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Time-Resolved Response of Fluorescent Alkali Ion Indicators and Detection of Short-Lived Intermediates upon Binding to Molecular Cavities

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Stopped-flow kinetic studies have been performed to determine the kinetic parameters of K^+ binding to the fluorescent cryptand F222 and of Na^+ binding to F221 at pH 8.0. The results clearly indicate that a comparatively stable intermediate is formed before the rate-limiting binding step occurs with a rate constant around 30 s^{-1} under the chosen experimental conditions. The conversion of the intermediate to the final cation complex is assigned to the final penetration of the already bound, but still partially solvated cation into the ligand's cavity. The main fluorescence intensity change found upon cation binding is attributed to the second reaction step, and not to the fast, initial binding reaction. The comparatively slow overall binding reaction is interpreted on the bases of a special solvate substitution mechanism which, in principle, can also account for the 1500 times slower binding of Ca^{2+} to F221. With regard to time-resolved analytical Na^+ and K^+ determinations, the response times under the chosen conditions are around 20 ms. Differentiation between Na^+ and Ca^{2+}, for example, is possible with F221 on the basis of completely different response times.

KEY WORDS: Alkali ion indicators; cryptands; fluorocryptands; fluorescence-stopped flow; cation binding mechanism; intermediate state detection.

INTRODUCTION

The fast and selective analytical detection of alkali ions in aqueous solution under physiological conditions in the form of time-resolved concentration changes is an important tool in biological research. Advanced techniques for the selective detection of alkali ions consist either of ion selective electrodes[1] or of suitable indicator systems. Among the considerable number of suggested ligands, only a few have been sufficiently characterized and exhibit large enough affinities and selectivities to be applied in aqueous media around pH 7 as well as in the presence of other cations.[2–4] Whereas electrodes are characterized by a comparative slow time response, dye indicators are expected to be suitable for considerably faster detection of concentration changes and thus could fulfill the expected requirements to sense, for example, the time dependence of ion currents or ion pulses in biological systems. The design of suitable alkali ion indicator dyes, however, appears to cause certain problems that are related to the special properties of this group of cations.

A low molecular weight ligand capable of recognizing an alkali ion, for example, in the presence of alkaline earth cations, is generally assumed to exhibit a cavity of distinct size with suitably positioned dipoles to provide cation interaction. If the ligand would contain coordinating groups exhibiting a negative charge, the divalent cation would be preferred over a monovalent one of similar size as a consequence of the resulting much

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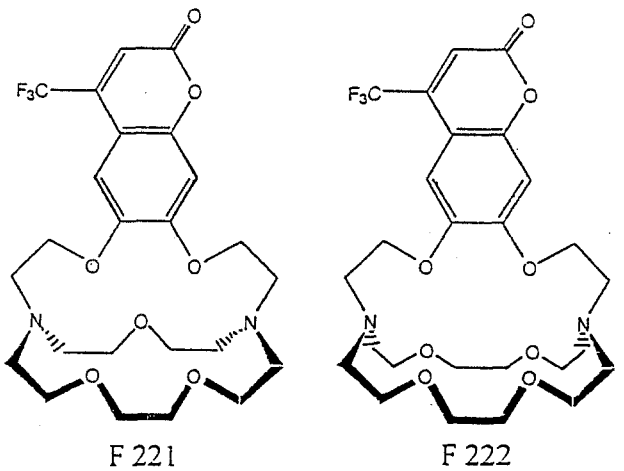

Fig. 1. Chemical structure of the fluorocryptands F221 (Na^+ selective) and F222 (K^+ selective).

higher electrostatic interaction energy. A second important aspect deals with the fact that cations are solvated in aqueous solution but usually are completely desolvated when high-affinity binding occurs. Most of the hitherto suggested alkali ion indicating ligands are of an open-chain or monocyclic nature and thus exhibit only weak binding because such ligands are generally not capable of fully substituting the cation's inner-sphere solvate molecules. Thus, bicyclic molecules such as cryptands appear to be more suitable for achieving high-affinity alkali ion binding and high selectivity.[5] However, this improvement of the properties with regard to monocyclic compounds is related to a particular kinetic problem. Binding of a solvated alkali ion to a cavity can be fast only provided that the ligand exhibits sufficient conformational flexibility to allow stepwise substitution of the solvate molecules around the cation by the coordinating groups of the ligand[6,7] such as carbonyl or ether oxygen and amine nitrogen atoms in the case of cryptands. If, however, a reaction step occurs where more than one solvate molecule has to dissociate in a reaction step preceding the final penetration of the cation into the cavity, an unusually high activation energy is considered to be involved, which will lead to a considerable reduction of the overall formation rate constant compared to the stepwise substitution process mentioned before.[8] In the former case, at least one intermediate state is expected to be significantly populated, and in addition, the formation rate constant could depend on the nature of the cation, for example, on its charge and size, which hitherto has not been observed in a marked manner for the stepwise substitution of alkali or alkaline earth cations, except for their smallest representatives.[6]

