Chemosensor Ensemble with Selectivity for Inositol-Trisphosphate

Kenichi Niikura, Axel Metzger, and Eric V. Anslyn*

*The Department of Chemistry and Biochemistry
The University of Texas at Austin
Austin, Texas 78712*

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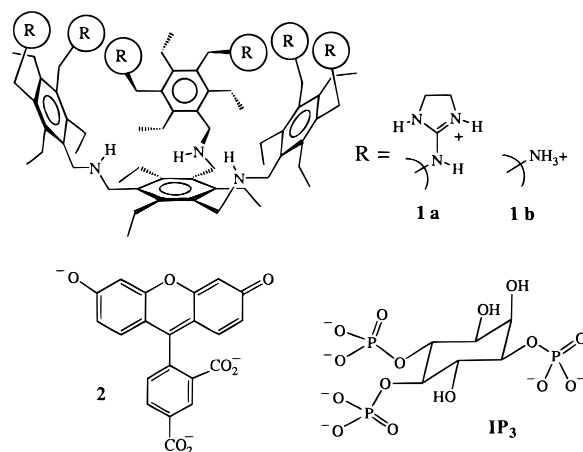
The development of optical sensors for biologically active molecules gives one the ability to monitor analytes during cellular processes.[1] For example, very effective sensors for the whole cell imaging of Ca^{2+} during cellular signaling events have been developed.[2a,b] In addition, capillary electrophoresis (CE) has been used to extract and analyze pL quantities of cytoplasm.[2c] One molecule that would be of interest to monitor by using CE is the polyanionic second messenger inositol-1,4,5-trisphosphate (IP_3).[3] However, because IP_3 does not have a chromophore, such an analysis has not yet been accomplished.

In the development of a chemosensor with selectivity for IP_3, a number of structural motifs can be considered for the receptor portion of the sensor. Synthetic receptors for anions in water typically fall into four categories: macrocyclic polyammoniums/guanidiniums,[4] clefts,[5] cyclophanes/calixarenes,[6] and porphyrins/sapphyrins.[7] The macrocyclic polyammonium receptors generally have the highest affinities, but manipulating their recognition selectivity is often limited to varying the cavity size. A cleft motif has an advantage since the shape is inherently more versatile, but few if any such receptors show strong binding of anionic guests in protic media. Strong binding is required for the development of a sensor for IP_3, whose intracellular concentration is typically in the nanomolar range.[8] Herein we report a cleft-like receptor that, when paired with an optical signaling molecule, can be used to quantitate IP_3 at nanomolar concentrations.

Although the structures of the natural IP_3 receptor sites have not been elucidated, arginine-modifying reagents block IP_3 binding,[9] thereby suggesting that guanidinium groups may be essential recognition elements for IP_3 in nature. We therefore focused upon the use of guanidiniums in our synthetic receptor.[10] Steric gearing[11] was used to impart a preference for six guanidiniums to be oriented toward the interior of a cavity (**1a**).



Receptor **1a** was synthesized by allowing 1,3,5-tris(bromomethyl)-2,4,6-triethylbenzene to react with 1-aminomethyl-3,5-[bis(4,5-dihydro-1H-imidazol-2-yl)aminomethyl]-2,4,6-triethylbenzene in CH_2Cl_2. Compound **1a** was isolated as a white powder after gel filtration chromatography. To test if ammoniums would be more effective recognition elements than guanidiniums, receptor **1b** was synthesized in a similar fashion.

