Selective detection of ATP and ADP in aqueous solution by using a fluorescent zinc receptor.

UNIVERSITÀ DEGLI STUDI DI TORINO

Selective Detection of ATP and ADP in Aqueous Solution by using a Fluorescent Zinc Receptor

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We report on the successfully use of a new zinc complex for the selective fluorescent detection of ADP and ATP in water. This is achieved by the complementary coordination of the phosphate groups to the metal centre and hydrogen bonding of the adenosine with the coordinated ligand.

Anions play a key role in a variety of environmental and biological processes. In particular among all anions, phosphate and molecules featuring this group are widely studied because of their ubiquitous presence in a range of life processes spanning from energy storage and signal transduction to gene construction.[1]

Lately several optical sensors for monitoring anions have been devised.[2;3] When designing phosphate receptors, possibility of sensing in an aqueous solution should be considered. The solvation of these substrates strongly competes with the process of complex formation, making the recognition of phosphate anions a challenging area of research. Another important point is the selectivity of recognition. Altough some examples of fluorescent sensors which are selective over inorganic phosphate anions, such as phosphate and pyrophosphate have been developed[3-8] the recognition and fluorescent sensing of adenosine di- or tri-phosphates over various nucleotide polyphosphates remains as a challenghing problem.[9]

Among the various approaches, metal ion complexes are considered to be ideal for phosphate recognition in aqueous solutions.[10] Many reported phosphate sensors involve fluorescent complexes with lanthanoid metals such as Eu(III), Tb(III) and Yb(III), having long lifetime of fluorescence and large Stokes' shifts.[11-13] Alternatively, the use of a transition metal offers the advantage of inducing fluorescence enhancement which is more desirable in sensing than fluorescence quenching. Recently Schiff base zinc complexes have been reported as efficient fluorescent sensors for adenosine nucleotides.[14] In this contest, we thought of synthesizing a Zn complex featuring a Schiff base ligand with alcoholic functions on the ligand skeleton that could be able to establish intramolecular hydrogen bonds with the guest of interest. It is expected that additional functionalities for multiple non-covalent interactions with the substrates can contribute to the stability of the host-guest adduct leading to an enhancement of specificity and selectivity of detection. Our Schiff base ligand of choice is a derivative of pyridoxal with ethylenediamine (*pyr*$_2$*en*H$_2$, = N,N'-ethylenebis(pyridoxylideneiminato), Fig. 1).[15]

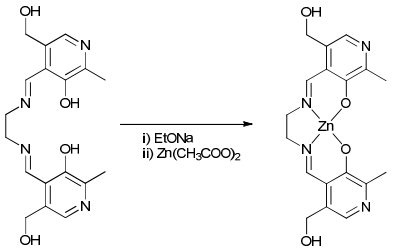

Fig. 1. Synthesis of *pyr*$_2$*en*Zn

The target zinc complex (*pyr*$_2$*en*Zn) was obtained in good yield by mixing equimolar amount of deprotonated ligand and $Zn(CH_3COO)_2$ in MeOH at room temperature. The stoichiometry was confirmed by ESI-MS where the major peak at 421.17 *m*/*z* units corresponds to the mononuclear $[(pyr_2enZn)H]^+$ species. No evidence for binuclear species in solution was found. The metal complex is soluble in dimethyl sulfoxide and acidic water. The ^{1}H NMR spectrum of *pyr*$_2$*en*Zn in DMSO-d$_6$ indicated the presence of sharp signals consistent with the expected symmetric coordination of the ligand. Diagnostic resonances at 7.39 and 8.86 ppm for the iminic and aromatic protons, respectively, together with a singlet at 3.80 ppm for the protons on the diimine bridge were observed. These assignments were based on literature data[15] and corroborated by means of mono- and bidimensional NMR experiments.