To determine experimentally the response time of two recently prepared fluorescent cryptands, F221 and F222[4,9] (cf. Fig. 1), which act as selective alkali ion indicators in aqueous solution, a fluorescence stopped-flow kinetic study has been carried out. It is hoped that this investigation will also contribute to a more detailed understanding of the mechanistic aspects mentioned above because the involvement of a fluorophore within the bridge of a cryptand should provide a direct and sensitive system to sense and report the approach of a cation during the binding process. Thus, these cryptand-type ligands are expected to be ideally suited to detect intermediate states that are populated during the multi-step cation binding process. Because ligands for alkali ions always exhibit also a certain affinity to alkaline earth cations, it was investigated whether the rate constants for mono- and divalent cations are considerably different, so that their analytical differentiation will be possible on the basis of different dynamic properties.

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical or Suprapur grade and were purchased from Merck, Fluka or Aldrich. The fluorescent cryptands were synthesized and characterized according to Ref. 9.

Spectrofluorometry. The fluorescence properties of F222 and F221 have been reported previously.[4] Spectrofluorometric equilibrium pH and cation titrations were carried out at 25°C on a Spex Fluorolog 212 spectrofluorometer. All evaluations are based on a 1:1 complex formation model. The equilibrium constants are given as apparent stability constants K' referring to the chosen pH value. These apparent stability constants depend on pH because only the fully deprotonated cryptand is assumed to bind the cation strongly. K' is related to the pH-independent stability constant K of the fully deprotonated state by Eq. (1), where K_{H2} and K_{H1} are the dissociation constants of protolysis of the fully and monoprotonated states of the ligand,

$$K' = \frac{K}{1 + ([H^+]/K_{H1}) + ([H^+]^2/K_{H1}K_{H2})} \quad (1)$$

Experimentally determined pK_{H2} values are about 5.9 for F222 and 7.0 for F221.[9] pK_{H1} values of about 9.5 for F222 and 11.0 for F221 have been estimated from the pH dependence of K' for different cations.[9]

Kinetic Studies. Stopped-flow experiments[10] were been performed at 25°C on an Applied Photophysics Model 13–106 equipped with a fluorescence detection.

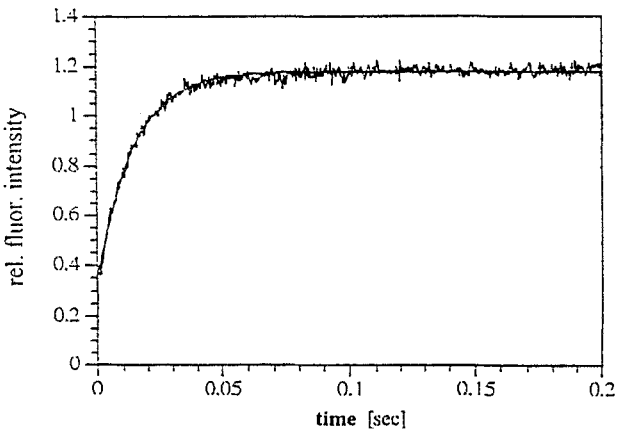

Fig. 2. Fluorescence stopped-flow, experiment at 25°C. Rapid mixing of 15 μ*M* F222 with 20 m*M*KCl (end concentrations: 7.5 μ*M* F222, 10 m*M* KCl) in 50 m*M* Tris/HCl, 0.1 m*M* EDTA, pH 8.0 (λ_{exc} = 366 nm λ_{emi} > 455 nm). The solid line is the result of a monoexponential fit with k_{exp} = 62 s^{-1}. Error bars calculated from standard deviation of 5 experiments.

