Implementation of anion-receptor macrocycles in supramolecular tandem assays for enzymes involving nucleotides as substrates, products, and cofactors†

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A supramolecular tandem assay for direct continuous monitoring of nucleotide triphosphate-dependent enzymes such as potato apyrase is described. The underlying principle of the assay relies on the use of anion-receptor macrocycles in combination with fluorescent dyes as reporter pairs. A combinatorial approach was used to identify two complementary reporter pairs, *i.e.* an amino-γ-cyclodextrin with 2-anilinonaphtalene-6-sulfonate (ANS) as dye (fluorescence enhancement factor of 17 upon complexation) and a polycationic cyclophane with 8-hydroxy-1,3,6-pyrene trisulfonate (HPTS) as dye (fluorescence decrease by a factor of more than 2000), which allow the kinetic monitoring of potato apyrase activity at different ATP concentration ranges (μM and mM) with different types of photophysical responses (switch-ON and switch-OFF). Competitive fluorescence titrations revealed a differential binding of ATP (strongest competitor) *versus* ADP and AMP, which constitutes the prerequisite for monitoring enzymatic conversions (dephosphorylation or phosphorylation) involving nucleotides. The assay was tested for different enzyme and substrate concentrations and exploited for the screening of activating additives, namely divalent transition metal ions (Ni^{2+}, Mg^{2+}, Mn^{2+}, and Ca^{2+}). The transferability of the assay could be demonstrated by monitoring the dephosphorylation of other nucleotide triphosphates (GTP, TTP, and CTP).

Introduction

Numerous supramolecular receptors for nucleotides have already been introduced with the general aim to achieve a high selectivity and the ultimate goal to construct highly specific biological chemosensors.[1–19] But applications to actual biological systems have remained scarce. With the tandem assay strategy, we have recently developed a supramolecular application, which reduces the key requirement for a receptor from the specificity for a single analyte to the mere necessity to show a differential binding between two analytes: the substrate and the product of an enzymatic reaction.[20–23] This affords convenient label-free enzyme assays, in which the enzymatic conversion is monitored through a reporter pair composed of a fluorescent dye and a macrocycle, which responds to the concentration changes affected by the enzymatic reaction. This involves, drawing inspiration from the indicator-displacement principle,[24,25] either a continuous displacement of the dye from the macrocycle by the higher affinity product (product-selective assay), or its uptake into the host, facilitated by the depletion of the competing substrate (substrate-selective assay).

At least two designed chemosensors with covalently incorporated chromophores have been previously demonstrated to be principally suitable for following enzymatic reactions in a non-continuous manner.[1,2] But in order to monitor an enzymatic reaction, it is in fact not required to employ a highly specific receptor with a covalently attached fluorophore, because the specificity of the reaction is determined through the enzyme itself. This principal insight is similar to that reached by Matile and coworkers, who have followed enzymatic reactions by using multifunctional synthetic pores embedded in vesicles,[26,27] with the difference that supramolecular tandem assays allow direct continuous monitoring in homogeneous solution.

While our previous examples of tandem assays were limited to cationic analytes in combination with cation-receptors,[20–23] the investigation of anionic metabolites such as nucleotides, and particularly ATP, requires the use of macrocycles with anion-receptor properties,[28] which we have now investigated.

Results and discussion

We have first screened a combinatorial library consisting of a set of 10 water-soluble anion receptors, which in part were already known to bind to nucleotides,[11–15] in combination with a set of 9 anionic and neutral water-soluble fluorescent dyes (Charts 1 and 2). To develop practical tandem assays, our aim was to obtain *i*) a large fluorescence response upon dye complexation, *ii*) a strong macrocycle-dye binding, and *iii*) a large differentiation in binding between nucleotide tri- and monophosphates. Our best (and new) reporter pair consists of the amino-γ-cyclodextrin **1** (the amino groups of which are positively charged near neutral pH) in combination with 2-anilinonaphtalene-6-sulfonate (ANS) as dye. A complementary reporter pair, originally suggested for the selective recognition of GTP,[10,11] consists of the cyclophane **2** with 8-hydroxy-1,3,6-pyrene trisulfonate (HPTS) as dye.

