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FULL PAPER

Fluorescence lifetime sensitive probes for monitoring ATP cleavage

Daniel Hammler[a], Andreas Marx[a,*] and Andreas Zumbusch[b,*]

Abstract: Adenosine triphosphate (ATP) probes modified with fluorescence dyes that change their fluorescence properties upon cleavage are an interesting tool for monitoring enzymatic ATP turnover. As a readout parameter, fluorescence lifetime is attractive since it is nearly independent of concentration. In our study we synthesised and investigated eleven different ATP analogues, in which the fluorophores were attached to the γ-phosphate of ATP. All analogues showed distinctly different fluorescence lifetimes compared to the corresponding values of the free fluorophores. Both fluorescence lifetime increases and decreases upon attachment to ATP were observed. In order to shed light on the photophysical processes governing the lifetime changes we performed photoelectron spectroscopy in air (PESA) to determine HOMO energy levels and time-resolved fluorescence spectroscopy to obtain rate constants. We present evidence that fluorescence quenching in the compounds tested is dynamic and due to photoinduced electron transfer (PET), whereas fluorescence lifetime increases are caused by stacking interactions between chromophore and the nucleobase reducing non-radiative relaxation. Finally, we demonstrate that enzymatic cleavage of the ATP analogues presented can be followed by continuous monitoring of fluorescence lifetime changes.

Introduction

Adenosine triphosphate is the main energy transporter in cells and plays a central role for the function of a variety of enzymes, such as kinases[1], ligases[2], RNA polymerases[3], motor proteins like kinesins[4], and ATPases[5]. The energy is released by the hydrolysis of the α/β- and, or β/γ-phosphate bond and can be enzymatically coupled to endergonic processes to drive them thermodynamically uphill[6]. This makes the number of enzymes using ATP as a substrate almost uncountable[7]. To investigate processes in which ATP hydrolysis is involved, several methods exist, e.g. the use of radioactively labelled ATP[8], spectroscopic detection of released phosphate or pyrophosphate[9], or enzyme-coupled assays[10]. These methods are laborious or do not allow direct and continuous measurements. As an alternative, we have recently reported the development of a time-resolved ATPase sensor (TRASE) assay that can be used for the continuous monitoring of the ATP consumption of single enzymes in vitro[11].

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This assay is based on doubly labelled ATP analogues, where one fluorescence dye (donor) is attached to the γ-phosphate and the second fluorescence dye (acceptor) is covalently attached to the nucleoside. Before enzymatic conversion of the TRASE, the two different dyes undergo Förster resonance energy transfer (FRET), such that excitation of the donor dye results in the emission of the acceptor dye. Upon cleavage, emission of the donor can be observed which makes this assay suited for direct measurements of enzymatic activity. However, since the second modification is attached to the nucleoside, for each experiment it has to be considered which modification is enzymatically accepted. While it was shown that different classes of ATP consuming enzymes accept various modifications on the nucleoside of ATP[12], there is no universal position for modification on the nucleoside scaffold that would allow a general application for a variety of enzymes. Moreover, it is obvious that the higher the number of modifications of ATP, the less likely it will be accepted by a broad variety of enzymes.

In this work, we report the synthesis and spectroscopic characterisation of ATP probes which carry only one fluorophore (Fig. 1a). These novel probes shall enhance the acceptance of the modified ATP probes by the target enzymes. As a read-out for hydrolysis, we employed fluorescence lifetime, τ_{fl}, measurements of the label (Fig. 1c). Fluorescence lifetime measurements are more robust than intensity or absorption read-outs because they are independent of the fluorophore concentration, the sample absorption, the sample thickness, the method of measurement, the fluorescence intensity, photo bleaching, and the excitation intensity[13]. Accordingly, it is not surprising that fluorescence lifetime experiments have become an important tool in the discovery of new drug candidates[14] and inhibitors[15] in recent years. Fluorescence lifetime changes are also increasingly used