In DMSO-d$_6$ solution, the methylenic protons of the CH_2OH group are equivalent and show scalar coupling with the hydroxyl proton, the CH_2OH group gives rise to a doublet and a triplet at 4.55 and 5.20 ppm with a vicinal coupling constant $^3J_{HH}$ of 4.7 Hz (Fig. S1, ESI†). When switching to D_2O, the same methylenic protons produce an AB pattern consisting of two doublets at 5.33 and 5.19 ppm with a geminal coupling constant $^2J_{HH}$ of 13.8 Hz (Fig. S2, ESI†). Most likely, this change indicates a dependence of the speed of rotation of the CH_2OH group upon the intermolecular interactions that occur in solution. *Pyr*$_2$*en*Zn was further characterized via Uv-vis and fluorescence spectroscopy. The electronic absorption spectra of the free ligand and of *pyr*$_2$*en*Zn were measured both in DMSO and in MilliQ water (Fig. S5-S6, ESI†). In DMSO an intense band was observed at 378 nm. In H_2O *pyr*$_2$*en*Zn displayed a more structured spectrum with a major peak at 223 nm and less intense absorption bands at 315 and 363 nm due to $\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$ intraligand transitions.[16;17]

The capability of *pyr*$_2$*en*Zn to act as recognition element for phosphate anions in aqueous solutions was first studied by ^{31}P NMR spectroscopy. Addition of the nucleoside ADP, ATP, GTP, CTP, UTP or TTP to a saturated D_2O solution of *pyr*$_2$*en*Zn

resulted into a clear and significant down field shift in the ^{31}P NMR spectrum with respect to the signals of the free anions. In the case of ADP the β-P-atom was shifted of 3.66 ppm whereas the α-P-atom underwent smaller shift (Δδ = 0.99 ppm, Fig. 2). For ATP, the β-P-atom and γ-P-atom underwent consistent shifts (respectively Δδ = 1,13 and 1,50 ppm) whereas the shift for α-P-atom was relatively small (Δδ = 0,19 ppm) strongly suggesting that the α-P center was not interacting with the zinc center. Similar shifts were observed for GTP, CTP, UTP and TTP (Fig. S7-S10, Table S1, ESI†). These behaviors are fully consistent with the coordination of the tested nucleosides to the metal centre.[14;18;19]

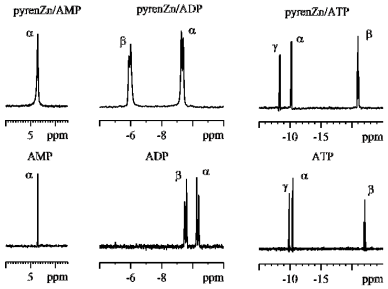

Fig 2. ^{31}P NMR spectra of Na_2AMP, Na_2ADP and Na_2ATP and after addition of *pyr₂en*Zn (rt, D_2O). [*pyr₂en*Zn] = 25 mM.

Significantly, when adding AMP or an inorganic phosphate (PO_4^{3-}, $P_2O_7^{4-}$, HPO_4^{2-}, $H_2PO_4^-$) to a saturated D_2O solution of *pyr₂en*Zn no difference could be detected in the ^{31}P NMR spectra of the phosphate salt (Fig. 2 and Fig. S11-S13, ESI†). Further insights for the interaction of selected nucleosides with *pyr₂en*Zn were gained from ^{1}H NMR studies. While the addition of AMP or inorganic phosphates to a saturated D_2O solution of *pyr₂en*Zn did not produce any relevant change in the ^{1}H NMR spectrum of *pyr₂en*Zn, the addition of ADP, ATP, GTP, CTP, UTP or TTP gave rise to an upfield shift in the ^{1}H NMR pattern of the complex. The largest shifts were observed for the aromatic C(*H*)N and imine *H*C=N resonances (Fig. 14-17, ESI†). For example, in the case of ADP, the aromatic C(*H*)N proton, resonated at 8.19 ppm in the case of *pyr₂en*Zn whereas it shifted at 7.82 ppm in the case of *pyr₂en*Zn /ADP.

