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Switching the recognition ability of a photoswitchable receptor towards phosphorylated anions

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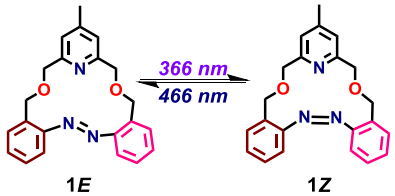
Munshi Sahid Hossain,[a] Sk. Atiur Rahaman,[a] Joydev Hatai,[b] Monochura Saha[a] and Subhajit Bandyopadhyay*[a]

An azobenzene based photoswitchable macrocyclic receptor displays different binding affinities in its *E* and *Z* forms towards various phosphorylated coenzymes under physiological conditions with remarkable selectivity for ATP in the *E*–form while selectivity towards GTP in the photoisomerized *Z*–form. Linear discriminant analysis clearly separated the analytes using the *E*–form. An application of this method enabled monitoring the progress of an enzymatic phosphorylation with a tyrosine kinase enzyme.

Photoswitchable molecules having efficient switching behavior can be exploited to regulate molecular functions reversibly using light.[1, 2] Light-driven switches toggle reversibly between two or more states, which gives rise to species having different electronic structures, sizes, and shapes.[3] Several photochromic systems are used for switching among which diarylethenes[4], spiropyrans[5, 6] acyclic as well as macrocyclic azobenzene[7–11] are the most frequently used.

The design and development of receptor for selective recognition of anions has received considerable attention in the recent years,[12, 13] not only for academic interest but also because of their applications in diagnostics, imaging, and environmental sciences.[14, 15] Among various anions, recognition of nucleotides, phosphates, pyrophosphates have significant role in a plethora of biological processes.[16, 17] Adenosine triphosphate (ATP) is a multi-functional nucleotide that plays an important role in energy transduction in organisms and controls several metabolic processes.[18–19] It also turns on and off the activity of a protein by phosphorylation. Selective recognition and discrimination of ATP and other phosphate containing coenzymes in aqueous media has emerged as an important area of research. Recently, a cationic pillar[6]arene that can selectively bind to ATP has been reported.[20] Selective recognition of ATP has also been reported.[21] A phosphate anion receptor was reported to achieve selectivity in affinity for ATP through π–stacking of pyrene-adenine-pyrene sandwitch.[22] An acyclic azobenzene based dipicolylamine modified γ-CyD–Cu^{2+} complexes that can recognize ATP has been reported to display a high selectivity over other phosphoric acid derivatives.[23] Competitive molecular recognition of GTP and ATP by adenylate kinase has also been reported.[24] Thus, there exists a number of excellent probes for each of these coenzymes separately. It would be beneficial if a single probe can detect and discriminate among ATP, ADP, AMP, P_i and GTP anions.[25] This motivated us to design a switchable receptor that can detect these coenzymes via a cross-reactive sensor array that can alter the selectivity upon external inputs like light which could be cost effective and beneficial in term of practical applications. Recently, photomodulation of anion recognition through non-covalent interaction using photoswitchable system was reported by Flood and by our group.[26, 27] A spirooxazine based photochromic sensor, differentiated among various metal ions via a cross-reactive sensor array.[28]

Here we report a macrocyclic azobenzene based fluorescent receptor **1** that can recognize ATP selectively, preferentially in the **1*E*** form and discriminate the rest of the anions as a cross-reactive sensor array. However, upon 366 nm light excitation, the photoisomerized **1*Z*** form of the receptor showed altered selectivity towards GTP. Thus, the photoswitchable cross-reactive receptor stands as a unique example through photoisomerization that detected various phosphorylated anions via that linear discriminant analysis (LDA).[29, 30]



Scheme 1. Photomodulation of the receptor **1**

[a] Department of Chemical Sciences, Indian Institute of Science Education and Research (IISER) Kolkata, Mohanpur, Nadia, West Bengal 741246, India. E-mail: sb1@iiserkol.ac.in
[b] Institute of Organic Chemistry, University of Duisburg-Essen, Universitatsstraße 745141, Essen, Germany. E-mail: joydev.hatai@uni-due.de