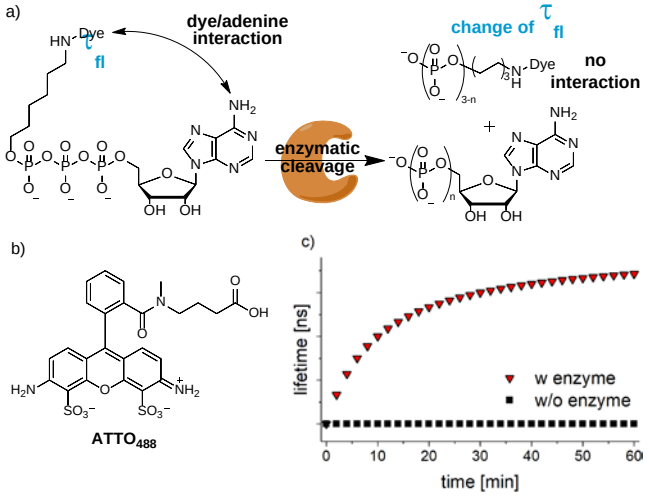

Figure 1. a) General scheme of dye-linked ATP: fluorescence lifetime of the attached fluorophore changes when it is in close proximity to the nucleobase. b) Structure of the rhodamine dye ATTO$_{488}$-COOH. c) Proposed change of τ_{fl} when modified ATP analogues are quenched by the nucleobase bevor cleavage.

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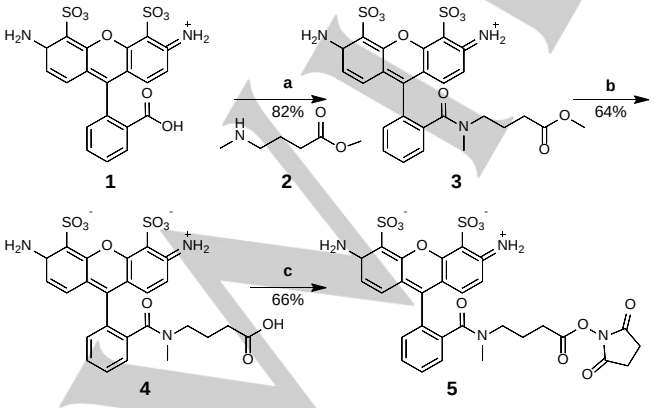
as a contrast in fluorescence lifetime imaging (FLIM)[16]. Moreover, there are also examples for fluorescence lifetime based nucleotide probes[17]. For fluorescence lifetime based assays, a dynamic quenching mechanism is preferred over a static complex formation on which would reduce the overall intensity without having an effect on the fluorescence lifetime[18]. In many instances both mechanisms are present, however, the contribution of the static complex can be diminished when stacking to a suitable quencher is supressed. An additional requirement is that the fluorescence lifetime of the dye must be long enough to be measured when quenched. We therefore based our experiments on derivatives of the highly hydrophilic fluorescent rhodamine dye 4',5'-disulforhodamine ATTO$_{488}$ (Fig. 1b) which has a fluorescence lifetime in aqueous solution of 4.1 ns[19] independent of the pH value over a broad range[20], and has got an exceptionally high photo- and thermal stability[21]. Furthermore, it is known that rhodamine derivatives are sensitive to their microenvironment due to the fact that polarity influences the equilibrium of planar and twisted conformations of the two amino functions which have different nonradiative relaxation rates[22]. These properties make ATTO$_{488}$ a well-suited fluorescence dye for the systematic investigation of fluorescence lifetime sensitive mono labelled ATP analogues.

Results and Discussion

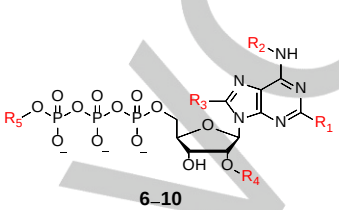
In order to design an efficient lifetime probe, we first investigated the fluorescence variations as a function of labelling position in mono labelled ATP analogues. For this purpose, we initially synthesised five modified ATP analogues bearing an ATTO$_{488}$-dye on different positions of the nucleobase, sugar, and γ-phosphate residue (Fig. 2).