Intriguingly when adding ADP or ATP to a saturated D_2O solution of *pyr₂en*Zn we observed a clear change in the multiplicity of the signal due the methylenic protons (at circa 5.3 ppm) of the C*H*$_2$OH group (Fig. 3). In addition to the geminal coupling, a scalar correlation with the imine (J = 5.1 Hz) and with the aromatic C(*H*)N protons (J = 8.5 Hz) was observed. The ^{1}H-^{1}H COSY spectrum of the *pyr₂en*Zn /ADP adduct establishing the coupling partners is also displayed in Figure 3. Similar results were obtained with ATP (Fig. S18, ESI†). This finding suggests that the OH groups on the ligand framework participate within the interaction process between *pyr₂en*Zn and ADP or ATP. The hydrogen-bonding between the OH and the nucleosides made the rotation of the CH_2OH group particularly hindered. On successive dilution the initial signal multiplicity is gradually restored, suggesting the reversibility of the recognition/interaction process. Significantly, when adding GTP, CTP, UTP or TTP to a saturated D_2O solution of *pyr₂en*Zn, the multiplicity of the CH_2OH remained unchanged (Fig. S19-S22, ESI†).

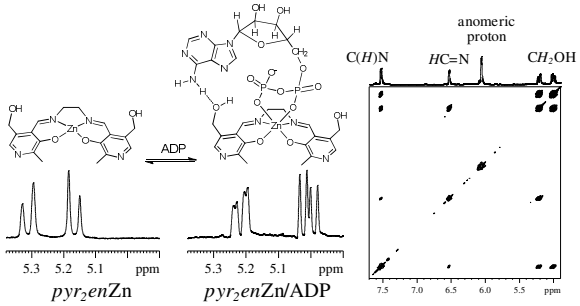

Fig. 3. Change in multiplicity for the CH_2OH group and ex-panded region of 2D COSY spectrum of *pyr₂en*Zn/ADP. ([*pyr₂en*Zn] = 25 mM [Na_2ADP]= 70 mM, rt, D_2O).

To gain insights into the mode of binding of phosphorylated anions to the Zn complex and to assess the contributions of the alcoholic functions to the binding, we studied the interaction of *pyr₂en*Zn with ADP, ATP and, for comparison, $P_2O_7^{4-}$ anions by Density Functional Theory, making use of the M06 functional. In the obtained adducts, the metal ion binds two hard phosphate oxygens of the phosphorylated species saturating its coordination sphere, the calculated O-Zn bond distances are 2.10 Å (Fig. S23-25, ESI†). ADP and ATP form structurally similar complexes: the organic moieties of the nucleotides are located above the coordinated ligand. In particular in the *pyr₂en*Zn /ADP adduct (Fig. 4) the amino group of the adenosine fragment (the hydrogen bond donor site) is close to the hydroxyl group of the ligand (the hydrogen bond acceptor site) interacting with it.

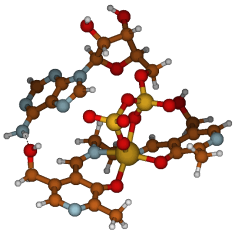

Fig. 4. Optimized structure of *pyr*$_2$*en*Zn/ADP adduct. The water molecules were omitted for clarity of representation.

The distance between -NH_2 and OH, peculiar to hydrogen bonds, is about 2.1 Å. The bonding between the phosphates and hydroxyls in these adducts is not feasible because of the large distance between the two groups. The interaction between these groups is mediated by an hydrogen bonded water molecule.

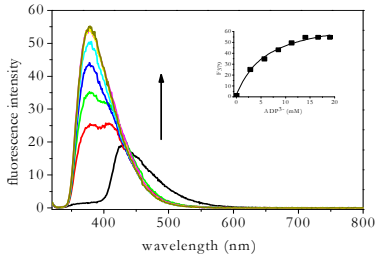

Fig. 5. Emission spectra of *pyr*$_2$*en*Zn (λ_{exc} 315 nm) after progressive additions of Na_2ADP (rt, MOPS). [Zn] = 5.5 mM.

