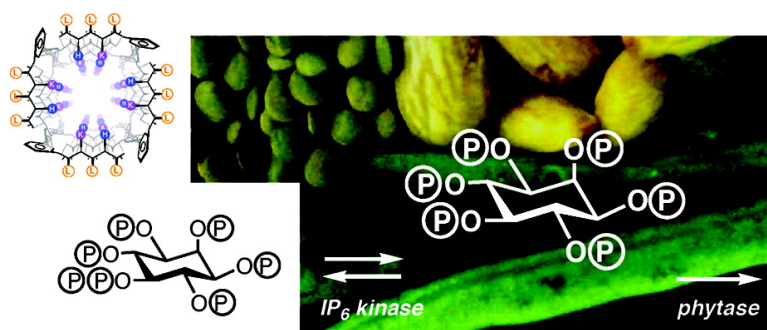## Communication

Fluorometric Detection of Inositol Phosphates and the Activity of Their Enzymes with Synthetic Pores: Discrimination of IP and IP and Phytate Sensing in Complex Matrices

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Fluorometric Detection of Inositol Phosphates and the Activity of Their Enzymes with Synthetic Pores: Discrimination of IP7 and IP6 and Phytate Sensing in Complex Matrices

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The use of synthetic ion channels and pores[1] as multianalyte sensors in complex matrices[2] is a topic of current scientific concern.[3] Here, we introduce synthetic pore **1** as a fluorometric, noninvasive (label-free), and user-friendly "naked-eye" sensor for inositol phosphates (IP_n's) and their enzymes, as well as for sensing in samples from the supermarket such as soybeans, almonds, and lentils (Figure 1). These are significant findings as IP_n's play numerous important roles in nutrition,[4] environmental sciences,[5] and biological processes such as cell signaling and bioenergetics[6] but do not contain an intrinsic chromophore for label-free sensing in multiwell assays. For example, the majority of phosphorus in cereal and legumes is present as phytate (inositol hexaphosphate, IP_6, Figure 1), and phytate profiling as well as phytase bioengineering are of interest to identify and liberate this otherwise lost source of nutritional phosphorus.[4,5] Less phosphorylated inositols are central in cellular signaling, and the more recent discovery of the "overphosphorylated" IP_7 (Figure 1) isomers is attracting attention for many reasons including the possibility of reversible IP_6/IP_7 interconversion as an ADP/ATP equivalent in bioenergetics and in protein diphosphorylation.[6]

Synthetic multifunctional pore **1** (Figure 1) was selected to elaborate on IP_n sensing and was synthesized as reported previously.[3a] This pore is formed by a rigid-rod β-barrel.[3] These versatile barrel-stave supramolecules are constructed from rigid *p*-octiphenyl rods that are brought together by short antiparallel β-sheets. In barrel **1**, the peptide sequence (LKLHL) was selected to produce a hydrophobic outer surface (LLL) and functional group arrays at the inner surface (KH). Molecular recognition within pore **1** can be easily seen, with the naked eye, if desired, as pore inactivation. The ability to discriminate between blockage by substrates and products identified pore **1** as an optical transducer of reactions, and the combination with enzymes as specific signal generators provided access to sensing.

The synthesis of the IP_7 stereoisomer (i.e., D-*myo*-5-PP-IP$_5$, Figure 1) will be reported elsewhere, and all other IP_n blockers were commercially available. The responsiveness of pore **1** to inositol phosphates was determined under standard conditions (Figure 2A).[3,7] Namely, egg yolk phosphatidylcholine large unilamellar vesicles (EYPC LUVs) were loaded with 5(6)-carboxyfluorescein (CF) at concentrations high enough for self-quenching. The CF efflux in response to the addition of pore **1** then turns on CF fluorescence. To detect molecular recognition, the same experiment is repeated in the presence of pore blockers at various concentrations (Figure S1), and the resulting changes in pore activity are summarized as dose response curves (Figure 2).