The target molecules were obtained by transferring a synthesis strategy developed for modified rhodamine 6G[23] to a five step synthesis of the commercially available fluorescence dye ATTO$_{488}$-NHS ester which could be coupled to the corresponding modified ATP analogues (scheme 1). The first two steps were already described in the literature by Ioffe et al.[24] and Mao et al.[25]. The amide formation with the peptide coupling reagent *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetra-methyluronium hexafluorophosphate (HATU) and the methyl 3-(*N*-methylamino)butyrate



Scheme 1. Synthesis of ATTO$_{488}$-NHS: a) methyl 4-(*N*-methylamino) butyrate, HATU, DMF, Et$_3$N, 0 °C, 3 h, b) 2.5 eq. LiOH, 4 °C, 48 h, c) TSTU, Et$_3$N, DMF, rt., 2.5 h.

(**2**) yielded compound **3** in 82% yield, followed by saponification with lithium hydroxide to give ATTO$_{488}$ (**4**). The choice of LiOH as a base was crucial in this step, since the use of sodium hydroxide led to the starting material 4',5'-disulforhodamine 110 (**1**) due to amide hydrolysis. The activation to the NHS ester was achieved with *N,N,N',N'*-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU). After freeze-drying and storage at - 20 °C we did not observe decomposition over extended time periods (> 12 months). More synthetic details and the spectroscopic data of the products are contained in the Supplementary Information. With the ATTO$_{488}$-NHS ester in hand we completed the synthesis of the nucleoside modified ATP analogues (Fig. 2 (**6 – 9**), syntheses details shown in the Supplementary Information).



(**6**): R_1 = -NH-$(CH_2)_6$-NH-ATTO$_{488}$, R_2, R_3, R_4, R_5 = H
(**7**): R_2 = -$(CH_2)_6$-NH-ATTO$_{488}$, R_1, R_3, R_4, R_5 = H
(**8**): R_3 = NH-$(CH_2)_6$-NH-ATTO$_{488}$, R_1, R_2, R_4, R_5 = H
(**9**): R_4 = -$(CH_2)_6$-NH-ATTO$_{488}$, R_1, R_2, R_3, R_5 = H
(**10**): R_5 = -$(CH_2)_6$-NH-ATTO$_{488}$, R_1, R_2, R_3, R_4 = H

Figure 2. Five synthesised ATTO$_{488}$ labelled ATP analogues.

Fluorescence lifetime measurements of compounds **6 – 9** reveal that in all cases, the lifetimes are lower (ranging from 2.07 – 3.33 ns) than that of the unbound dye, for which a fluorescence lifetime of 4.28 ns was determined. This means that the fluorescence of ATTO$_{488}$ is significantly quenched when bound to any of the positions at the nucleoside tested. The observation of multi-exponential fluorescence decays for all compounds suggests the existence of various conformations with different quenching rates. To investigate further whether the triphosphate moiety has an influence on the fluorescence lifetime, compounds **6 – 9** were treated with the α/β anhydride cleaving phosphodiesterase I from *Crotalus adamanteus* (Snake Venom Phosphodiesterase, SVPD), before the determination of fluorescence lifetimes (Fig. S1, left). We found that the cleavage of the anhydride bond does not have any influence on the fluorescence lifetimes. Therefore, attachment of the label ATTO$_{488}$ to the nucleoside appeared not to be suited for the development of a general strategy to obtain fluorescence lifetime probes for ATP hydrolysis.

Since the phosphate groups had no influence on the fluorescence lifetimes, while ATTO$_{488}$ was strongly quenched when attached to the nucleoside, we next attached the ATTO$_{488}$-dye via phosphoester linkage to the γ-phosphate of ATP and measured the fluorescence lifetime. The synthesis was built upon commercially available ATP which was alkylated[26], followed by azide reduction and subsequent NHS ester coupling (scheme 2, synthetic details described in the Supplementary Information).

As for the compounds **6 – 9**, the fluorescence lifetime was also reduced for ATTO$_{488}$-γATP (**10**) before phosphodiesterase treatment (from 4.28 ns to 3.64 ns). However, for compound **10** we found that cleavage of the α/β anhydride bond has a strong effect on the fluorescence lifetime, which raised to the value of the free dye due to the spatial separation after cleavage (Tab. 1).

