CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200601509

Europium Tetracycline as a Luminescent Probe for Nucleoside Phosphates and Its Application to the Determination of Kinase Activity

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Abstract: The determination of enzyme activities and the screening of enzyme regulators is a major task in clinical chemistry and drug development. A broad variety of enzymatic reactions is associated with the consumption of adenosine triphosphate (ATP), including, in particular, phosphorylation reactions catalyzed by kinases, formation of adenosine cyclic monophosphate (cAMP) by adenylate cyclases, and ATP decomposition by ATPase. We have studied the effect of a series of adenosine (ATP, ADP, AMP, cAMP) and guanosine (GTP, GDP) phosphoric esters, and of pyrophosphate (PP) on the fluorescence emission of the europium tetracycline (EuTC) complex. We found that these compounds have strongly different quenching effects on the luminescence emission of EuTC. The triphosphates ATP and GTP behave as strong quenchers in reducing the fluorescence intensity of EuTC to 25% of its initial value by formation of a ternary 1:1:1 complex. All other phosphate esters showed a weak quenching effect only. The applicability of this fluorescent probe to the determination of the activity of phosphorylation enzymes is demonstrated by means of creatine kinase as a model for non-membrane-bound kinases. In contrast to other methods, this approach does not require the use of radioactively labeled ATP substrates, additional enzymes, or of rather complex immunoassays.

Keywords: ATP · fluorescent probes · kinases · lanthanides · phosphorylation

Introduction

Complexes of Eu^{3+} with various ligands have attracted remarkable interest as luminescent probes and labels for proteins, antibodies, and DNA.[1] Applications include staining of gels or blots, cellular histochemical staining,[2] time-resolved fluoroimmunoassays (TRFIA),[3] and time-resolved Foerster resonance energy transfer (TRFRET) assays.[4] Europium–chelate complexes have a hypersensitive linear emission due to ligand charge-transfer, evoked by the originally forbidden transition $^5D_0 \rightarrow {}^7F_2$.[5] Lanthanide complexes undergo reversible coordination with certain additional ligands, and can form ternary complexes in which the additional ligand has a strong effect on the charge-transfer efficiency. The intrinsic hypersensitive fluorescence was used for the detection of chelating agents such as hydrogen carbonates, acetates, lactates, citrates, and amino acids.[6] In addition, non-enzymatic assays for the determination of hydrogen peroxide were developed by using the europium(III)–tetracycline complex (EuTC) as a fluorescent probe. These are based on the report from Rakicoglu et al.[7] who found that fluorescence intensity is increased 15 times if hydrogen peroxide is added to the fluorescent EuTC system. Consequently, EuTC was applied to monitor the activity of glucose oxidase, catalase, and peroxidase, whose activities are associated with the production or consumption of H_2O_2.[8] It was found that the luminescence intensity and lifetime of EuTC also responds to analytes such as citrate and its metabolic products,[9] heparin,[10] phosphate,[12] and nicotine adenine dinucleotide phosphate (NADP).[13] Because of the strong ligand effect on the luminescence lifetime of EuTC, detection schemes include time-resolved fluorescence imaging.[14] Europium complexes with antibiotics from the tetracycline family, such as doxycycline, metacycline, or oxytetracyline, have been used for the spectrofluorimetric determination of human serum albumin (HSA),[14] lysozyme,[15] or nucleic acids,[16] respectively. Recently, it was demonstrated that europium oxytetracyline EuOTC can be used to determine the concentration of adenosine triphosphate (ATP) from 80 nmol L^{-1} to 1.5 μmol L^{-1} in a buffered solution.[17] The authors postulate that fluorescence enhancement occurs

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from intramolecular energy transfer in the ternary complex [Eu(OTC)(ATP)].

The increasing number of publications related to europium complexes as luminescent probes for the detection of quite different analytes emphasizes their large versatility. On the other hand, it demonstrates the drawbacks of these methods: the lack of selectivity and the high sensitivity towards interferences. Therefore, it is doubtful that europium probes can be used to quantify the concentration of ATP (or any other of the analytes listed above) in complex biological samples. Rather, their applications are limited to situations in which the specificity of the probe is not crucial and the amount of interferences can be minimized. These conditions are met, first of all, in pharmaceutical high-throughput screening. Here, only a single and well-defined compound is tested for its interaction with a target molecule, usually in a well of a microplate under controlled conditions. If the consumption of a particular analyte such as ATP can be monitored, the assay can be used to screen enzyme activities and the efficiency of their inhibitors or activators. A huge variety of fluorescent probes for ATP and pyrophosphate anions have been reported over the last years. These are based on either metal–ligand complexes[17,18] or artificial receptors such as macrocyclic polyamines or oligopeptides.[19] Lack of selectivity and considerable synthetic effort are the drawbacks of most of these molecular chemosensors.