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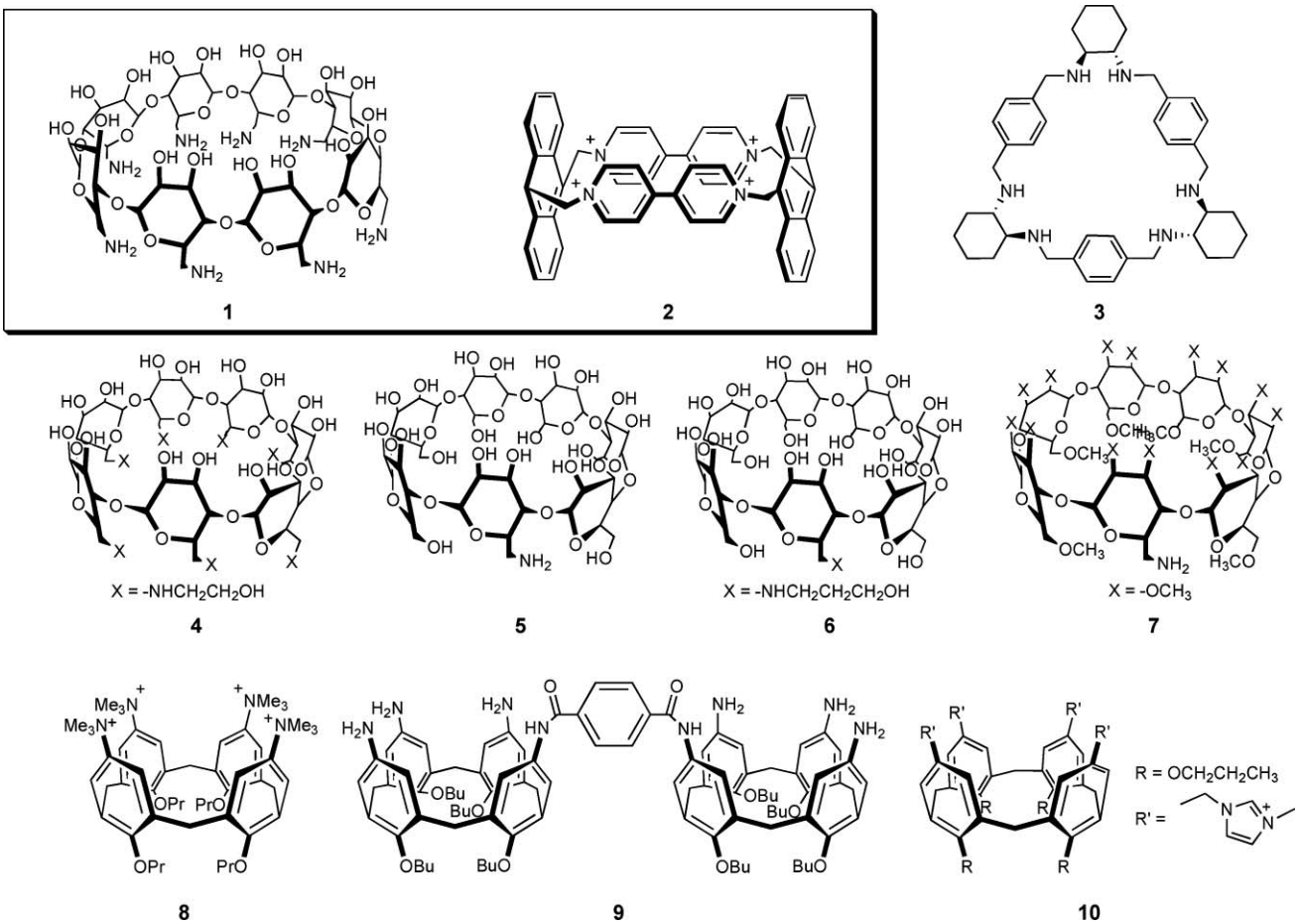


Chart 1 Investigated library of macrocycles with anion-receptor properties.

