission was between 474 and 480 nm (Table I). However, the emission intensities depend markedly on the nature of the cation (cf. Table II). For example, the fluorescence intensity increased strongly upon binding of K^+ and NH_4^+ and, on the other hand, decreased markedly upon binding of Na^+, Mg^{2+}, Ca^{2+}, and Ba^{2+} (Table II).

The position and intensity of the main absorption band of F[bpy.bpy.2] at about 299 nm remained essentially unchanged upon the binding of monovalent cations. In the case of bound Ca^{2+} and Ba^{2+}, the extinction coefficient was higher (cf. Table I). Also, the properties of the second absorption band around 364 nm were not changed drastically in the cation-bound state, except for the Ba^{2+} cryptate, where this band was shifted to a markedly lower wavelength. If excited at 366 nm, the fluorescence emission maxima were also characterized by only small shifts (Table I). The observed emission intensity, however, increased strongly as a consequence of cation binding, except for Tl^+. The increase, compared to the fluorescence intensity of the uncomplexed ligand, was largest for the binding of Ba^{2+}, smaller for the binding of Ca^{2+} and K^+, and again smaller for that of Na^+. The K^+-induced fluorescence change found under the conditions of our titrations, as specified under Experimental and in Table II, was twofold. However, the quantum yield increased by a factor of 3.4, which is essentially consistent with the values reported in earlier studies [5,18]. On the other hand, the effect of Na^+ was larger under our experimental conditions. In general, the absorption changes observed upon cation binding of CD222 are larger than those found for F221 and F222 [6,7], which contain the same fluorophore.

Cation Binding Affinities

The interaction between our fluorescent ligands and cations were investigated quantitatively at pH 8.0 by carrying out spectrofluorimetric titrations for the determination of the stability constants (cf. Fig. 4). Whenever possible, the ionic strength was kept constant during the titration at 0.186 *M* (adjustment with choline chloride). Titrations were performed by adding small volumes of the chloride salt of the chosen cation (nitrate in the case of Tl^+). A constant set of excitation and emission wavelengths was used for each of the investigated ligands (cf. Table II). The evaluation of the titration data was based on a 1:1 binding model.

Concerning CD222, according to our study K^+, and not Na^+, as reported earlier [5], is the monovalent cation that is bound the most strongly (Table II). Our affinity for K^+ corresponds to that of the study mentioned before [5,19]. Li^+ is bound very weakly. Although CD222 is obtained as the Li^+ salt, the cation was present only at very low concentrations under the conditions of our titrations. Its presence here does not lead to a reduction in the stability constant of the cations that are bound strongly. The comparatively large Cs^+ is bound very weakly. With regard to alkaline earth cation binding, only Ba^{2+} is bound with a high affinity. For CD222, the pH dependence of K^+ binding was investigated. Additional

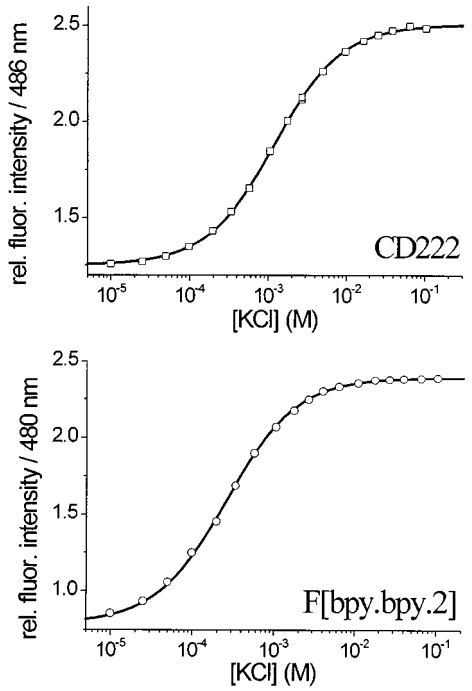

Fig. 4. Cation binding of CD222 and of F[bpy.bpy.2]. Spectrofluorometric K^+ titration in 100 m*M* Tris/HCl with a total ionic strength of 0.186 *M* (adjusted with choline chloride) of 15 μ*M* CD222 (top) at pH 9.0 (excitation, 398 nm) and of 4 μ*M* F[bpy.bpy.2] (bottom) at pH 8.0 (excitation, 366 nm) with KCl. Emission intensities are dilution corrected. The solid lines correspond to theoretical titration curves with log*K* values of 2.95 and 3.60 for the K^+ cryptates of CD222 and F[bpy.bpy.2], respectively. Further details are given under Experimental and in the text.