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As a potential application, this system was investigated for the easy monitoring of a phosphorylation reaction. Post-translational modifications of proteins or peptides upon phosphorylation regulate cellular signalling pathways and protein-protein interactions. Generally progress of the reaction could be monitored by mass spectrometry in a biological experiment.[31] However, recently a few luminescent probes having metal ions have also been employed for monitoring the kinase activity.[32] In this work, a metal free probe, **1*E*** has been used as an ATP-specific sensor to monitor the phosphorylation of tyrosine. This enabled fast and real-time *in vitro* monitoring of a kinase activity.

Initially, the receptor **1** was synthesized and characterized by spectroscopic and spectrometric methods and also via single crystal X-ray diffraction (CCDC number-1941103, see SI for detailed experiments and characterisation). The **1*E*** ⇌ **1*Z*** photoswitching behavior of the receptor was performed via UV-Vis as well as ^{1}HNMR spectroscopy, displayed characteristic of azobenzene photo switching properties (Fig. S1-S3).

The optical sensing of ATP, ADP, AMP, P_i and GTP anions were demonstrated by the fluorescent response of the receptor **1*E*** as well as **1*Z*** due to its high sensitivity over UV absorption (Fig. S4-S5). The influence of the anions (sodium salts) on the fluorescent emission spectrum of receptor **1** in both form (10 μM) was investigated in an aqueous solution of Tris (1 mM, buffered at pH 6.8) at 25 ^{o}C. Upon addition of ATP to an aqueous solution of **1*E***, the emission spectrum shifted (15 nm) towards the longer wavelengths. An increase in the ATP concentration from 0 to 10 equivalents resulted in a ~7 fold fluorescence enhancement. However, the addition of 10 equivalents of ADP and GTP to **1*E*** showed only a 3.5 fold and 3.1 fold enhancement accompanied by a 16 nm and an 8 nm red shift respectively. In the case of AMP and P_i receptor, **1*E*** showed only a subtle emission intensity enhancement (~2 fold and ~1.5 fold respectively) but with large red shifts (32 nm and 30 nm respectively) (Fig. 1A and Fig. S6 and S8A). The degree of fluorescence enhancement was ascertained to follow the order ATP> ADP> GTP> AMP> P_i. On the contrary, no appreciable spectral changes were observed upon addition of pyrophosphate ($P_2O_7^{4-}$) and a whole host of other common anions Cl^-, AcO^-, NO^{3-}, NO^{2-}, N^{3-}, F^-, HCO_3^-, CO_3^{2-}, S^{2-}, SO_4^{2-} to the **1*E*** isomer (Fig. S9). Therefore, it was interesting to note that the degree of enhancement along with the spectral shift of each of these phosphate species served as a unique fingerprint for their detection (see Table 1). This led us to the multivariate analysis of our data using linear discriminatory analysis (see later: multivariate analysis). Interestingly, upon exposure to the 366 nm light, ATP⊂**1*E*** complex underwent conversion to the ATP⊂**1*Z*** system. The switching from the ***E*** to the ***Z*** complex was marked by a significant decrease in the emission intensity. Similar, reduction of the fluorescence intensity was also observed for the ADP⊂**1*E***, AMP⊂**1*E***, GTP⊂**1*E*** as well as P_i⊂**1*E*** complexes upon exposure to the UV light, although the extent of the decrement in the intensity was much lesser. Quantitative measurement of the binding of the anions with **1*Z*** was also checked by systematic titration. It was found that the addition of 10 equivalents of ATP and GTP