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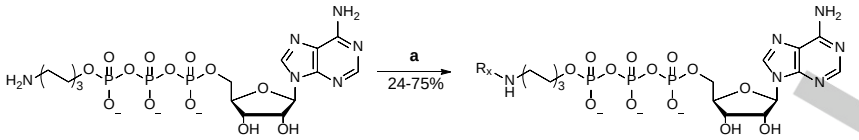

(**12**) R_1: 2',7'-DifluoroATTO$_{488}$	36% (two steps)	(**15**) R_5: ATTO$_{532}$	55%	(**19**) R_9: Bodipy FL	21% (two steps)
(**13**) R_2: 2',7' DichloroATTO$_{488}$	24% (two steps)	(**16**) R_6: Bodipy R6G	57%	(**20**) R_{10}: rhodamine R6G	60%
(**10**) R_3: ATTO$_{488}$	50%	(**17**) R_7: ATTO$_{465}$	46%	(**21**) R_{11}: ATTO$_{495}$	68%
(**14**) R_4: ATTO$_{514}$	67%	(**18**) R_8: ATTO$_{520}$	75%		

Scheme 2. Synthesis of γ-phosphoester- (dye amidohexyl)-adenosinetriphosphates: a) Dye-NHS ester, aqueous 0.1 M $NaHCO_3$, pH = 8.7, 1 h, rt. Detailed yields are given next to the compound list.

Attachment of the chromophore to the γ-phosphate therefore presents a way to generate mono-labelled ATP analogues which exhibit pronounced fluorescence lifetime changes upon ATP hydrolysis. In order to study the quenching effect in more detail, we prepared a series of ATP analogues in which different chromophores were attached to the γ-phosphate (Scheme 2, Fig. 3). With the exception of two BODIPY derivatives, the chromophores chosen are cyanine dyes similar to ATTO$_{488}$, but substituted with different electron withdrawing and donating groups.

Fluorescence lifetime measurements for all the synthesised ATP analogues with different chromophores at the γ-phosphate are summarised in table 1. For all compounds, we determined the fluorescence lifetimes of the intact ATP analogues and the fluorescence lifetimes after cleavage of the anhydride bond with SVPD. The latter values are referred to as the values of the unbound chromophore. The full cleavage of every single ATP probe was monitored by analytical HPLC and the cleavage products verified by MS.
The data show that attachment of the chromophores to the γ-phosphate of ATP can lead to both, reductions but also increases in fluorescence lifetimes. A closer inspection of the data reveals, that an increase in fluorescence lifetime was only observed for chromophores which did not obtain any sulfonate groups. All the respective ATP analogues also exhibited slight bathochromic shifts as compared to the unbound chromophores. For this reason, the ATP analogues with sulfonate substituted chromophores are discussed separately from those without sulfonate groups.

A possible mechanism explaining the observed fluorescence lifetime reductions of the chromophores containing sulfonate groups is photoinduced electron transfer (PET). In the first step of this process, an acceptor chromophore is electronically excited, which leads to singly occupied highest occupied and lowest unoccupied molecular orbitals (HOMO and LUMO, respectively, cf. Fig. 4). If the HOMO of a neighbouring donor molecule is higher in energy than the HOMO of the acceptor, an electron of the donor's HOMO can be transferred to the excited acceptor's singly occupied HOMO. The cycle is closed by transferring the electron from the acceptor's LUMO to the donor's HOMO. PET competes with radiative relaxation and quenches the fluorescence emission of the dye. It is only efficient, if the organic fluorophore and the quenching moiety come into close contact, typically on the scale of a few nanometer[27]. PET has been widely used for fluorescence sensors in sensing of e.g. inorganic salt[28] and nucleotides[29]. Until now, tryptophan and guanine are the only amino acid and nucleobase described to be potent quenchers which are able to reduce, e.g. cumarine[30] and rhodamine fluorophores[31].
Several fluorescently modified guanosine triphosphate (GTP) analogues are commercially available[32]. To the best of our knowledge, however, a fluorescently modified ATP instead of GTP for monitoring enzyme activity based on PET has not been reported yet. This is most likely due to the fact that the oxidation potential for adenine is higher than that of guanine[33]. However, a couple of the modified ATP analogues described in this work are commercially available too and may be directly used for cleavage experiments.