titrations were carried out at pH 5.5, 6.0, 6.5, and 7.2 (100 m*M* BisTris/HCl adjusted with choline chloride to a total ionic strength of 0.186 *M*) and at pH 9.0 as well as pH 10.0 (100 m*M* Tris/HCl adjusted accordingly). All determined log*K* values were within the value of 3.0 ± 0.1 and thus independent of pH in the investigated range.

The cation affinity of F[bpy.bpy.2] is markedly higher than that of CD222. Among the monovalent cations, Tl^+ was bound the most strongly, followed by K^+ (cf. Table II). Also here, Li^+ and Cs^+ were characterized by very low affinities. With regard to alkaline earth ion binding, Mg^{2+} is bound very weakly. Surprisingly, Ca^{2+}, with an ionic radius similar to that of Na^+, exhibited a higher affinity than Ba^{2+}, characterized by a radius close to that of K^+, which was the strongest bound alkali ion.

Dynamic Efficiency

Indicators are often applied for time-resolved measurements. Their respective response time is therefore of interest for a particular investigation. For this reason, different fluorescent alkali ion indicators including F221 and F222 [6,20] were compared under suitable conditions by carrying out stopped-flow experiments around neutral pH. For this comparison, the most tightly bound alkali ion was chosen for every ligand.

The experiments were carried out at pH 7.75 in 100 m*M* Nem/HCl and 100 m*M* imidazole/HCl buffer. The concentration of the alkali and alkaline earth cation chloride after mixing was 20 m*M*. The fastest reaction was found for CD222 because only a fast amplitude change, and no time-resolvable process, could be observed in both buffer systems (Fig. 5). The corresponding time constant for K^+ binding must thus be shorter than 1 ms. The time constants for Na^+ binding to F221 were 20 and 3 ms; those for K^+ binding to F222, 19 and 1.5 ms in the applied Nem/HCl and imidazoleHCl buffers, respectively; and that of K^+ binding to F[bpy.bpy.2], 15 ms in the Nem/HCl buffer.

In contrast to this comparatively fast binding of alkali ions, the divalent alkaline earth cations were bound considerably more slowly, as indicated earlier for parent cryptands [17]. Under similar conditions, the binding of Ca^{2+} and Ba^{2+} to CD222 was characterized by time constants of about 1 s and 10 ms, respectively. For the binding of Ca^{2+} to F221 and of Ba^{2+} to F222, the determined time constants, again under similar conditions, were about 40 s and 200 ms, respectively.

DISCUSSION

According to our measurements, CD222 and F[bpy.bpy.2] are K^+ selective (Table II). In an earlier

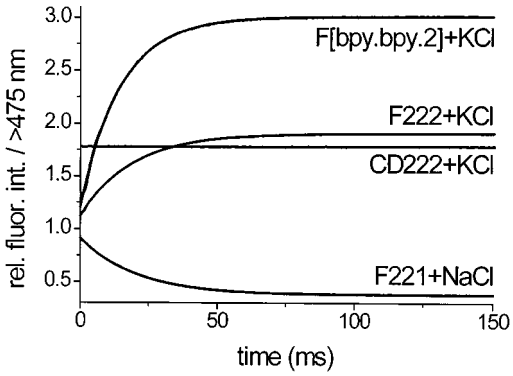

Fig. 5. Dynamics of cation binding to different fluorescent alkali ion indicators. Fluorescence stopped-flow experiments with CD222, F221, and F222 (15 μ*M*) as well as F[bpy.bpy.2] (6 μ*M*) in 100 m*M* Nem/HCl, pH 7.75, with 20 m*M* chloride salt of the chosen alkali ion (concentrations refer to conditions after mixing). Plot of relative fluorescence emission intensity (level of the uncomplexed ligand is set at 1.0) versus time. The k_{obs} values (reciprocal decay times) according to the fit with a monoexponential function were >500 s^{-1} for CD222 + KCl, 49.5 s^{-1} for F221 + NaCl, 53.0 s^{-1} for F222 + KCl, and 68.5 s^{-1} for F[bpy.bpy.2] + KCl.