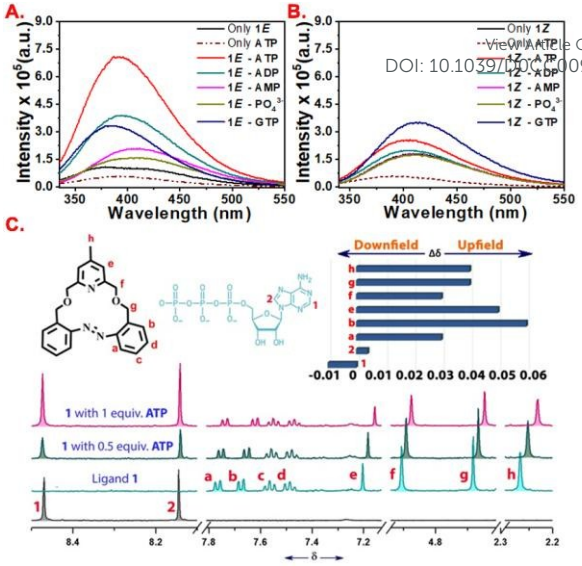

Fig. 1 Emission intensity of (A) **1*E*** (10 μM) (B) **1*Z*** (10 μM) with phosphate anions (10 equiv. each) in a Tris buffer of pH 6.8. The same scale have been maintained in all the figures for the ease of comparison. (C) Systematic ^{1}H NMR titration of **1*E*** (5 mM) with ATP (0 to 1 equivalent).

to an aqueous solution of **1*Z*** displayed only a modest enhancement in the emission intensity (1.5 fold and 1.9 fold respectively). However, addition of ADP, AMP and P_i (10 equivalents each) did not influence the emission spectra (Fig. 1B and Fig. S7-S8B). Therefore, these fluorescence enhancement and spectral shifts showed that the receptor **1*E*** can easily detect and differentiate among these phosphate containing anions simultaneously through a combination of Δλ and ΔF values (see Table 1) and at the same time display altered selectivity towards GTP upon isomerization. The binding of the ATP with **1*E*** was also studied by ^{1}H NMR titration in deuterated solvent. Addition of ATP upto 1 equivalent displayed significant upfield shift of the aromatic as well as aliphatic protons of the **1*E*** to some extent (Fig. 1C). This clearly demonstrated that the **1*E*** form binds strongly to the anions. The Job's plot showed 1:1 binding stoichiometry ($\chi_{[ATP]}$ = 0.53) between **1*E*** and the ATP (Fig. S10, binding constant for the analytes are given in the Table S1 and Fig. S11-S15).

The geometry of the host receptor **1** (in the ***E*** isomer as well as in the ***Z*** isomer), negative charge of the guest anions and the structure of the phosphate derivatives dictate the mode of interactions of the receptors with the various anions. Crystal structure of **1*E*** (crystalized from 1:1 v/v CH_2Cl_2/MeOH, CCDC No.-1941161) showed that it exists as a protonated form through the pyridine nitrogen even at a neutral pH (protonated form captured in presence of ClO_4^- counter anion, Fig. 2). Furthermore, the coenzymes ATP, ADP, AMP and GTP also exists as ATP^{4-}, ADP^{3-}, AMP^{2-} and GTP^{4-} respectively under neutral pH condition.[33] Receptor **1*E*** experiences strong affinity towards ATP^{4-} most likely via the coulombic interaction between the negatively charged oxygen of the anions and the positively charged protonated form of the receptor. In addition, H–bonding interaction of the guest anion with the amine group of the adenosine moiety and the –N=N– group of

the receptor is also possible (Fig. S18). The binding mode of

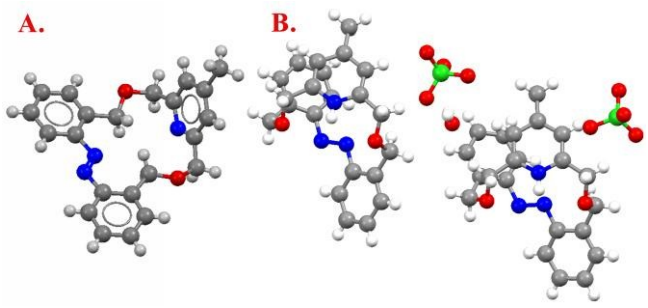

Fig. 2 (A) Single crystal X-ray diffraction of **1*E*** and (B) protonated **1*E*** at 50% ellipsoid probability.