These considerations prompted us to investigate the luminescent response of the EuTC complex (in a stoichiometry of 1:1) towards different nucleoside phosphates that are associated with enzymatic phosphorylation reactions. Among these, adenosine-5′-triphosphate (ATP) plays a central role as a chemical-energy carrier in the cell and as a (co)substrate for enzymes such as kinases, adenylate cyclases, DNA polymerase, ATPase, or luciferase. The conversion of ATP to ADP is the key step in all kinase-catalyzed reactions. In the case of protein kinases, the terminal phosphate group is transferred to a reactive tyrosine, serine, or threonine unit of a protein. Phosphorylation of a protein is an important reaction in intracellular signal transduction, regulatory processes associated with gene expression, metabolic processes, transduction of hormonal signals, and information processing in the nervous system. Transmembrane protein kinases are one of the major drug targets in cancer therapy.

Screening of inhibitors is performed mostly with immunological assays combined with chemiluminescent, radiometric, or colorimetric detection, or by measuring fluorescence resonance energy transfer or fluorescence polarization.[20] Analysis of kinase activities also plays an important role in clinical chemistry. The determination of creatine kinase (CK) is a diagnostic routine test for suspected myocardial infarction or muscular diseases. An assay for the determination of glycerol in blood is based on glycerol kinase. All these diagnostic tests are monitored through the conversion of ATP by means of secondary enzymatic reactions.

Adenosine-3′,5′-cyclic monophosphate (cAMP), on the other hand, is an important cellular messenger and activator for protein kinase A. It is formed from ATP by the adenylate cyclases. Inhibitors of adenylate cyclase activity are also promising candidates in drug development.[21]

Hence, this study is focused on the adenosine phosphate series ATP, ADP (adenosine-5′-diphosphate), AMP (adenosine-5′-monophosphate), and cAMP. Furthermore, we report on the effects of the guanosine phosphates GTP (guanosine-5′-triphosphate) and GDP (guanosine-5′-diphosphate), and of pyrophosphoric acid (PP) on the emission spectra of EuTC. The applicability of this approach for the determination of enzyme activities is verified by means of a creatine kinase (CK) assay.

Results and Discussion

The spectra of the europium–tetracycline system have been described previously.[14] In this study, we use the change in emission intensity of the hypersensitive Eu^{3+} main band with its maximum at 616 nm ($^5D_0 \rightarrow {}^7F_2$). The excitation wavelength was 405 nm throughout. Addition of a small excess of ATP, ADP, AMP, cAMP, and PP ($c = 25\ \mu mol\,L^{-1}$, respectively) to probe solutions with final concentrations of $21\ \mu mol\,L^{-1}$ of EuTC gives a general impression of the response of the fluorescence emission to the different adenosyl phosphates (Figure 1). Some interesting results can be

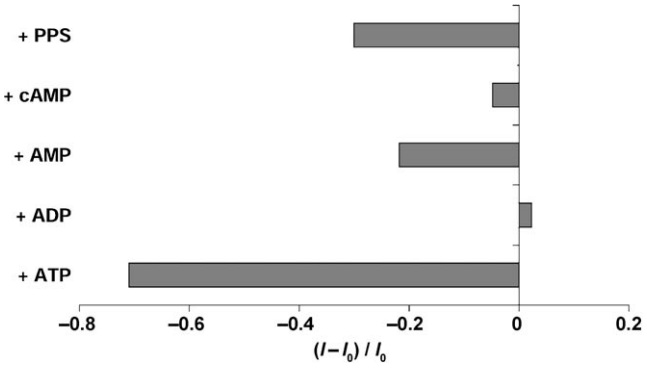

Figure 1. Referenced fluorescence response $(I-I_0)/I_0$ of EuTC, $c = 21\ \mu mol\,L^{-1}$, in MOPS buffer (pH 7.4) in the presence of $25\ \mu mol\,L^{-1}$ of ATP, ADP, AMP, cAMP, and PP, respectively. I_0 represents the fluorescence of EuTC in the absence of phosphate.

deduced from these experiments: ATP quenches nearly 75% of the initial fluorescence of EuTC. AMP and PP induce only a 20–30% decrease in fluorescence intensity, whereas cAMP and, rather unexpectedly, ADP, have no significant effect.