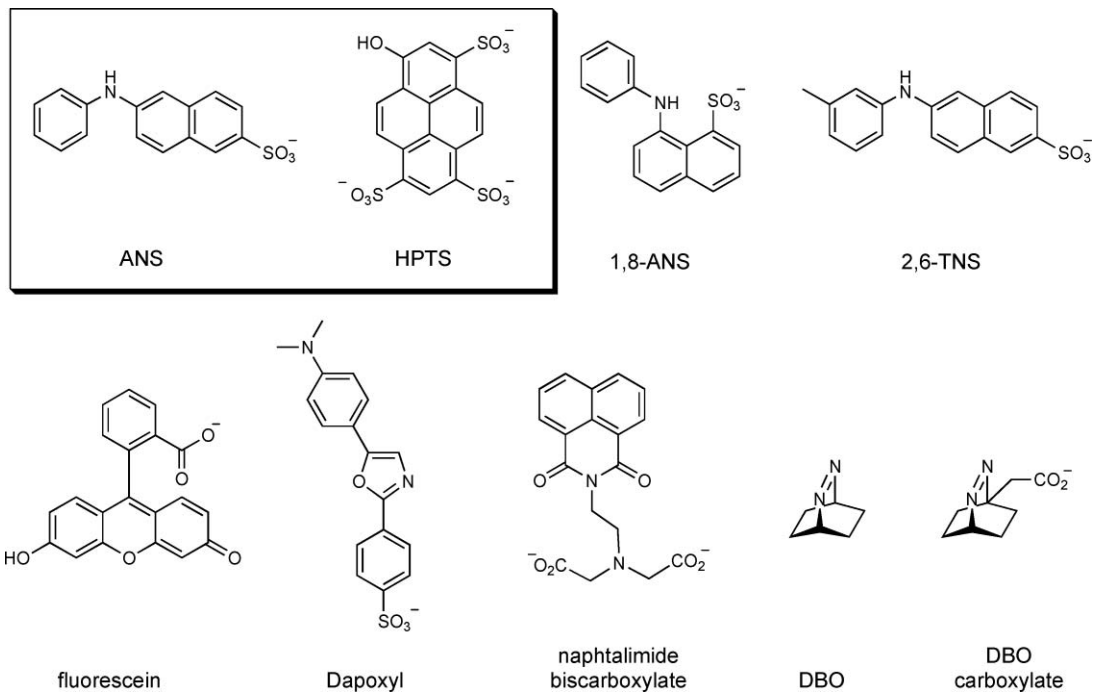


Chart 2 Investigated library of anionic and neutral fluorescent dyes studied in combination with the macrocycles in Chart 1.

Table 1 Binding constants of **1** and **2** with the substrate as well as intermediary and final enzymatic products

Guest	$K/10^6$ M^{-1} (**1**)[a]	$K/10^3$ M^{-1} (**2**)[b]
ATP	100 ± 60	4.5 ± 1.0
ADP	2.8 ± 0.8	< 1
AMP	0.011 ± 0.003	< 1
Orthophosphate	0.05 ± 0.01	< 0.1

[a] Determined by competitive fluorescence titration using **1**/ANS in 50 mM NaOAc buffer, pH 5.5, *cf.* Fig. 2a. The binding constant of **1**/ANS is $(1.7 \pm 0.1) \times 10^4$ M^{-1} *cf.* Fig. 1a. [b] Determined by competitive fluorescence titration using **2**/HPTS in 10 mM phosphate buffer, pH 7.4. The binding constant of **2**/HPTS was determined as $(7 \pm 3) \times 10^6$ M^{-1}, *cf.* Fig. 1b.

Successive addition of macrocycle to dye solutions resulted in a strong fluorescence enhancement by a factor of 17 (λ_{exc} = 318 nm, λ_{obs} = 462 nm, for **1**/ANS) or quenching by a factor of more than 2000 (λ_{exc} = 425 nm, λ_{obs} = 512 nm, for **2**/HPTS), from which dye binding constants of 1.7×10^4 and 7×10^6 M^{-1} were determined (Fig. 1). Addition of different nucleotides reversed these fluorescence changes due to competitive binding (Fig. 2 and Fig. 3). Both systems showed a charge-dependent differentiation of nucleotides (*e.g.*, between ATP, ADP, AMP, and orthophosphate),[10] which is the sufficient and necessary criterion for our presently explored application (*cf*. binding constants in Table 1). Specifically, we implemented the **1**/ANS and **2**/HPTS