As outlined above, a pivotal parameter for PET to occur is the relative energetic position of the HOMOs of acceptor and donor. Most often, the respective energies are derived from cyclic voltammetry (CV) experiments. In our studies, however, the poor

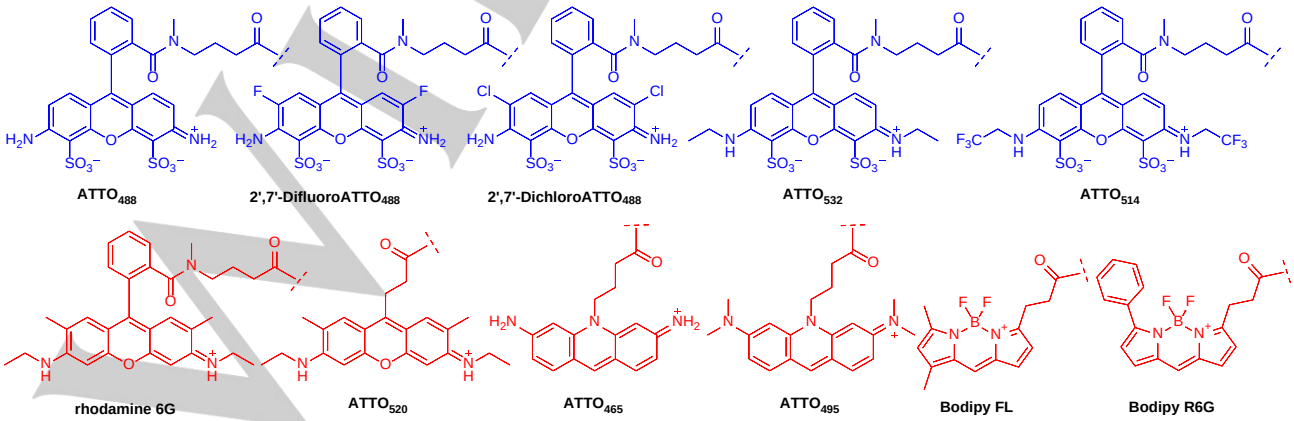

Figure 3. Hydrophilic ATTO$_{488}$ core structure (top blue) and hydrophobic (bottom red) fluorescence dyes.

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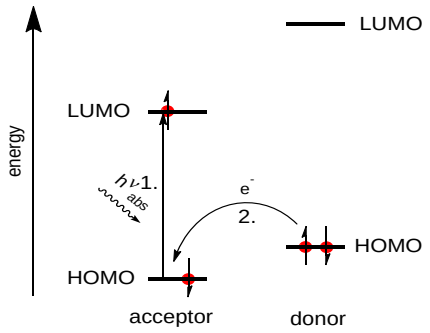

Figure 4. The efficiency of PET is governed by the relative energies of donor and acceptor orbitals.

solubility of the ATP analogues in suitable solvents prevented us from obtaining reproducible results for the redox potentials from CV experiments. We therefore employed photoelectron spectroscopy in air (PESA) to determine the HOMO energies of the chromophores from dry thin films (Fig. 5). As a reference, we first determined the ionisation energy of ATP by PESA with a value of -5.78 ± 0.02 eV (dashed line in Fig. 5).

Only chromophores with HOMO energies below this level should be able to undergo PET. The data obtained for the HOMO levels of the different chromophores indeed show that the reduction in fluorescence lifetimes are directly related to the differences in HOMO energies relative to the HOMO of ATP. This shows that PET most likely is the mechanism responsible for the reduction of fluorescence lifetimes in ATP analogues with sulfonate groups containing chromophores. In order to investigate whether this quenching is dynamic or static, one can compare the ratios of fluorescence intensities with the free or nucleoside-bound chromophore, I_0 and I, respectively, and the corresponding fluorescence lifetimes τ_0 and τ. For purely dynamic quenching, one expects that $I_0/I = \tau_0/\tau$, whereas $I_0/I >> \tau_0/\tau$ for static quenching. We find that τ_0/τ lies between 0.67 to 0.82 times I_0/I, indicating that the quenching is mostly dynamic. This finding is further corroborated by the fact, that no spectral shifts between bound and unbound chromophores have been observed (data shown in SI). This hints at very little interaction between the chromophore and the π-electron system of the nucleobase most likely due to the high hydrophilicity of the sulfonate substituted chromophores.

