Real-Time Assay of Inorganic Pyrophosphatase Using a High-Affinity Chelation-Enhanced Fluorescence Chemosensor

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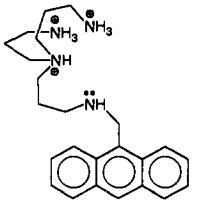
Received June 14, 1993

While an ability to monitor the concentrations of simple anions in real time is surely as desirable as that for metal ions, there is almost no literature on the chemosensing of monomeric, inorganic anions in water. Batch methods have been described for fluorimetric chemodosimetry of several halides,[1] but *sensing* methods (i.e., nondestructive and reversible) reported to date all rely on the ability of the analyte (e.g., iodide) to quench collisionally the fluorescence of compounds such as rhodamine 6G.[2] Thus, it would seem useful to apply the substantial literature on anion recognition to this purpose.[3] As a signal type, fluorescence is both more sensitive than absorption spectroscopies and more amenable to the conception of signal transduction mechanisms on the molecular level. We have reported previously that the aqueous complexation of anions such as phosphate and sulfate to anthrylpolyamines (e.g., **1**) results in chelation-enhanced fluorescence (CHEF) signaling.[4] Compound **1** proved to be the



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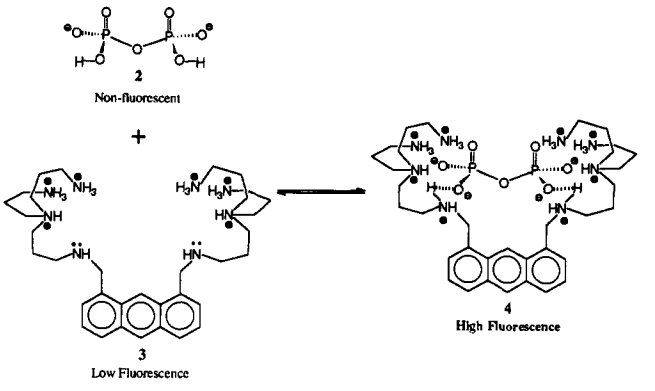
first fluorescent chemosensor for an inorganic anion that does not rely on an inherent quenching property of the anion. Even so, it is a conception far from usable form. For example, chemosensor **1** binds phosphate at pH 6 with K_d = 150 mM; in other words, the midpoint of the decade-wide titration occurs at a phosphate concentration of 150 mM, which is very high for many applications. In an effort to improve upon the conceptual design of anion sensors, we have synthesized a convergent chemosensor demonstrating anion binding in a more useful concentration range (i.e., micromolar K_d) and designed to discriminate between phosphate (P_i) and pyrophosphate (PP_i) ions on the basis of size. The resulting chemosensor binds pyrophosphate over 2000 times more tightly than phosphate, permitting the real-time monitoring of pyrophosphate hydrolysis.

Chemosensor **3**, prepared by the reaction of 1,8-bis(bromomethyl)anthracene[5] with excess tris(3-aminopropyl)amine (TRPN),[6] yields a pH–fluorescence titration with pK_a^{obsd} = 6.7; thus, anion binding could be measured at pH 7, even though anion binding using **1** (pK_a^{obsd} = 5.4) could not.[7] Besides possessing a high charge density at neutral pH, the anion receptor groups of **3** are geometrically disposed to bind both sides of pyrophosphate simultaneously. Our previously reported rationale for CHEF signaling of phosphate[4] applies similarly to the current situation, as shown in Scheme 1. We anticipated that the binding of pyrophosphate (nonfluorescent) to chemosensor **3** (low fluorescence intensity at pH 7 consistent with photoinduced electron transfer) would yield enhanced fluorescence as a result of intracomplex amine protonation. Anion titrations were thus carried out in pH 7 solution containing 4 μM **3** and excess cyclen (1 mM), which does not itself bind to **3** but does chelate adventitious transition metal ions. We were gratified to observe (Figure 1) that the binding of pyrophosphate to **3** occurs with fluorescence enhancement (λ_{ex} 368 nm, λ_{em} 414 nm) and that the 1:1 complexation occurs with K_d = 2.9 μM under these conditions.[8] By contrast, the association of **3** to phosphate occurs with a K_d of 6.3 mM, which is only 13 times tighter binding than that of phosphate with 1-(TRPNmethyl)anthracene (not shown; K_d = 82 mM). Thus, chemosensor **3** displays a pyrophosphate/phosphate discrimination of 2200-fold at pH 7.[9]

The ion selectivity, which is also readily apparent in Figure 1, allows for real-time assay of pyrophosphate hydrolysis. For example, the fluorescence of a reaction containing 20 μM pyrophosphate would be expected to decrease (by 54%) on conversion at constant pH to 40 μM phosphate (Scheme 2). This reaction is catalyzed by inorganic pyrophosphatase, a Mg(II)-