Interactions between *pyr*$_2$*en*Zn with ADP, ATP, AMP, UTP, CTP, TTP, GTP, PO_4^{3-}, $P_2O_7^{4-}$, HPO_4^{2-}, $H_2PO_4^-$, Cl^-, F^- in MilliQ water solutions were also studied by means of fluorescence spectroscopy. When exciting an aqueous solution of *pyr*$_2$*en*Zn at the maximum of its absorption (λ = 315 nm) and adding an excess of ADP a fast and consistent enhancement (~80%) of the initial fluorescence intensity was observed. When repeating the same experiment in the same conditions as before and adding an excess of ATP instead of ADP an enhancement of ~75% of the initial fluorescence intensity value was registered. On the other hand, adding an excess of UTP, CTP, TTP, GTP, resulted in a 75%, 35%, 75%, 82% fluorescence quenching respectively (Fig. S26, ESI†). Addition of an excess of an inorganic phosphate, either HPO_4^{2-} or $H_2PO_4^-$ or $P_2O_7^{4-}$ or PO_4^{3-}, resulted in a 15%, 20%, 40% or 51% fluorescence quenching respectively (Fig. S27, ESI†). Addition of an excess of Cl^- or of F^- or of AMP did not result in any significant change of the fluorescence intensity.‡

The selective fluorescent detection of adenosine di- or tri-phosphate over other nucleotides and inorganic phosphates should include the contribution of the hydrogen-bonding interactions between OH groups on the ligand framework and ATP or ADP. A possible explanation for this finding is the interruption of photoinduced electron transfer (PET) of electronic density from the lone pairs on the oxygen atom to the π-system of the fluorophore resulting in luminescence enhancement.[20] This explanation is also supported by the ^{1}H NMR data by which we found that the multiplicity of the CH_2OH signal changes only in the case of addition of ATP and ADP.

To determine the binding affinity of *pyr*$_2$*en*Zn for adenosine di- or tri-phosphates, ADP and ATP titrations were performed while monitoring the fluorescence intensity of the system. Figure 5 shows the ADP fluorescence titration of *pyr*$_2$*en*Zn in the range of 0 - 3.5 eq. A similar fluorescence trend was obtained when titrating *pyr*$_2$*en*Zn with ATP (Fig. S28, ESI†). The apparent equilibrium constants of association (K_a) were 158,7 ± 1.4 M^{-1} for the *pyr*$_2$*en*Zn /ADP system and of 434,8 ± 0.5 M^{-1} for the *pyr*$_2$*en*Zn /ATP construct. The greater interaction of ATP is probably due to the greater electronic charge density on the phosphate groups than that for ADP.

The detection limit of the proposed system for ADP and ATP was also determined and found to be in the micromolar range (Fig. S29-30 ESI†).

In summary a Zn^{2+}-pyridoxal derivative has been synthesized and properly characterized via Uv-vis, fluorescence and NMR spectroscopy. Its potential in molecular recognition of adenosine phosphates over various nucleoside polyphosphates (UTP, CTP, TTP, GTP) and inorganic phosphates ($P_2O_7^{4-}$, PO_4^{3-}, $H_2PO_4^-$ and HPO_4^{2-}) has been investigated by means of ^{31}P NMR, ^{1}H NMR and fluorescence spectroscopy. Clear evidences that *pyr*$_2$*en*Zn can be successfully used for the selective fluorescent detection of ADP and ATP have been provided. It was shown that the interaction of the nucleoside polyphosphate with *pyr*$_2$*en*Zn occurs through the complementary chelation of the phosphate groups to the metal centre and the hydrogen bonding between the adenosine and the alcoholic groups on the ligand framework. In order to gain independent evidence for the binding of ADP/ATP to *pyr*$_2$*en*Zn and to model the adducts formed, DFT calculations were undertaken.

The OFF-ON switching of fluorescence together with the possibility of performing the measurements in aqueous solution add advantages to our system.

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Notes and references

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‡ No significant changes in the fluorescence intensities were ob-served in the absence of phosphates or when aqueous solutions of free phosphates were excited at 315 nm.

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