A competition assay using an ensemble of 5-carboxyfluorescein (**2**) and receptors **1a**/**1b** was used to measure binding constants. A similar competition assay for the quantification of citrate in beverages using a synthetic receptor and **2** was recently reported.[12] This type of assay, using a colorimetric/fluorescent probe, is very useful for monitoring a guest that has no chromophore, such as citrate or IP_3. Moreover, competition assays are extensively used in sensing schemes for biologically relevant analytes employing antibodies.[13]

UV/vis spectroscopy was employed to determine the binding constants for the complexes formed between **1a**/**1b** and **2**. The addition of **1a** or **1b** to **2** resulted in a red shift of the absorption of **2** (Figure 1A). Monitoring the absorption at 502 nm, followed by analysis of the data using the Benesi-Hildebrand method,[14] we obtained affinity constants of 2.2×10^4 M^{-1} and 1.2×10^4

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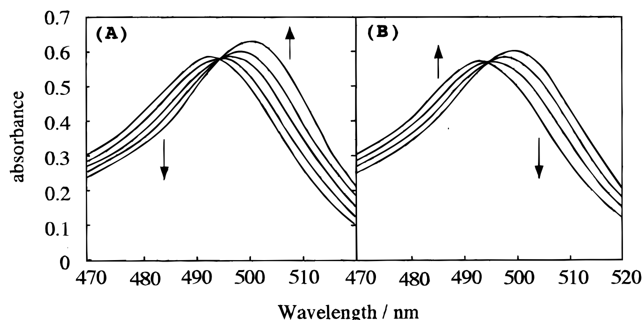

Figure 1. (A)UV/visible absorption spectra of **2** upon addition of **1a** (0, 3.5, 10, 50, 80 μM). (B) IP_3, (0, 26, 42, 60 μM) in the presence of **1a** (75 μM) and **2** (10 μM). All are in 10mM HEPES buffer (pH = 7.4).

Table 1. Association Constants (K_a) of Receptor **1a** and **1b** for Anionic Guests or Inositol at 20 °C, 10 mM Hepes Buffer (pH 7.4)[a]

guest	$K_a/10^5$ M^{-1}	
	1a	**1b**
2	0.22	0.12[b]
IP_3	4.7	5.0[b]
benzene-1,3,5-trisphosphate	5.0	5.5[b]
phytic acid	7.5	not determined[b,c]
ATP	0.231.	1.6[b]
fructose-1,6-diphosphate	0.22	1.6[b]
citrate	0.08	0.45[b]
EDTA	0.02	0.2[b]
inositol	<0.005	<0.005[b]

[a] All K_a were obtained by a UV/Vis competition method [**1a**] = 75 μM, [**1b**] = 150 μM, [**2**] = 10 μM. [b] There are no isosbestic points. [c] The absorbance change was not saturated as a function of the guest concentration. Error is <10% for **1a** and <30% for **1b**.

M^{-1} for **2** binding to **1a** and **1b**, respectively. The fluorescence of **2** behaved similarly upon the addition of **1a** or **1b**.

Addition of IP_3 as well as that of other anionic guests (listed in Table 1) to a solution of the complexes formed between **1a**/**1b** and **2** (75 μM **1a** or 150 μM **1b** and 10 μM **2**) resulted in displacement of **2** and a subsequent blue shift (Figure 1B). The absorption curves showed a clean isosbestic point indicating smooth conversion of a 1-to-1 complex between **1a** and **2** to a 1-to-1 complex of **1a** and anion. This stoichiometry was confirmed with a Job plot for the binding of **1a** with IP_3 using ^{1}H NMR. However, not all competition assays with **1b** gave clean isosbestic points. This, along with the occasional observation of cloudiness with **1b**, indicated that some form of aggregation was occurring. Binding constants for the anions to both **1a** and **1b** were calculated using the typical mathematical linearization method applied to competition experiments.[14]

As indicated in Table 1, IP_3 and the similar structure benzene-1,3,5-trisphosphate bound to receptor **1a** with higher association constants than all the other anions tested except for phytic acid (a hexaphosphate). All of the guests had nearly equal or higher binding constants for **1b** than for **1a**. However, the selectivity in the recognition process was much less pronounced and the effect of ionic strength was much more pronounced with **1b**. With the addition of 50 mM NaCl, the K_a for **1a** and IP_3 remained high (8.2×10^4 M^{-1}), but that of **1b** decreased significantly (<1.0×10^4 M^{-1}). This salt effect indicates that the electrostatic attraction involved in the ion pairing between ammoniums and simple salts is stronger than with guanidiniums,[15] resulting in an increase in nonspecific complex formation with ammoniums. Similar nonselective binding by ammoniums has also been observed in the comparison between the binding of polylysine