Scheme 1



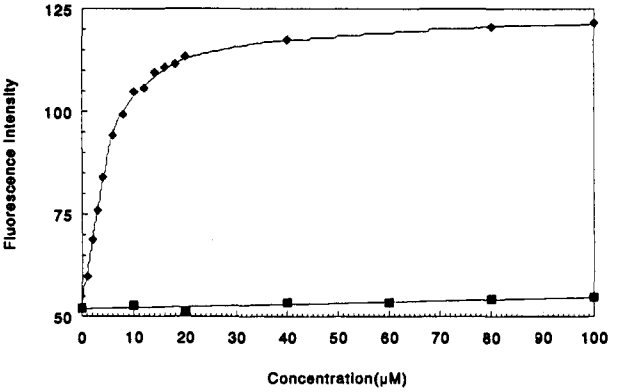


Figure 1. Chemosensor **3** titrations with Pi and PPi. 1 mM cyclen, 50 mM HEPES, pH 7. ◆, PPi; ■, Pi.

(1) Guilbault, G. G. In *Practical Fluorescence*; Guilbault, G. G., Ed.; Marcel Dekker, Inc.: New York, 1990; Chapter 5.

(2) Wyatt, W. A.; Bright, F. V.; Hieftje, G. M. *Anal. Chem.* **1987**, *59*, 2272.

(3) Lehn, J.-M. *J. Inclusion Phenom.* **1988**, *6*, 351. For an excellent overview of the great variation achievable in the design of anion receptors, see: Schmidtchen, F. P. *Nachr. Chem., Tech. Lab.* **1988**, *8*, 10.

(4) Huston, M. E.; Akkaya, E. U.; Czarnik, A. W. *J. Am. Chem. Soc.* **1989**, *111*, 8735. The general issue of multiple ionization states at pH 7 has been addressed in footnote 12 of this paper. An expansion on the subject of intramolecular PET in (aminomethyl)anthracenes is available: Beeson, J. C.; Huston, M. E.; Pollard, D. A.; Venkatachalam, T. K.; Czarnik, A. W. *J. Fluorescence* **1993**, *3*, 65.

(5) Akiyama, S.; Nakagawa, M. *Bull. Chem. Soc. Jpn.* **1971**, *44*, 3158–3160.

(6) A description of the synthesis of compound **3**, together with all characterization data (UV, ^{1}H and ^{13}C NMR, mass spectroscopy, and microanalysis), is included in the supplementary material.

(7) While the fluorimetrically determined pK_a^{obsd} for **1** describes its +3 → +4 protonation, pK_a^{obsd} for **3** likely is an aggregate of two +3 → +4 protonations, one at each TRPN group. The 9,10-isomer (ref 4) cannot be used for sensing at pH 7 owing to its substantially lower pK_a^{obsd}.

(8) Binding constants were determined by using the computer program ENZFITTER, available from Elsevier-BIOSOFT, 68 Hills Rd, Cambridge, CB2 1LA U.K. Due to formation of a low affinity **3**-$(PP_i)_2$ complex, K_d for the **3**-PP_i complex required an estimation of the 1:1 infinity fluorescence intensity.

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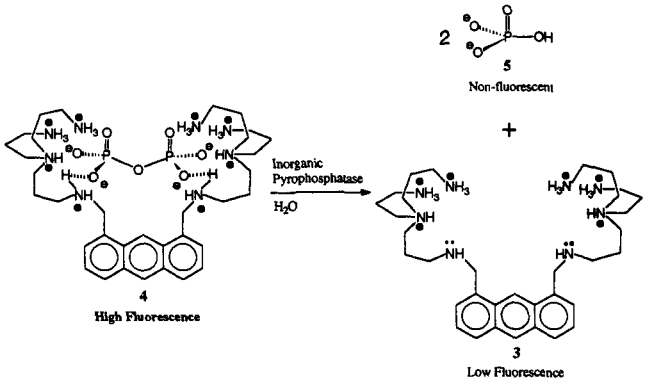
Scheme 2



dependent[10] hydrolase that serves to drive reactions dependent upon PP_i release by further hydrolysis to P_i.[11] Previously reported assay methods for this enzyme include phosphomolybdate analysis, paper electrophoresis, starch gel electrophoresis, isotopic labeling, pH stat analysis, and enzymic UV coupling.[12] To test the use of chemosensor **3** for this purpose, we prepared solutions containing 50 mM HEPES, 1 mM cyclen, 20 μM $Na_4P_2O_7$, 20 μM $Mg(ClO_4)_2$, and 4 μM **3**. Inorganic PPase from baker's yeast was added, and, after a brief mixing period (30 s), fluorescence intensity (λ_{ex} 368 nm, λ_{em} 414 nm) was monitored as a function of time. As shown in Figure 2, the hydrolysis of pyrophosphate generates the predicted fluorescence response, with the rate of hydrolysis increasing as does the enzyme concentration (curves shown are not calculated).