Response of EuTC luminescence to nucleoside phosphates: dynamic ranges and limits of detection: The concentration of EuTC was $21\ \mu mol\,L^{-1}$ in all experiments, unless otherwise stated. EuTC responds to ATP concentrations within a dynamic range of 1–50 $\mu mol\,L^{-1}$. From the referenced intensity plots it can be concluded that EuTC can bind maximally one ATP molecule. Additional ATP causes no further de-

crease in the intensity. The 1:1 stoichiometry of Eu to ATP in the ternary [Eu(TC)(ATP)] complex was confirmed by a Job plot[22] (results not shown). A similar response can be observed in the case of AMP, but with a lower quenching efficiency. On addition of PP, constant signals were already obtained at a ratio of 1:4 of PP to EuTC. This indicates that complexes are formed with two europium ions coordinated to each phosphate unit of the pyrophosphate anion. Interestingly, this correlates well with previous results in which phosphate anions showed a maximum quenching at a ratio of 1:2 (phosphate to EuTC) with a 50% quenching efficiency.[11] The addition of cAMP and ADP has no effect on the luminescence intensity within this concentration range. A distinct decrease in fluorescence can be observed only in the case of a 25-fold excess of cAMP and ADP over EuTC. Figure 2 shows the resulting plots. All data points can be fitted by a second-order exponential-decay function according to the general equation [Eq. (1)]:

$$I = I_0 + A_1 e^{-c/k_1} + A_2 e^{-c/k_2} \quad (1)$$

in which I_0 is the fluorescence intensity of EuTC, and I is the fluorescence intensity in the presence of a phosphate concentration c. A_1, A_2, k_1, and k_2 are numerical calibration factors. Resulting χ^2 values are around 1.38 in all cases.

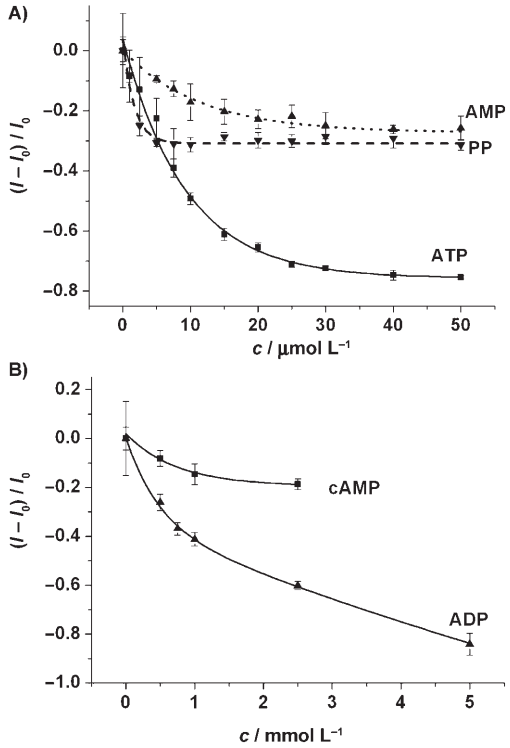

Figure 2. Referenced luminescence intensity $(I-I_0)/I_0$ of EuTC, $c=21\ \mu mol\,L^{-1}$, in MOPS buffer (pH 7.4) in the presence of various concentrations of (top) ATP (■), PP (▼), AMP (▲); and (bottom) ADP (▲), cAMP (■), after a reaction time of 10 min. All curves were fitted by a second-order exponential-decay function. The error bars represent the standard deviations as calculated from four wells containing equal concentrations.

A Stern–Volmer type of plot (I_0/I vs. quencher concentration $[Q]$) gives a linear fit for concentrations of up to $25\ \mu mol\,L^{-1}$ of ATP. Similar fits can be obtained for the other phosphate esters. Figure 3 shows plots for ATP and

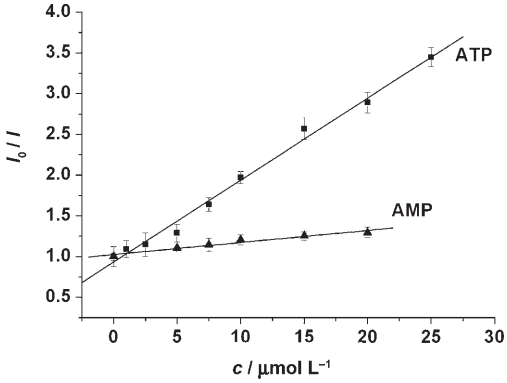

Figure 3. Linear Stern–Volmer type plots (I_0/I) versus quencher concentration $[Q]$, shown for ATP (■) and AMP (▲). The error bars represent the standard deviations as calculated from four wells containing equal concentrations.