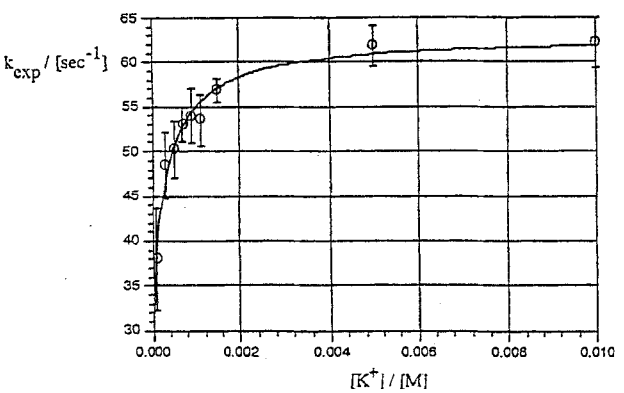

Fig. 3. Dependence of the observed rate constant k_{exp} versus KCl concentration for F222 in 50 m*M* Tris/HCl, 0.1 m*M* EDTA, pH 8.0, at 25°C (experimental conditions as in Fig. 2). The solid line was calculated according to Eq. (2); parameters are given in the text.

Evaluation of the kinetic experiments (a single monoexponential kinetic reaction phase) was done employing the manufactor's program. The corresponding reciprocal time constant τ is denoted k_{exp}. In the case of a two-step binding reaction [a bimolecular binding step, characterized by the preequilibrium constant K_1, is followed by a monomolecular interconversion step, characterized by the kinetic rate constants k_{23} and k_{32} and the equilibrium constant $K_2 = k_{23}/k_{32}$] under conditions where the binding proceeds much more rapidly than the subsequent interconversion and where the total cation concentration is much higher than that of the cryptand ($[Me^{n+}]_{total} = [Me^{n+}]_{free} = [Me^{n+}]$), the following expression [Eq. (2)] holds for τ^{-1} of the resolvable, slower process of a corresponding stopped-flow experiment:

$$\tau^{-1} = k_{exp} = \frac{k_{23} K_1 [Me^{n+}]}{1 + K_1 [Me^{n+}]} + k_{32} \qquad (2)$$

Equation (2) corresponds to that of chemical relaxation experiments under similar conditions.[11,12] The expression of the initial, faster binding process does not need to be considered here because only one time-resolvable reaction phase was detected. Under pseudo-first-order conditions a linear dependence of k_{exp} on the metal ion concentration would be expected to characterize the fast binding step. Because the kinetic stopped-flow experiments were also performed at a given pH, the parameters that can be determined according to Eq. (2) are also denoted as apparent ones, namely, K_1', K_2', k_{23}', and k_{32}'. According to previous kinetic experiments carried out above pH 7,[13] the rate of cation dissociation from cryptates has generally been assumed to be pH independent.

RESULTS

At pH 8.0 logK' values of 2.0 for Na^+, 3.5 for K^+, 1.7 for Ca^{2+}, and 5.6 for Ba^{2+} in the case of F222 and 3.6 for Na^+, 1.2 for K^+, 3.2 for Ca^{2+}, and 3.0 for Ba^{2+} with F221 in 100 m*M* Tris/HCl buffer at 25°C were determined by spectrofluorometric titrations. The ionic radius of Na^+ is similar to that of Ca^{2+}, whereas the ionic radius of K^+ corresponds approximately to that of Ba^{2+}. The unusually high Na^+/K^+ selectivity of F221 has already been emphasized before.[4]

The result of a typical stopped-flow fluorescence experiment on F222 with KCl at pH 8.0 is shown in Fig. 2. The transient curve corresponds to a monoexponential dependence and is characterized by a particular k_{exp} value.

The resolvable fluorescence increase is only slightly smaller than the change found under the same conditions by employing standard spectrofluorometry, which exhibits a much lower time resolution. This implies that a second, faster kinetic phase [step I in Eq. (3)] of a low amplitude, not resolvable by the stopped-flow method, may exist. If the k_{exp} values determined at different KCl concentrations are plotted as shown in Fig. 3, a pronounced saturation behavior is observed, as expected according to Eq. (2). The maximum observable time constant at high KCl concentrations under these conditions is about 15 ms.