the anions with the receptor can be speculated from the crystal structure as well as from the optimized geometry of the receptor. The interactions mentioned earlier are most likely with the **1*E*** form because of its open structure (Fig. 2A) which the guest anions can access. This can lead to H–bonding interactions between the lone pair of the azo –N=N– of the host and the NH moieties of the adenosine unit. The coulombic interaction plays a major role is intuitively envisaged since the host and the guest bear the opposite charges under the working pH of 6.8. The columbic interactions bring the guests and the host in the close proximity and thereby facilitate the H–bonding interactions between them. The weaker interaction of the GTP compared to ATP with **1*E*** could stem from the lack of the H–bonding between the adenosinyl-NH_2 and the lone pair of the –N=N– of the host that is present for the later. This was supported by the DFT studies at the B3LYP level using the polarizable continuum model (PCM) (Fig. S18). Interestingly the **1*Z*** form does not allow both the interactions because of the geometrical constrains. Therefore, the molecular recognition of **1*E*** isomer with the guest anions is stronger than the corresponding **1*Z*** isomer (Fig. S17).

The unique spectral shift as well as change in fluorescent intensity in the presence of each of the anions further enabled the discrimination among the analytes. For a qualitative discrimination among the coenzymes, the fluorescence intensities of **1*E*** (10 μM) upon the addition of analytes in aqueous media were also recorded (λ_{ex} = 292 nm, Tris-buffered at pH 6.8 at 25 °C) (Fig. 1). The changes in the fluorescence intensities at different wavelength led to the generation of unique optical fingerprints (Fig. 3A) for each of the coenzymes. The emission pattern thus obtained subjected to the chemometric methods (LDA) to discriminate among these patterns. The LDA mapping clearly indicated that sensor can detect and discriminate among the analytes. Even the discrimination between AMP and P_i that is difficult using univariate analysis, the LDA plot showed a clear discrimination with 100% cross-validation (Fig. 3D). The LDA plot was used to identify the 23 of 25 unknown samples of the analytes (ATP, ADP, AMP, GTP and P_i), indicating 92% accuracy (Table S5).

The two-dimensional score plot for the **1*E*** exhibited a distinct separation among ATP, ADP and GTP with rest of the observables (AMP or P_i) but the clustered data of AMP and P_i in a close proximity. These observations were in conformity with the previous results for the **1*E*** and hence, reinforced the capacity of differentiating among the phosphate based analytes with the azobenzene system (Fig. 3B). The introduction of another factor to the score plot enhanced the separation among the observables in the 3D plot which enabled a better separation. This result confirmed spectacular ability of the receptors to recognize and differentiate among the various analytes of biological importance (Fig. 3C).

The biological phosphate transfer reaction uses kinases and an ATP as the coenzyme producing a phosphorylated protein and the ADP. Since our system can detect and discriminate among the phosphate derivatives, to explore the real-life use of our system, a biological kinase reaction was carried out to monitor the kinase activity with the receptor **1*E***. Addition of tyrosine by itself to the receptor **1*E*** (2.5 μM, in Tris–buffered at pH 6.8) did not show any significant fluorescence signal prior to the addition of ATP. Upon addition of ATP (25 μM), a drastic increase in the fluorescence signal with a sevenfold enhancement of the fluorescence intensity at 390 nm was observed. This is consistent with the response of **1*E*** in the presence of ATP. For real-time monitoring of the kinase activity, the enzyme c–**Src** kinase was subsequently added and the time course of the fluorescence spectra of the mixture showed a gradual decrease at 390 nm corresponding to a

Table 1. Fluorescence enhancement and wavelength shift of receptor **1** in ***E*** form as well as in the ***Z*** form upon addition of the anions.