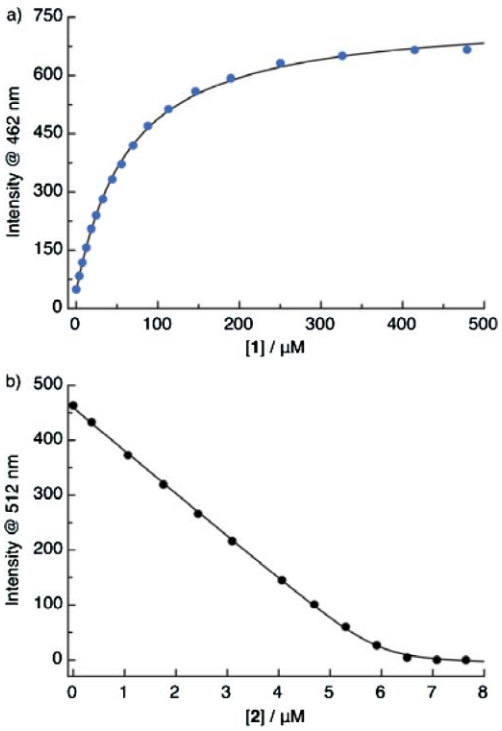


Fig. 1 Plots of the fluorescence intensity of a) 10 μM ANS with increasing concentrations of **1** in 50 mM NaOAc buffer, pH 5.5 (λ_{exc} = 318 nm, λ_{obs} = 462 nm) and b) 6.3 μM HPTS with increasing concentrations of **2** in 10 mM phosphate buffer, pH 7.4 (λ_{exc} = 425 nm, λ_{obs} = 512 nm). The nonlinear fittings were made by assuming the formation of a 1 : 1 complexation model. It should be noted that the fluorescence of HPTS increased again slightly at higher macrocycle (**2**) concentrations, suggesting the formation of higher-order complexes. An equimolar mixture of **2** and HPTS was consequently employed as reporter pair.

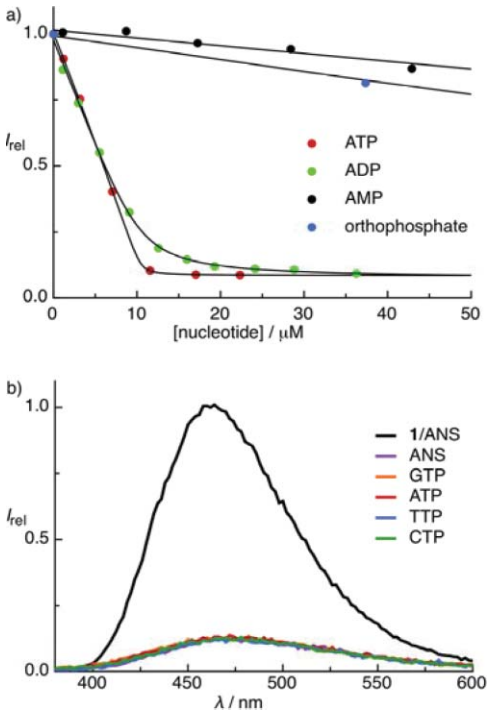


Fig. 2 Changes in fluorescence intensity and spectra of the **1**/ANS reporter pair (10 μM **1** and 25 μM ANS, λ_{exc} = 318 nm) upon displacement by a) varying concentrations of ATP, ADP, AMP, and orthophosphate (λ_{obs} = 462 nm) and b) different nucleotide triphosphates (16 μM), in NaOAc buffer, pH 5.5.

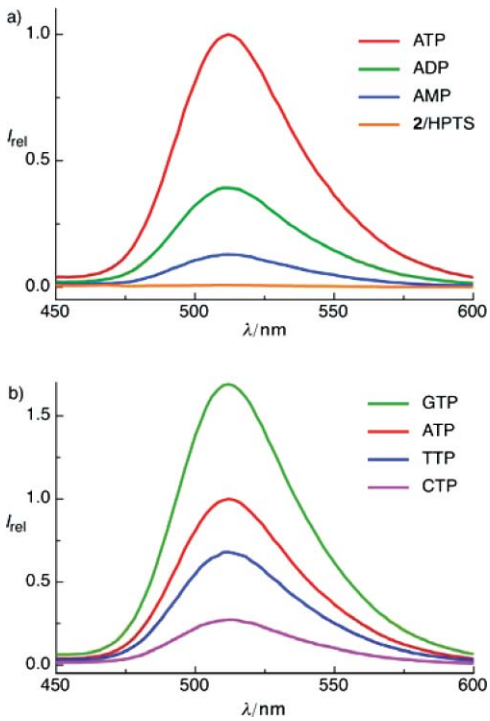


Fig. 3 Changes in fluorescence spectra of the **2**/HPTS reporter pair (both 6.3 μM) upon addition of a) ATP, ADP, and AMP (each 4 mM) and b) different nucleotide triphosphates (4 mM), in succinate buffer, pH 6.5; λ_{exc} = 403 nm.

reporter pairs into supramolecular tandem assays for potato apyrase, an ectoenzyme of the class of ATP-diphosphohydrolases (E.C. 3.6.1.5) which convert ATP to AMP through a stepwise dephosphorylation with ADP as an intermediate.[29–32] Apyrases regulate extracellular nucleotide levels (in animal tissues) and the biosynthesis of starch and cell walls (in plants).