If a one-step binding process, as hitherto postulated,[13] occurred, a simple linear dependence should result. Thus, the following, simplest kinetic reaction scheme must be assumed (F denotes the fluorescent

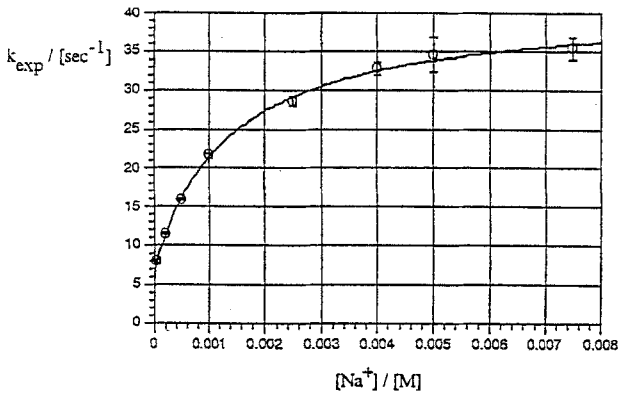

Fig. 4. Dependence of the observed rate constant k_{exp} versus NaCl concentration for F221 in 2 m*M* Veronal/TMAOH, 0.1 m*M* EDTA, pH 8.0, at 25°C (experimental conditions and evaluation as in Fig. 3).

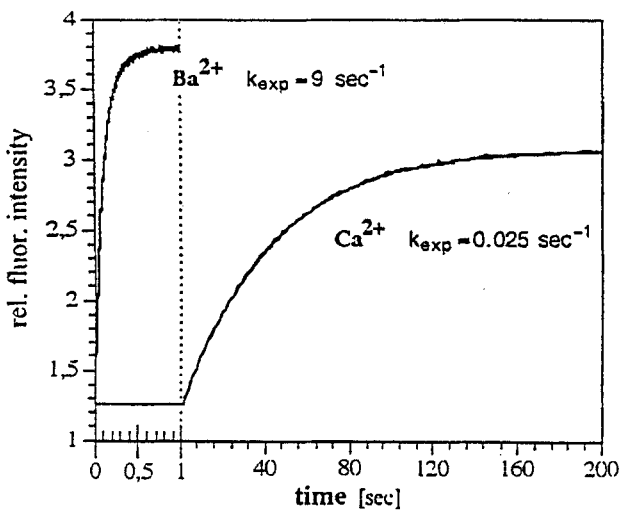

Fig. 5. Stopped-flow traces of Ca^{2+} and Ba^{2+} binding to F221 (15 μ*M*) in 50 m*M* Tris/CF_3SO_3H, pH 8.0 (λ_{exc} = 366 nm, λ_{emi} > 455 nm). Concentrations of $CaCl_2$ and $BaCl_2$ were 100 m*M*.

cryptand),

$$Me^{n+} + F \underset{I}{\overset{K_1'}{\rightleftharpoons}} F\cdot\cdot Me^{n+} \underset{II}{\overset{k_{23}'}{\underset{k_{32}'}{\rightleftharpoons}}} F - Me^{n+} \qquad (3)$$

where $F\cdot\cdot Me^{n+}$, represents a comparatively stable intermediate binding state. The time-resolvable process shown in Fig. 2 is unambiguously assigned to the slower, second reaction step II, which is coupled to the preceding, much faster equilibrating binding step I. Therefore, the fluorescence intensity change shown in Fig. 2 must be attributed to the conversion of the intermediate to the final binding state [step II in Eq. (3)]. Fitting Eq. (2) to the experimental results provides the parameters $\log K_1' = 3.5$, $k_{23}' = 31\ s^{-1}$, and $k_{32}' = 32\ s^{-1}$ for the binding of K^+ to F222 at pH 8.0. From the kinetic parameters we can calculate K_2', which is about 1. An analogous dependence is observed for the stopped-flow experiments of F221 with NaCl under similar experimental conditions (Fig. 4). For this system, the maximum observable time constant is about 25 ms under the chosen conditions, which is slightly slower than that reported before for K^+ binding to F222. Here the determinable parameters are $\log K_1' = 2.9$, $k_{23}' = 34\ s^{-1}$, and $k_{32}' = 7\ s^{-1}$, and accordingly K_2' is about 5. Also, here a marked population of the intermediate state is observed.