AMP as examples. The quenching constants (K_{SV}) were calculated according to the Stern–Volmer equation [Eq. (2)]:

$$I_0/I = 1 + K_{SV}[Q] \quad (2)$$

The results are listed in Table 1, along with the corresponding limits of detection (LODs). These were calculated according to LOD = 3 × SD (blank EuTC) divided by the slope of the linear fit. Concentrations above these linear ranges show significant deviations from the Stern–Volmer relationship with a saturation of the quenching effect. This can be expected if static quenching is predominant and all binding sites of the europium ion are occupied.

Table 1. Quenching constants (K_{SV}) as calculated from the respective linear parts of Stern–Volmer plots, with c[EuTC] = 21 μmol L^{-1}.

Quencher	Linear range [μmol L^{-1}]	R[a]	K_{SV} [mol]$^{-1}$	LOD [μmol L^{-1}]
ATP	0–25	0.996	1.0×10^5	1.5
AMP	0–20	0.978	1.5×10^4	2.5
PP	0–5	0.961	8.8×0^4	1.0
ADP	250–2500	0.995	5.9×10^2	250
cAMP	250–1000	0.978	2.3×10^2	700
GTP	0–20	0.998	1.2×10^5	1.8
GDP	250–2500	0.986	5.2×10^2	200

[a] R = correlation coefficient of linear fit.

Additional experiments revealed the following features of the adenosine phosphate assay:

1) the best sensitivity is achieved with the europium–tetracycline 1:1 complex

2) it can be used over a pH range of 6–8.5 without affecting the fluorescence properties or the dynamic range of the response to ATP
3) it requires a 5–10-min incubation time to obtain maximal signal changes
4) in terms of response and reproducibility, it performs best with time-resolved detection of fluorescence (gated detection); in the microwell-plate experiments presented here, a lag time of 40 μs after the excitation pulse and an integration time of 60 μs was applied
5) the presence of interferences has to be considered thoroughly; only buffers free of phosphate (such as MOPS (3-(*N*-morpholino)propanesulfonic acid), HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), or TRIS (2-amino-2-hydroxymethyl-1,3-propanediol)) can be used
6) the temperature has to be kept constant throughout the assay, as the fluorescence of europium complexes is highly sensitive towards temperature effects
7) due to stoichiometric complex formation with ATP, any change in the concentration of EuTC is accompanied by a shift of the dynamic range and the corresponding LOD.

Because the fluorescence of EuTC has a quantum yield of only 0.3%,[9] the sensitivity of the assay is strongly influenced by the instrumental set-up. With a commercially available time-resolving microplate reader, the LOD for ATP determination can be lowered to 0.1 μmol L^{-1} within a dynamic range of 0.1–1 μmol L^{-1} of ATP by using a probe concentration of 1 μmol L^{-1}. The choice of the EuTC concentration not only determines the LOD and the dynamic range of the assay, but also has an effect on K_{SV}.

Surprisingly, it was found that luminescence lifetime is not a useful parameter for the determination of ATP. Assuming a triple-exponential decay as was found in previous studies,[9] the weighted-average lifetime $<\tau>$ of the EuTC complex in a stoichiometry of 1:1 is 44 μs, as obtained by means of a time-correlated single photon counting (TCSPC) instrument. Analysis with TCSPC and the rapid lifetime determination (RLD)[23] method revealed a stepwise decrease in lifetime only in the case of low ATP concentrations of up to 10 μmol L^{-1} ($<\tau>=31$ μs). Thus, in the presence of excess EuTC dynamic ("collisional") quenching is predominant. ATP in concentrations higher than 10 μmol L^{-1} causes an increase in the average lifetime again. In the presence of 50 μmol L^{-1} of ATP the lifetime of EuTC reaches its initial value of around $<\tau>=45$ μs. Now, static quenching seems to be the dominating mechanism of deactivation. Complexation of the ATP ligand apparently prevents the europium center from collisional quenching. The same tendencies can be observed for the other phosphate esters studied.