Table 1. Spectroscopic properties and intrinsic fluorescence lifetimes of γ-O-(Dye amidohexyl)-adenosine triphosphate (scheme 2) before and after treatment with SVPD. All fluorescence lifetimes represent mean ± standard error of triplicates.

R_x-γ-ATP	Δτ[ps]	E_{HOMO} [eV]	SVPD	τ_{fl} [ps]	λ_{abs} [nm]	λ_{em} [nm]	Φ	k_r [ms^{-1}]	k_{nr} [ms^{-1}]
2',7'-Difluoro	- 817	- 5.92	-	3438 ± 49	501	520	0.57	167	124
$ATTO_{488}$ (12)	± 52	± 0.01	+	4255 ± 17	502	520	0.90	210	24
2',7'-Dichloro	-666	- 5.88	-	3350 ± 29	511	527	0.55	164	135
$ATTO_{488}$ (13)	± 57	± 0.03	+	4016 ± 48	511	527	0.84	210	39
$ATTO_{488}$ (10)	- 576	- 5.87	-	3661 ± 16	502	520	0.54	148	126
	± 43	± 0.02	+	4225 ± 39	501	521	0.86	203	33
$ATTO_{514}$ (14)	- 534	- 5.78	-	3554 ± 32	513	531	0.87	243	38
	± 52	± 0.02	+	4088 ± 41	512	530	0.71	173	72
$ATTO_{532}$ (15)	+ 4	- 5.71	-	3995 ± 19	534	552	0.82	205	46
	± 29	± 0.02	+	3998 ± 22	533	552	0.84	210	41
Bodipy R6G (16)	+ 274	- 5.75	-	5877 ± 35	529	550	0.84	143	27
	± 40	± 0.01	+	5603 ± 20	525	546	0.79	141	38
$ATTO_{465}$ (17)	+ 494	- 5.68	-	5609 ± 11	460	508	0.54	96	82
	± 29	± 0.02	+	5115 ± 27	454	507	0.50	98	98
$ATTO_{520}$ (18)	+ 365	- 5.56	-	4260 ± 29	527	555	0.87	203	32
	± 55	± 0.02	+	3895 ± 47	523	552	0.87	223	33
Bodipy FL (19)	+ 513	- 5.55	-	6544 ± 15	504	514	0.86	131	21
	± 50	± 0.01	+	6030 ± 48	503	510	0.94	156	10
Rhodamine 6G (20)	+ 470	- 5.47	-	4731 ± 64	539	559	0.86	143	27
	± 67	± 0.03	+	4262 ± 20	534	556	0.94	141	38
$ATTO_{495}$ (21)	+ 1028	- 5.32	-	2094 ± 55	502	525	0.33	156	323
	± 70	± 0.02	+	1067 ± 44	498	525	0.18	164	771

The picture is more complicated for the group of analogues in which the chromophores contain no sulfonate groups. We interpret the fact that all these compounds exhibit slight bathochromic shifts as a sign for a weak interaction between the π-systems of the chromophore and the adenine. Compared to their sulfonated counterparts, the chromophores in these ATP analogues are more hydrophobic. It can therefore be expected that the hydrophobic effect will lead to a stacking interaction between the similarly hydrophobic adenine and the chromophore. While this interaction only leads to minor spectral changes, it seemingly has a stronger influence on the relaxation rates of the excited chromophores. In order to get more detailed insight into this phenomenon, we determined fluorescence quantum yields Φ for all the compounds exhibiting an increase in the fluorescence lifetimes upon attachment of the chromophore to ATP (cf. Tab. 1).