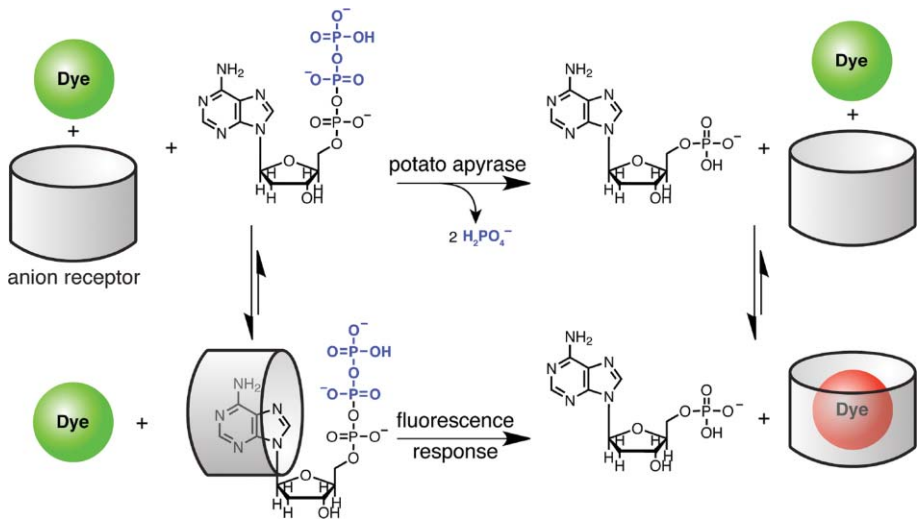
The working principle is illustrated in Scheme 1. Our assay is based on the propensity of the highly positively charged hosts **1** and **2** to form self-assembled complexes with negatively charged guest molecules, particularly ANS and HPTS, as well as ATP. The dye ANS is weakly fluorescent in its uncomplexed form, but experiences an efficient fluorescence enhancement upon complexation with **1**, due to its sensitivity towards environmental polarity.[33] In contrast, the dye HPTS is highly fluorescent in its free form, but suffers from a strong fluorescence quenching when complexed with **2**, presumably by charge-transfer-induced quenching.[10]

Behaving as a strong competitor, ATP displaces the dyes from the complexes, which results in a low or high initial fluorescence intensity, respectively (Fig. 2 and Fig. 3). Upon enzymatic dephosphorylation, AMP and orthophosphate are formed as final products, which behave as weak competitors (due to their lower net negative charge) and thereby allow a successive complexation of the fluorescent dyes with the macrocycles. This results in a switch-ON (for **1**/ANS) or switch-OFF (for **2**/HPTS) fluorescence response,[20] which allows a highly sensitive monitoring of the enzymatic activity. Moreover, since the guest-macrocycle exchange equilibria are much faster than the enzymatic reactions, the response of the system to concentration changes is immediate and allows for real-time continuous kinetic monitoring. It is thus possible to follow the ATP depletion as a result of an enzymatic reaction or, conversely, the enzymatic reaction through the depletion of ATP. Conceptually, the resulting tandem assays can be qualified as substrate-selective assays because the substrate binds more strongly than the product.[22]

The two reporter pairs are complementary in the sense that they show μM or mM affinities to the different nucleotides, respectively (Fig. 2 and Fig. 3). This determines the substrate concentration range of the tandem assays, which were run with 25 μM ATP for **1**/ANS and 2.3 mM for **2**/HPTS. Addition of ATP to the pre-formed reporter pair partially recovered the fluorescence of the uncomplexed dyes through competitive displacement and an approximately 10-fold fluorescence reduction (for **1**/ANS) or more than 100-fold enhancement (for **2**/HPTS) was observed. Addition of apyrase to a solution containing ATP as substrate along with one of the reporter pairs led to a time-resolved rise (factor of 4 for **1**/ANS) or decay (factor of 12 for **2**/HPTS) of the fluorescence intensity with a plateau being reached within 1–2 h (black traces, Fig. 4). In accordance with Scheme 1, we assigned the time-resolved fluorescence response to the enzymatic conversion, thereby establishing a convenient supramolecular tandem assay for apyrases, and generally, for the conversion of ATP. The occurrence of the enzymatic reaction on the same time scale was independently monitored by ^{31}P NMR (Fig. S1 in ESI†). The assay could also be conducted with ADP as substrate (see Fig. S2 in ESI†), but for AMP no significant fluorescence change was noticed, as no dephosphorylation is possible (negative control, data not shown).