The dose response curves obtained for inositol phosphates revealed increasing blocker efficiency with increasing blocker
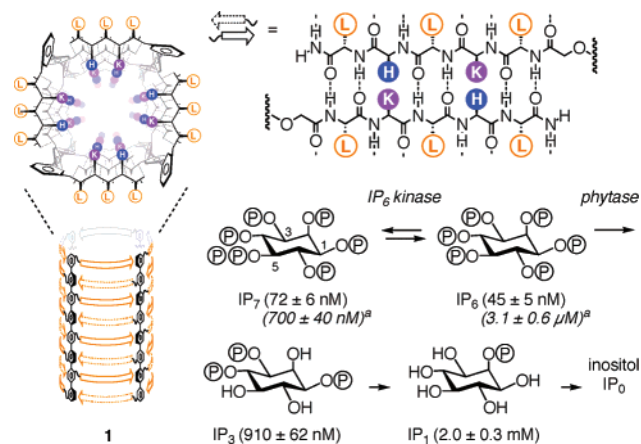

Figure 1. Rigid-rod β-barrel pore **1** and inositol phosphate blockers (IP_n, P = $PO_3H_n^{-(2-n)}$ or PP = $P_2O_6H_n^{-(3-n)}$) with their IC_{50}'s in parentheses; [a]IC_{50} in the presence of $\sim$30 μM $ZnCl_2$. β-Sheets are given as solid (backbone) and dotted lines (hydrogen bonds, *top*) or as arrows (N $\rightarrow$ C, *bottom*); external amino acid residues are dark on white (L, leucine), and internal ones, white on dark (K, lysine; H, histidine).
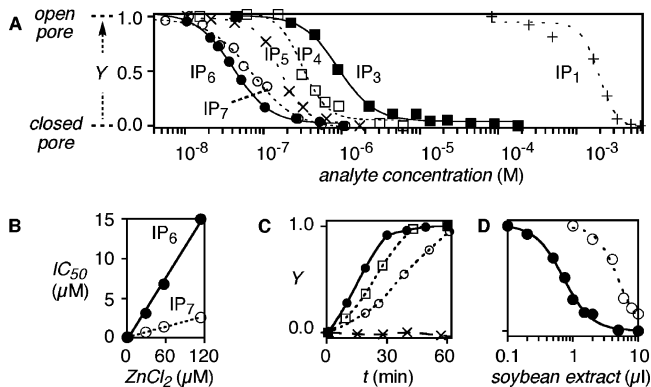

Figure 2. (A) Dependence of the fractional activity Y of pore **1** on the concentration of inositol phosphates ($\bigcirc$, IP_7; $\bullet$, IP_6; $\times$, IP_5; $\square$, IP_4; $\blacksquare$, IP_3; +, IP_1). (B) Dependence of the IC_{50} of IP_7 ($\bigcirc$) and IP_6 ($\bullet$) on $ZnCl_2$. (C) Time course of IP_6 conversion with varying concentrations of phytase ($\bullet$, 0.66; $\square$, 0.44; $\bigcirc$, 0.22; $\times$, 0 units/mL), as measured by Y. (D) Pore **1** blockage with soybean extract before ($\bigcirc$) and after ($\bullet$) incubation with phytase (0.97 units/mL, 30 min). Determined from fractional change in CF emission I (λ_{ex} 492 nm, λ_{em} 517 nm) in response to the addition of samples and **1** to CF-loaded vesicles.[7]

charge (Figure 2A). The IC_{50} of D-*myo*-2-IP$_1$ (i.e., the IP_1 concentration needed to cause 50% pore blockage) was nearly negligible. With an IC_{50} of 45 $\pm$ 5 nM, pore blockage by IP_6 was nearly 4 orders of magnitude more effective. This substoichiometric binding relative to the β-barrel concentration (375 nM monomer, $\leq$94 nM tetrameric pore) is well below the 1 μM limit, where the

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Table 1. Phytate Sensing in Complex Matrices with Pore **1**[a]

entry	sample[b]	expected (mg/g)[c]	found (mg/g)[a]
1	soybeans	10−28	9.5 ± 1
2	almonds	21	19 ± 2
3	lentils	2.3	1.5 ± 0.5