study of CD222, a slightly higher affinity was reported for Na^+ than for K^+ [5]. There is consistency between our value for the K^+ binding constant and those of other studies under slightly different conditions [5,19]. Compared to the parent cryptand [2.2.2] at pH 8, calculated according to Refs. 21 and 22, F[bpy.bpy.2] exhibits the same K^+ affinity, whereas CD222 shows a lower one. Both ligands, however, bind Na^+ more strongly than [2.2.2], which evidently is the expression of a comparatively low Na^+/K^+ selectivity. This can be easily understood if we assume that the presence of aromatic residues reduces the size of the cavity of these ligands, thus leading to more suitable geometric conditions for Na^+ binding. It is thus not surprising that the interaction between both fluorescent ligands and the large Ba^{2+} is not as strong as for [2.2.2]. On the other hand, the affinity of Ca^{2+} to F[bpy.bpy.2] is unexpectedly high (Table II). In general, the fluorescence intensity changes upon cation binding, measured under our excitation conditions, are larger in the case of F[bpy.bpy.2] than for CD222 (Tables I and II).

If one or two bridgehead nitrogens of cryptands are protonated, the apparent cation affinity is very low but increases with decreasing degree of protonation. Although unstable above pH 8.5, the deprotonated form of F[bpy.bpy.2] is expected to exhibit unusually high alkali ion affinities, because its second pK_a is, in agreement with that determined for [bpy.bpy.2] [9,10], assumed to be very high ($>$10.5). Since a relatively high affinity for K^+ was already found at pH 8, this implies that the structure of F[bpy.bpy.2] is likely to be better preorganized than that of the parent cryptand.

In contrast to the pH dependence of the stability constant of bipyridyl cryptands [9,10], the stability constant of the K^+ complex of CD222 was independent of pH between pH 5.5 and pH 10. Thus, the higher pK_a value of the bridgehead nitrogens must be lower than 5.5. This is consistent with the general fact that aromatic amines show considerably lower pK_a values than aliphatic ones. With regard to the assignment of the experimentally determind pK_a values of CD222, we conclude that the pK_{a3} and pK_{a4} values of 6.3 and 7.5 can be attributed essentially to the deprotonation of the two aromatic carbonic acid residues. Consequently, the pK_{a1} and pK_{a2} values of 3.0 and 4.15 can be assigned to the deprotonation of the two bridgehead nitrogens. With regard to fluorescence, similar to the protolysis of F221 and F222 [6], the pK_{a2} of F[bpy.bpy.2] is likely to represent primarily the deprotonation of the first bridgehead nitrogen, whereas the pK_{a1} value will characterize the pyridin nitrogens, which is consistent with data on a simpler compound [23].

The different dynamic efficiency of the two alkali ion indicators can also be explained on the basis of this dissimilar protolytic behavior. At neutral pH, CD222 binds cations in less than 1 ms, which is considerably faster than F[bpy.bpy.2], because both bridgehead nitrogens of CD222 are already deprotonated. Since the pK_a value of the second bridgehead nitrogen of F[bpy.bpy.2] must be high, buffer-assisted deprotonation has to occur prior to cation coordination, which leads to a slowing-down of the reaction rate. This is the reason why cation binding by F[bpy.bpy.2] occurs in the millisecond time range and is slower than that of CD222. The dynamic efficiency of cation binding also depends on the nature of the buffer compound and on its concentration. As observed for both fluorescent ligands, alkali ion binding occurs much more rapidly than that of alkaline earth cations. If these indicators are applied for time-resolved studies, for example, by employing the stopped-flow technique, it is possible to determine easily not only the alkali ion concentration but also that of a simultaneously occurring alkaline earth cation on the basis of different time responses.

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