Enzyme assays are employed in pharmaceutical-industrial high-throughput screening. Previously, we have demonstrated the potential of tandem assays in the screening of inhibitors,[22] and the apyrase assay offered the complementary opportunity to screen for activators, namely, divalent metal ions (Ni^{2+}, Mg^{2+}, Mn^{2+}, and Ca^{2+}). In all cases, we observed rate enhancements between a factor of 1.3 to 17, with Ca^{2+} and Mn^{2+} being the most potent activators of potato apyrase (Fig. 4),[34] consistent with literature findings obtained by more laborious and complex multi-step colorimetric and radioisotope-based assays.[30–32] Consequently, all enzymatic reactions were subsequently performed with Mn^{2+} (for **1**/ANS) or Ca^{2+} (for **2**/HPTS), which reduced the assay times typically to 10–20 min.

One could argue that the **1**/ANS pair is 2 orders of magnitude more "sensitive" since it allows the conversion of μM instead of mM amounts of ATP to be followed. However, one has to recall that the substrate (or cofactor) concentration range is not the limiting factor for an assay, as it could be for a sensor. Rather, the substrate concentration can be adjusted at will to the desired time and the specific activity of the enzyme, such that even assays with mM substrate concentrations are common.[26] For example, the reported K_M values of potato apyrase ranges



Scheme 1 Working principle of a supramolecular tandem assay for monitoring dephosphorylation of ATP.

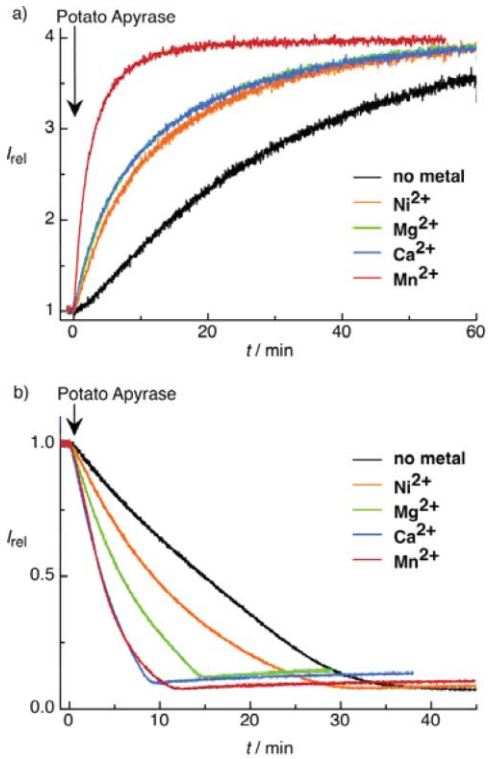


Fig. 4 Evolution of normalized fluorescence intensity monitoring potato apyrase activity with and without activating metal ions (1.7 mM). Assays were initiated by addition of a) 25 μg ml^{-1} enzyme to ATP (25 μM) with the **1**/ANS reporter pair (25 μM both) and b) 100 μg ml^{-1} enzyme to ATP (2.3 mM) with the **2**/HPTS reporter pair (6.3 μM both).