	1*E*		**1*Z***	
Anions	**Fold (F/F_o)**	**Shift (Δλ)**	**Fold (F/F_o)**	**Shift (Δλ)**
ATP	6.5	+15 nm	1.5	-6 nm
ADP	3.55	+16 nm	1.1	-3 nm
AMP	1.9	+32 nm	-	-
P_i	1.4	+30 nm	-	-
GTP	3.1	+8 nm	1.9	+4 nm

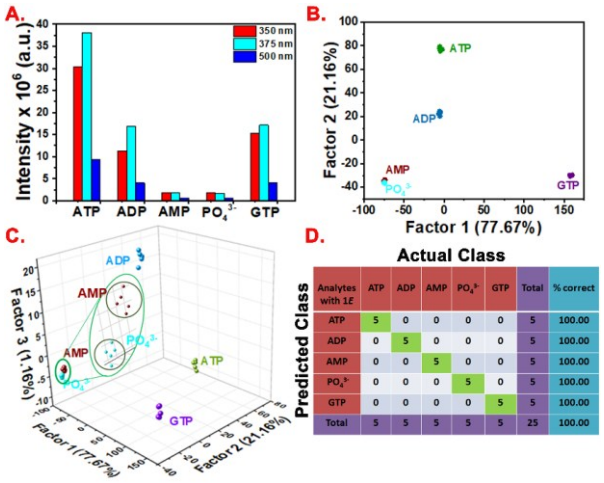

Fig. 3 (A) Recognition patterns for the analytes (10 equiv. each) based on the variation of fluorescence intensity of **1*E*** (10 μM) at different wavelengths; (B) 2D and (C) 3D score plot for an array of **1*E*** discriminating the analytes; (D) The confusion matrix obtained (using LDA classifier) as a classification accuracy trained on the full data set for all the analytes in aqueous solution of Tris (buffered at pH 6.8) at 25 °C.

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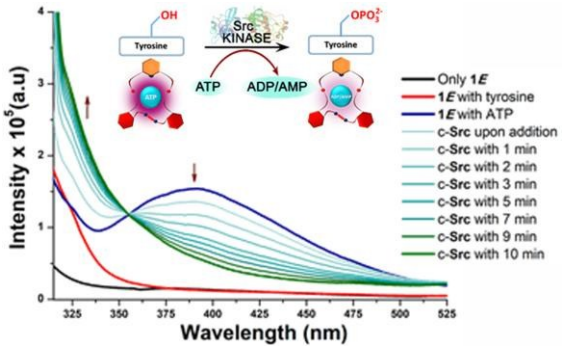

Fig. 4 Scheme of kinase activity with probe **1*E*** (top); Kinase study of **1*E*** (2.5 μM) with ATP (25 μM) in presence of Tyrosine upon addition of c-**Src** kinase (1 μM) in Tris (buffered at pH 6.8) at 25 °C (bottom).

decrease in the concentration of ATP (Fig. 4).

In summary, an azobenzene based macrocyclic probe in the ***E***–isomer showed strong binding affinity towards ATP whereas the selectivity towards GTP was displayed by the ***Z***–isomer. All the species showed distinct patterns in terms of the enhancement of their emission intensity (ΔF_λ values) and the spectral shifts (Δλ). Therefore, taking a combination of these fluorescence enhancements at various wavelengths and their spectral shifts with the receptors **1*E***, these phosphate containing anions can easily be detected and differentiated using the linear discrimination analysis method. On the contrary, the other photoisomerized form **1*Z*** exhibited very poor discrimination ability towards the anions. Thus, one can tune the recognition and discrimination ability of the receptor **1** via light. The practical application of this probe was demonstrated with **1*E*** as a fluorescent probe for the monitoring of the progress of an enzymatic phosphate` transfer reaction using a tyrosine kinase enzyme (c–**Src** kinase) which enabled a real time monitoring of the *in vitro* kinase activity.

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Conflicts of interest

There are no conflicts to declare.

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