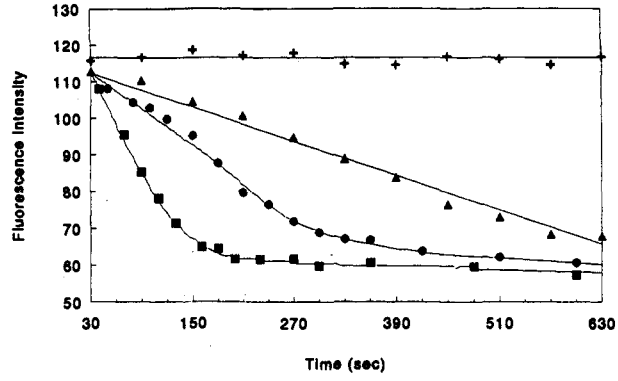


Figure 2. PPase assay using chemosensor **3**. +, 0 units; ▲, 0.4 units; ●, 0.8 units; ■, 1.6 units.

In summary, we report (1) the synthesis of a new fluorescent chemosensor with convergent anion reception; (2) micromolar affinity for pyrophosphate, a monomeric, inorganic anion; and, (3) a nondestructive, real-time assay for inorganic pyrophosphatase. These results may provide further incentive for the development of anion-selective host molecules whose straightforward conjugation to fluorophores will yield new chemosensors for anion monitoring.

Acknowledgment. D.H.V. is a National Needs Fellow of The Ohio State University; we thank Profs. Weldon Mathews and Daniel Leussing for their efforts in making this resource available. FT-NMR spectra were obtained using equipment funded in part by NIH Grant No. 1 S10 RR01458-01A1. A.W.C. thanks the A. P. Sloan and the Camille and Henry Dreyfus Foundations for support in the form of Fellowships and Eli Lilly and Co. for support in the form of a Granteeship.

Supplementary Material Available: Experimental methods for the syntheses of compounds **3** and 1-[(tris(3-aminopropyl)amino)-methyl]anthracene HCl (1-(TRPNmethyl)anthracene); pH–fluorescence profiles for compounds **3** and 1-(TRPNmethyl)-anthracene; fluorescence titrations of compounds **3** and 1-(TRPNmethyl)anthracene with P_i and with PP_i (4 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(9) It is of special interest to note that the closely related anthrylpolyamine 1,8-bis(cyclenylmethyl)anthracene (Vance, D. Ph.D. Thesis, The Ohio State University, Columbus, OH, 1993) displays *no* change *in* fluorescence intensity upon anion introduction. While this might result from a virtual lack of association even at very high anion concentrations, we believe instead that it results from a lack of signal transduction *upon* binding. The pK_a values of cyclen itself (<1, 1.15, 9.60, 10.53) guarantee that this potential chemosensor will exist with each cyclen in the dication form. A third protonation is prohibitive, meaning that intracomplex protonation as in Scheme 1 will not occur. The negative result in this case provides evidence in favor of our working mechanism for signal transduction.

(10) Mg(II) does not bind to TRPN with high affinity; in fact, fluorescence assay shows no measurable binding under these concentrations. Therefore, the use of a Mg(II)-containing assay solution is compatible with chemosensor **3**. Polyamine–metal ion complexation proves more of a problem when enzyme activities are evaluated in the presence of transition metal ions, for which a metal–ion insensitive chemosensor is preferable: Hong, S.-Y.; Czarnik, A. W. *J. Am. Chem. Soc.* **1993**, *115*, 3330.

(11) (a) Josse, J.; Wong, S. C. K. In *Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, NY, 1971; Vol. 4, p 499. (b) Butler, L. G. In *Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, NY, 1971; Vol. 4, p 529.

(12) (a) Josse, J. *J. Biol. Chem.* **1966**, *241*, 1938. (b) Moe, O. A.; Butler, L. G. *J. Biol. Chem.* **1972**, *247*, 7308. (c) Cooperman, B. S.; Chiu, N. Y.; Bruckman, R. H.; Bunick, G. J.; McKenna, G. P. *Biochemistry* **1973**, *12*, 1665. (d) Ryan, L. M.; Koxin, F.; McCarty, D. J. *Arthritis Rheum.* **1979**, *22*, 892. (e) Baltscheffsky, M.; Nyrén, P. *Methods Enzymol.* **1988**, *126*, 538. Only the pH stat method is nondestructive with real-time signaling, but it is not useful in the micromolar concentration range.