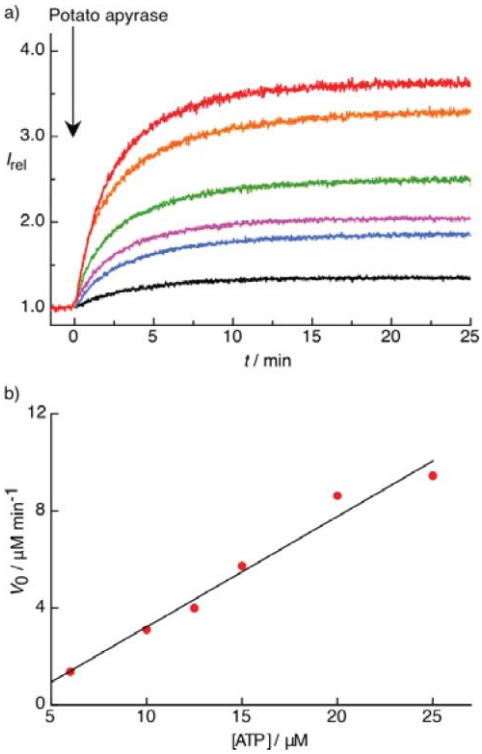


Fig. 5 a) Evolution of normalized fluorescence intensity monitoring potato apyrase activity (25 μg ml^{-1}) at different substrate (ATP) concentrations with the **1**/ANS reporter pair (both 25 μM, in NaOAc buffer, pH 5.5, with 1.8 mM Mn^{2+}, λ_{exc} = 318 nm, λ_{obs} = 462 nm). ATP concentrations (in μM) are, from bottom to top trace: 6, 10, 12.5, 15, 20, 25. b) Plot of the initial rates, v_0, *versus* substrate concentration and linear correlation line. The initial rates were obtained by linear fits of the normalized intensities (assuming a conversion linear with the fluorescence intensity and full conversion at the plateau region).

from 24 to 200 μM,[31,32] suggesting that the **1**/ANS and **2**/HPTS reporter pairs operate either below or above the K_M value. For the **1**/ANS reporter pair, the initial rates do in fact increase linearly with the substrate concentration (Fig. 5). A more detailed analysis of the enzyme kinetics is, however, not possible because the complexation of the substrate by the host lowers the effective substrate concentration, which presents a peculiarity of substrate-selective assays.[22] This was possible, however, for the **2**/HPTS reporter pair, for which a large excess of substrate was used and where the enzyme kinetics was expected to be zero-order with respect to the substrate. Indeed, determination of the initial reaction rates from the fluorescence decays at a fixed enzyme concentration (50 μg ml^{-1}) and 4 varying substrate concentrations (0.5–3.2 mM) afforded the same rate, within 10% error (0.17 μmol min^{-1}, expected from reported commercial activity: 0.19 μmol min^{-1}). This rate was assigned to the v_{max} value when working far above the K_M value. Finally, as shown for both reporter pairs (Fig. S3 in ESI†), the initial rates of ATP dephosphorylation increased linearly with the enzyme concentration.

Important to note, all nucleotide triphosphates caused a fluorescence decrease for the **1**/ANS reporter pair and a fluorescence enhancement of the **2**/HPTS reporter pair, by competitive displacement (see Fig. 2b and Fig. 3b). This shows that hosts **1** and **2** are actually unselective or at best moderately selective anion receptors, at least in the investigated concentration range. In tandem assays, such a "sloppy" molecular recognition[24,27] presents actually an advantage, because dephosphorylation of both nucleotide di- and triphosphates can be monitored quite generally (for example also GTP, TTP and CTP, Fig. 6), which opens screening possibilities in many directions.

Conclusions

In conclusion, we have introduced convenient supramolecular tandem assays for monitoring biocatalytic ATP dephosphorylation. The assay principle relies on the addition of an anion-receptor macrocycle and a fluorescent dye setting up a reporter pair. It is the high specificity of the enzyme itself which reduces selectivity considerations in the choice of a receptor to a minimum, bypasses the necessity to utilize designed chemosensors, but instead allows the use of simple, even if moderately selective, macrocycles. We demonstrated applications in the screening of activators and a broader applicability for other nucleotides and complementary reporter pairs. Our dephosphorylation assays should be transferable to other nucleotide-dependent enzymes.

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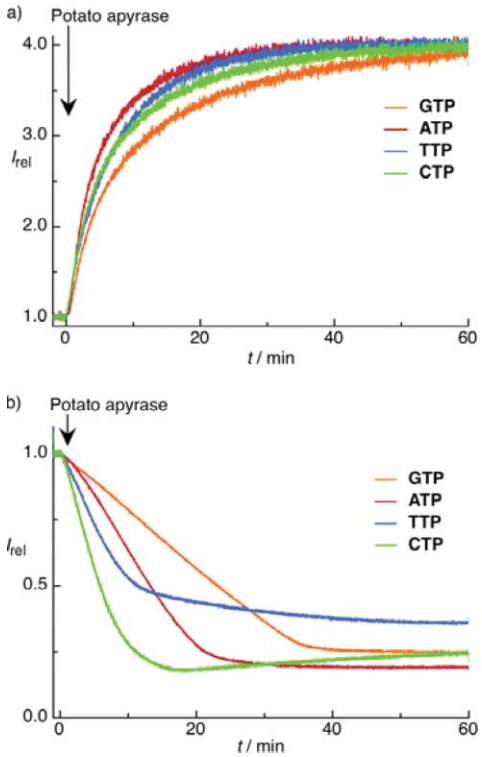

Fig. 6 Evolution of normalized fluorescence intensity monitoring dephosphorylation of various nucleotide triphosphates. Assays were initiated by addition of a) 12.5 μg ml^{-1} potato apyrase in the presence of **1**/ANS (25 μM both) to nucleotides (25 μM), and b) 100 μg ml^{-1} potato apyrase in the presence of **2**/HPTS (6.3 μM both) to nucleotides (4 mM).