[a] Determined from differential dose response curves for the blockage of pore **1** in fluorogenic vesicles by extracts that were or were not exposed to phytase (Figure 2D). [b] Routine sample preparation (solid−liquid extraction, ion exchange).[7] [c] From literature.[7]

onset of contributions from stoichiometric binding usually obscures any intrinsic sensitivities and selectivities.[3,8] This substoichiometric blockage indicated that phytate binds to unstable[9] supramolecular pores that act at ≤45 nM. Control experiments revealed that phytate blockage of pore **1** was 132 times better than the inactivation of synergistic polyarginine-dodecylphosphate (pR-DP) anion carriers[10] ($IC_{50} = 5.4 \pm 1.1\ \mu M$, Figure S11). This finding confirmed previous conclusions that pR-DP sensors are cost-effective alternatives for routine applications, whereas structured synthetic pore sensors show greater potential for sensitive and selective binding.[3,10]

The discrimination of ATP and ADP with the naked eye at low concentrations has been accomplished recently with pore **1** and was used for sucrose, lactose, and acetate sensing.[3] Compared to this challenge, fluorometric on−off discrimination of IP_6 and IP_7 appeared even more difficult. Under standard conditions, the IC_{50} for IP_7 was 72 ± 6 nM, thus precluding the IP_6/IP_7 discrimination necessary for fluorometric detection of IP_6 and IP_7 molecular conversion (Figure 1 and 2A). To liberate eventual pore selectivity from suppression by (sub)stoichiometric binding,[8] the dependence of IP_6/IP_7 discrimination on Zn^{2+}, a competitive phosphate binder, was determined. In the presence of ∼30 to 120 μM Zn^{2+}, IP_6 and IP_7 pore blockage was sufficiently weakened to reveal discrimination factors $D = 4−6$ (Figure 2B). This is sufficient to discriminate IP_7 and IP_6 at submicromolar concentrations and to detect the activity of their enzymes such as IP_6 kinase with the naked eye.[3] Zn^{2+}-mediated IP_6/IP_7 discrimination was reversible with EDTA and was not observed with Fe^{3+}, Ca^{2+}, and Mg^{2+} (Figures S5, S6).

The ability of phytate to block pore **1** decreased with increasing time of incubation with phytase (Figure 2C). Dependence of the velocity of this process on phytase concentration confirmed fluorometric detectability of the activity of this important enzyme family with pore **1**. To elaborate on sensing in complex matrices, soybean extracts were prepared following routine procedures.[7] These extracts were found to efficiently block pore **1** (Figure 2D, ●). Subtraction of the phytase-resistant contributions to pore blockage (Figure 2D, ○) and comparison with calibration curves gave a phytate content of 9.5 ± 1 mg/g in soybeans (Table 1, entry 1). In this approach, phytase substrates other than IP_6 are naturally recorded as false positives. With a content of less than 30% of that of IP_6 in food samples[4] and a 3.6 times weaker blockage efficiency (myo-1,3,4,5,6-IP_5, $IC_{50} = 160 \pm 7$ nM, Figure 2A), eventual contributions of IP_5 to the phytate content determined with pore **1** are small. Even poorer blockage efficiency and lower content in plant samples also excludes significant contributions from IP_4 (D-myo-1,4,5,6-IP_4, $IC_{50} = 310 \pm 30$ nM, Figure 2A) and lower IP_n's

to phytate sensing. The phytate content determined for soybeans, almonds, and lentils were in meaningful agreement with literature values and results from other assays (Tables 1, S4).

In conclusion, we demonstrate that synthetic pores can be used to detect the presence of inositol phosphates as pore closing and the activity of their enzymes as pore opening. Zn^{2+}-mediated discrimination of IP_7 and IP_6 demonstrates that the naturally poor selectivity of substoichiometric, nanomolar binding of privileged analytes can be overcome with the judicious use of additives. The availability of an assay for IP_7 with nanomolar sensitivity could have a significant impact on the study of protein diphosphorylation and cellular signaling pathways regulated by IP_7.[6] Sensing applications with synthetic pores rely on the selectivity of enzymes, where the difference in pore blockage before and after enzymatic treatment reveals the concentration of the substrate analyte. Here, the compatibility of this approach with complex matrices is confirmed with phytate sensing in almonds, soybeans, and lentils. Solutions to eliminate eventual false positives from insufficient substrate specificity must naturally come from enzymology as well. Possibilities include multienzyme cascade signal generators, enzyme engineering, or kinetic enzymatic discrimination. Preliminary results suggest that phytase alone may already be sufficient for kinetic discrimination of IP_7, IP_6, and IP_5 (Figure S2).

Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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