Again, these data were determined before and after application of SVPD as an agent for the hydrolysis of the anhydride bond. From the quantum yield and fluorescence lifetime measurements, we calculated the radiative and non-radiative relaxation rates k_r and k_{nr}, respectively, of the excited chromophore states using $\Phi=k_r/(k_r+k_{nr})$ and $\tau=1/(k_r+k_{nr})$. We find that in all cases, the attachment of the chromophore to ATP leads to no or only a slight decrease of k_r (+1% to -21%). This change in the radiative rate alone would thus lead to a small increase in the fluorescence lifetimes. However, as pointed out above, the latter also depends on the non-radiative rates. Here, we found a non-uniform behaviour with changes in k_{nr} ranging from +109% to -58% upon attachment of the chromophore to ATP. Interestingly, among the chromophores exhibiting an increase in fluorescence lifetime, the largest decrease in k_{nr} was observed for $ATTO_{495}$ which by far has the smallest fluorescence quantum yield of all chromophores used. The data can be rationalised in the following manner: for chromophores which in the unbound states have a high quantum yield, small decreases in the radiative rates lead to an increase in the fluorescence lifetimes upon binding to ATP. Since in these cases the non-radiative rates are much smaller, the minor changes in the latter will not have an important influence on the fluorescence lifetimes. For chromophores with low fluorescence quantum yields, however, the interaction of the chromophore with adenine will lead to an important reduction of the non-radiative rate k_{nr}. Since for these compounds k_{nr} is larger than k_r, the reduction of k_{nr} is the dominating effect explaining the increase of the fluorescence lifetime upon binding to ATP.

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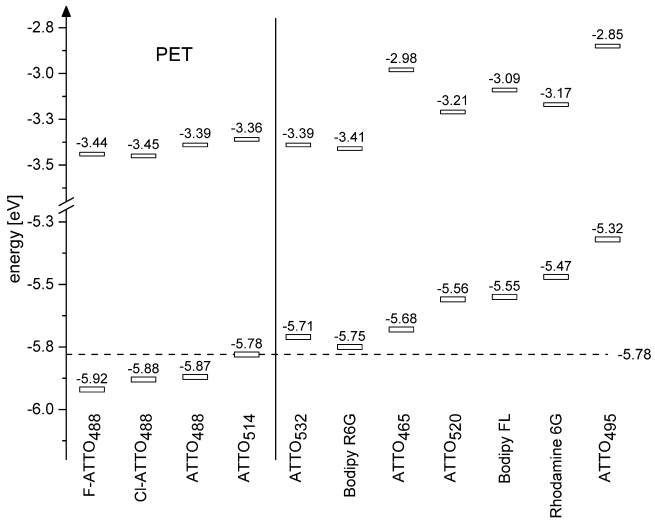

Figure 5. Measured ionisation potentials for eleven different carboxylic acid fluorescence dyes. The LUMO potentials are calculated by adding the absorption maximum. The dashed line corresponds to the HOMO of ATP.

Afterwards, we compared the cleavage kinetics of the hydrophilic dye $ATTO_{488}$ attached to ATP (**10**), the unsulfonated hydrophobic dye rhodamine B attached to ATP (**20**) and natural ATP with SVPD and monitored the ratio between cleaved and uncleaved probes by analytical HPLC over time. It is worthwhile to mention that the hydrophilic $ATTO_{488}$ analogue (**10**) is hydrolysed significantly faster than the hydrophobic rhodamine B (**20**) (results are shown in the SI, Fig. 2). This can be explained by the already mentioned stacking behavior between hydrophobic dyes and the nucleobase adenine leading to a stacked complex. This conformation is less tolerated and/or has to rearrange to an open conformation to be accepted by SVPD. Natural ATP is slower processed by SVPD than compound **10** but faster than compound **20**, thus taking an intermediate positon in this cleavage assay.

As a first test, whether the concept of using mono functionalised ATPs as more widely applicable fluorescence lifetime probes for enzyme dynamics can be realised, we continuously measured the hydrolysis of fluorescence lifetime sensitive mono labelled ATP analogues on a wide-field fluorescence lifetime imaging microscope (FLIM). $ATTO_{488}$-γATP (**10**) with a reduced fluorescence lifetime and $ATTO_{495}$-γATP (**21**) with an increased fluorescence lifetime before cleavage were both separately incubated with and without SVPD. Both control experiments without SVPD showed that the compounds are stable during the measurements, as indicated by the constant fluorescence lifetimes. For the SVPD experiments, the ATP analogues are rapidly processed, resulting in a change of the fluorescence lifetimes to the corresponding free uncoupled dyes (Fig. 6 a) and b)).