(15) Similarly, when using guanidinium- and ammonium-based receptors that are not designed to complement particular guests, the ammonium receptors typically have stronger interactions with phosphates and carboxylates. Dietrich, B.; Fyles, D. L.; Fyles; T. M.; Lehn, J-. M. *Helv. Chim. Acta* **1979**, *62*, 2763−2787.

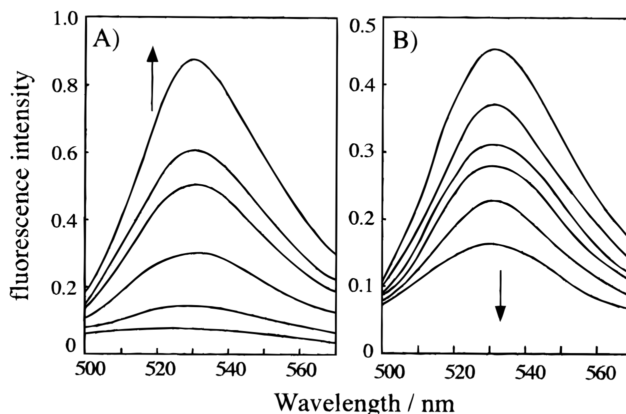

Figure 2. (A) Fluorescent spectra of **2** upon addition of receptor **1a** (0, 50, 150, 350, 500, 700 nM) in methanol. (B) IP_3 (0, 2, 4, 6, 8, 18 nM) in the presence of **1a** (150 nM) and **2** (16 nM). Excitation is at 450 nm.

and polyarginine peptides with TAR RNA.[16] In the comparison between **1a** and **1b,** the use of guanidiniums gives less nonspecific binding thereby allowing the shape and preorganization of the receptor to guide the recognition process.

To enhance the affinity of receptor **1a** for IP_3, we performed similar assays in methanol. In methanol, **2** prefers a cyclized form in which the 2-carboxylate has undergone an intramolecular conjugate addition to the quinoid structure. This form of **2** is colorless and nonfluorescent.[17] Upon addition of **1a**, the yellow color reappears as does the fluorescence (Figure 2A). The positive character of the receptor induces a ring opening to give the colored/fluorescent form of **2**. Using the Benesi-Hildebrand method, we obtained a binding constant of 1.2×10^5 M^{-1} for **1a** and **2.** As anticipated on the basis of the differences in the spectroscopy of **2** when it is bound to **1a** or free in solution, addition of IP_3 to a solution of **1a** and **2** resulted in a decrease of absorbance and fluorescence (Figure 2B) due to release of **2** into the methanol solution. Using this competition, we obtained a binding constant of 1.0×10^8 M^{-1} for IP_3 and **1a**. There was no advantage using **1b** in methanol since the binding constant between **1b** and IP_3 was found to be 6.3×10^5 M^{-1}.

Since fluorescence spectroscopy is a much more sensitive technique than UV/vis spectroscopy and the use of methanol gave significantly stronger binding between **1a** and **2** as well as between **1a** and IP_3, the monitoring of fluorescence was found to be the method of choice for sensing nanomolar concentrations of IP_3. In the absence of any competitive molecules beside 10 mM HEPES buffer, we found that the addition of IP_3 to an ensemble of **1a** and **2** in water can detect and quantitate IP_3 at a concentration as low as 1 μM. Importantly, in methanol a 2 nM IP_3 concentration was easily detected (see the fluorescence changes shown Figure 2B). A detection level in the nanomolar range is appropriate for the development of an assay using methanol as an eluent and CE to sample and fractionate cellular components. We are currently using our sensing ensemble to spectroscopically signal the presence of IP_3 during signal transduction, and our results will be reported in due course.

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