Experimental

Materials

The macrocycles and dyes employed in the initial screening are shown in Charts 1 and 2. 2-Anilinonaphtalene-6-sulfonic acid (ANS), and Dapoxyl were obtained from Molecular Probes. 8-Anilinonaphtalene-1-sulfonic acid (1,8-ANS), 8-hydroxy-1,3,6-pyrene trisulfonic acid (HPTS), 6-(*p*-toluidino)-2-naphtalene-sulfonic acid (2,6-TNS), fluorescein sodium, GTP, and ADP were purchased from Fluka. The naphtalimide biscarboxylate, 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO), and DBO carboxylate were synthesized according to the literature.[35–37] ATP, AMP, CTP, TTP, and apyrase (grade I, from potato, 7.7 U/mg) were from Sigma-Aldrich. The cyclodextrin samples octakis(6-amino-6-deoxy)-γ-cyclodextrin (**1**), heptakis(6-deoxy-6-*N*-(2-hydroxy)ethylamino)-β-cyclodextrin (**4**), 6-monoamino-6-monodeoxy-β-cyclodextrin (**5**), 6-monodeoxy-6-mono(3-hydroxy)propylamino-β-cyclodextrin (**6**), heptakis(2,3-di-*O*-methyl)-hexakis(6-*O*-methyl)-6-monodeoxy-6-monoamino-β-cyclodextrin (**7**) were obtained as hydrochloride salts from CycloLab, Hungary. The synthesis of the cyclophane (**2**) was performed according to the literature with minor modifications.[11] Trianglamine (**3**) was kindly supplied by Prof. N. Kuhnert, Jacobs University Bremen, Germany. The bis-calixarene (**9**) was synthesized according to the literature,[15] whereas calixarenes (**8**) and (**10**) were kindly supplied by Prof. J. Schatz, Universität Erlangen-Nürnberg, Germany.

Instrumentation

Fluorescence spectra, intensities, and time courses were recorded on a Varian Eclipse fluorometer at ambient temperature (for the titrations) or at 25.0 ± 0.1 °C (using an external Peltier thermostat, only for the enzyme assays). UV measurements were performed with a Varian Cary 4000 UV-Vis spectrophotometer. The dephosphorylation of nucleotides was additionally monitored by ^{31}P NMR using a Jeol JNM-ECX 400 spectrometer.

Fluorescence titrations

All fluorescence titrations were performed in 1.4 mL quartz cuvettes (Starna, Typ 29-F/Q/10 mm). The titration of host with fluorophore was performed by keeping the dye concentration constant. Accordingly, the titrant contained the same concentration of dye, and a much higher host concentration (approximately 10–100 times higher). The competitive titrations using different nucleotides were carried out similarly to the host-guest titrations, ensuring a constant host and dye concentration during the titration. Therefore, the titrant contained, besides a high concentration of competitor, host and dye in concentrations equal to those in the cuvette solution. All titrations were performed in 50 mM sodium acetate buffer, pH 5.5 when the **1**/ANS reporter pair was employed, and in 10 mM sodium phosphate buffer, pH 7.4, or 10 mM sodium succinate buffer, pH 6.5, when using the **2**/HPTS reporter pair.

Supramolecular tandem assays

Stock solutions of substrate (nucleotides), host (**1** or **2**), fluorescent dye (ANS or HPTS), and enzyme were required for performing the enzyme assays. The stock solutions were prepared in 50 mM sodium acetate buffer, pH 5.5, with 1.6–1.8 mM $MnCl_2$ (when using **1**/ANS reporter pair) and in 10 mM sodium succinate buffer, pH 6.5, with 1.6–1.8 mM $CaCl_2$ (when **2**/HPTS was employed). Careful control of the pH was required to obtain reproducible fluorescence intensities. The required volumes of all stock solutions were calculated to afford the final concentrations in 1 mL total assay volume. All assays were conducted using equimolar mixtures of host and fluorescent dye (25 μM for **1**/ANS and 6.3 μM for **2**/HPTS). The volumes of nucleotide stock solutions were calculated to obtain the respective concentrations. The enzymatic reactions were initiated by adding an aliquot from a stock solution of 1 mg mL^{-1} enzyme.

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