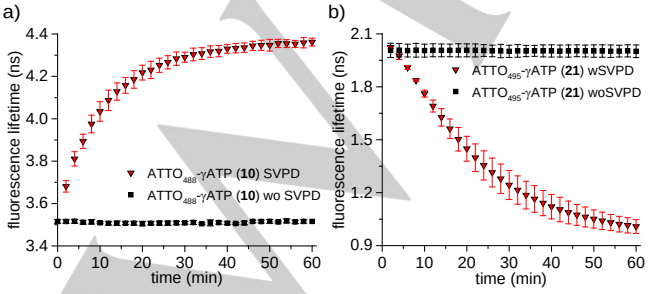

Figure 6. Fluorescence lifetime time course of a) γ-O- ($ATTO_{488}$ amidohexyl)-adenosine triphosphate and b) γ-O- ($ATTO_{495}$ amidohexyl)-adenosine triphosphate treated with and without SVPD at 25 °C. All data represent mean ± standard error of triplicates.

Conclusions

In conclusion, we synthesised 15 fluorescently labelled ATP analogues and determined their absorption spectra, emission spectra and fluorescence lifetimes. For the four nucleoside modified probes (**6** – **9**), we show that there are no influences of the polyphosphates on the fluorescence lifetime of $ATTO_{488}$. However, all four fluorescence lifetimes were lower than for the uncoupled dye. In contrast, when attaching $ATTO_{488}$ to the γ-phosphate of ATP, the fluorescence lifetime is still lower. We give evidence that the observed reduced fluorescence lifetime is dynamic in nature and occurs due to photoinduced electron transfer, where the fluorophores used are quenched by the adenosine moiety of the ATP. On the one hand, by introducing electron withdrawing groups into the fluorescence dye, it was possible to lower the HOMO energy level, which resulted in reduced fluorescence lifetimes which makes the probes more sensitive and supports our PET hypothesis. On the other hand, when attaching dyes like the acridines $ATTO_{465}$ and $ATTO_{495}$ to the γ-phosphate of ATP, longer fluorescence lifetimes were observed. Our fluorescence lifetime-sensitive γ-modified ATP probes can be used in monitoring enzyme activity in a continuous manner. These sensors work without any modification on the nucleoside which renders them more promising for applications compared to doubly labelled ATP probes. All these properties and the fact that the used fluorescence lifetime read-out is robust and less affected make the fluorescence lifetime sensitive γ-modified ATP probes a promising tool that has the potential to complement existing methods to study enzyme activity continuously.

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Keywords: ATP • modified nucleotide • photoinduced electron transfer • fluorescence lifetime • molecular probe

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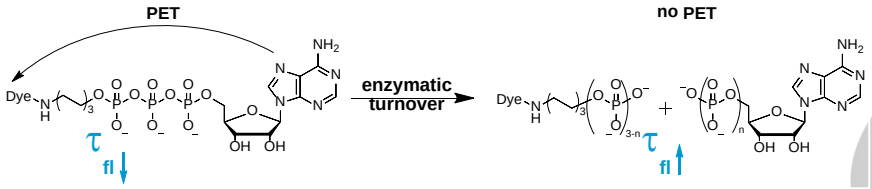

Monitoring of ATP consumption in real time: Fluorescently modified adenosine triphosphate (ATP) probes that change their fluorescence properties upon enzymatic turnover have been synthesised and applied in a simple enzymatic cleavage reaction. Both, fluorescence lifetime increases and decreases upon attachment to ATP were observed and we present evidence that fluorescence quenching in the compounds tested is dynamic and due to photoinduced electron transfer (PET), whereas fluorescence lifetime increases are caused by stacking interactions between chromophore and the nucleobase reducing non-radiative relaxation.

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Page No. – Page No.

Fluorescence lifetime sensitive ATP probes for monitoring enzyme activity