A quite different situation is found for the binding of alkaline earth cations of comparable size to these ligands. Whereas Ca^{2+}, which has an ionic radius similar to that of Na^+, is bound extremely slowly with a time constant around 40 s if present at high concentration, Ba^{2+}, similar in size to K^+, under identical conditions again exhibits a comparatively fast response, around 110 msec, for F221 (cf. Fig. 5).

Preliminary kinetic binding studies performed at a constant pH but at different buffer concentrations indicate that K_1' as well as the kinetic parameters related to equilibrium II in Eq. (3) depend on the buffer concentration. This result suggests that buffer catalysis is involved in cation binding. At higher buffer concentrations than employed here, considerably faster responses can be achieved.

DISCUSSION

The main fluorescence intensity change observed upon cation binding to F222 and F221 must be attributed to the transition of the intermediate to the final cation complex [cf. Eq. (3)]. Our fluorescent cryptands exist at pH 8.0 preferentially in the monoprotonated state, which is characterized by a very low quantum yield.[4] The initial binding of the cation apparently does not change the quantum yield significantly. The final cation complexes, however, exhibit fluorescence properties similar to those of the diprotonated states of the cryptands, which have been characterized by a comparatively high quantum yield.[4]

The intermediate state is thought to represent binding of the still partially solvated cation outside the central cavity such as within the surface region of the cryptand, as indicated schematically in Fig. 6B. Because the rate constants characterizing the final binding equilibrium [step II in Eq. (3)] depend on the buffer concentration, it appears to be likely that this intermediate

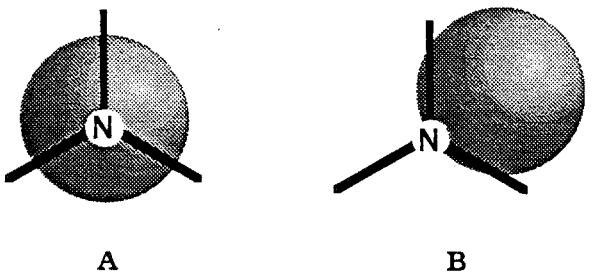

Fig. 6. Scheme of different arrangements of cation coordination to cryptants: (A) cation binding inside cavity (B) cation binding outside cavity.

state is still monoprotonated. Final penetration of the cation into the cavity could thus probably be accompanied by concerted deprotonation.

Previously, cation binding to cryptands has been analyzed on the basis of an assumed single-step reaction scheme[13] because only the dissociation rate constants could be determined experimentally. Therefore the corresponding formation rate constants have been calculated simply employing the separately determined binding constant. Due to their suitable fluorescence properties, our cryptands allow much more detailed detection and analysis, leading to at least a two-step kinetic reaction scheme [Eq. (3)], as applied for the analysis of other types of alkali ion binding ligands.[8,12,14] The conversion of the intermediate to the final binding state observed here is, however, much slower than, for example, in the case of valinomycin. In principle, this could be attributed to a low rate of nitrogen inversion (e.g., upon deprotonation). This seems to be very unlikely because experimental evidence has been presented indicating that these processes are very fast.[15] Thus, the rate-limiting step of the transition of the intermediate to the final complex could tentatively be attributed to a reaction step where more than one solvate molecule has to dissociate from the already bound but still partially solvated cation prior to its penetration into the cavity, as discussed in the Introduction and suggested earlier.[8] Based on this concept and because the hydration energy of divalent cations is much higher than that of monovalent ones, it would be understandable why Ca^{2+} binds about 1500 times more slowly than Na^+, although it is similar in size. According to their dimensions, both cations could be assumed to be bound inside the cavity as indicated schematically in Fig. 6A. Ba^{2+}, which is too large to be completely bound inside the cavity, again exhibits much faster binding than Ca^{2+}. The larger alkaline earth cation may thus be bound in a state intermediate to those shown in Figs. 6A and B. In such circumstances the bound Ba^{2+} could remain partially solvated and the special desolvation step discussed for Ca^{2+} may not occur to the same extent under these conditions.

As a consequence of the special mechanistic aspects involved here, the time response for analytical alkali ion detection is around 20 ms at pH 8.0 employing usual buffer concentrations and can be in the range of seconds for certain alkaline earth cations. However, even if the change of the spectral properties observed upon binding of, for example, Na^+ and Ca^{2+} to F221 were exactly the same, an analytical differentiation in a mixture of both cations would still be easily possible because the response to both cations occurs in completely different time ranges.

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