GTP is essential for cellular signal transduction and energy transfer. GTP-binding proteins such as protein biosynthesis factors or G proteins play an important role in the activation of enzymes. The intracellular level of GTP and GDP is controlled by the GTPase cycle. If GTP and GDP are probed with EuTC, the same responses, dynamic ranges, and LODs can be observed as in the case of ATP and ADP, correspondingly. This indicates that the nucleoside moiety has no effect on the binding to EuTC. The resulting quenching constants are similar to those of ATP and ADP (Table 1). Figure 4 shows the response curve for the GTP

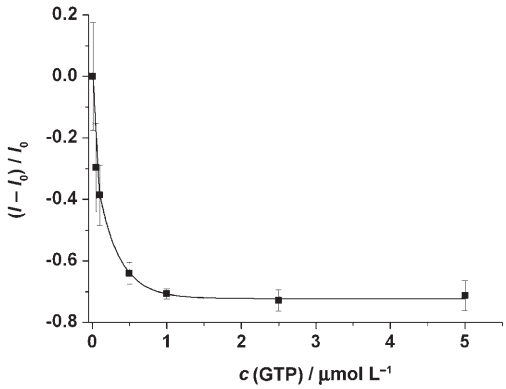

Figure 4. Referenced luminescence intensity $(I-I_0)/I_0$ of EuTC, $c=$ 420 nmol L^{-1}, in MOPS buffer (pH 7.4) in the presence of various concentrations of GTP after 10-min reaction time, fitted by a second-order exponential-decay function.

assay obtained at a probe concentration as low as 420 nmol L^{-1}. A Stern–Volmer type of plot showed linearity from 50–1000 nmol L^{-1} with a K_{SV} of 1.82×10^6 mol^{-1} at a linear correlation coefficient of 0.997. This represents the maximum sensitivity that could be achieved with the europium probe by means of time-resolved fluorescence detection using a microwell-plate reader.

Calibration plots for ATP/ADP and ATP/cAMP systems: As outlined, protein kinases (PKs) transfer the terminal phosphate (P) group from ATP to hydroxylated amino acid units (serine, tyrosine) according to the following scheme [Eq. (3)]:

$$\text{Protein}-\text{OH}+\text{ATP}\xrightarrow{(\text{PK})}\text{Protein}-\text{O}-\text{P}+\text{ADP} \qquad (3)$$

Because ATP and ADP exert different quenching effects on EuTC, we investigated the impact of different mole fractions (x) of ATP and ADP on the europium luminescence. The resulting calibration plot (Figure 5A) shows a significant increase in fluorescence emission as the fraction of ATP decreases. Analogous plots were obtained for the GTP/GDP system. Phosphorylated peptides or proteins have a negligible quenching effect and do not interfere significantly. A phosphorylated 13-mer peptide (sequence: GAEEKEpYHAEGGK) with $c=20$ μmol L^{-1} reduced the fluorescence intensity by only 10%.

Adenylate cyclase (AC) converts ATP into cAMP and pyrophosphate (PP) according to Equation (4):

$$\text{ATP}\xrightarrow{(\text{AC})}\text{cAMP}+\text{PP} \qquad (4)$$

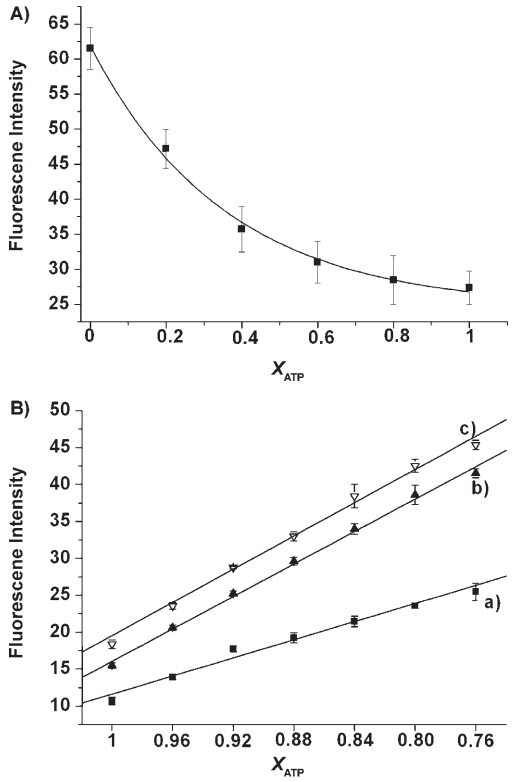

Figure 5. A) Effect of varying mole fractions x_{ATP}, in which $x_{ATP}=c[ATP]/(c[ATP]+c[ADP])$, on the relative luminescence intensity (arbitrary units) of EuTC with $c=21\ \mu mol\,L^{-1}$ (MOPS buffer, pH 7.4; reaction time: 10 min at 30 °C). The overall concentration of ATP and ADP is constant at $25\ \mu mol\,L^{-1}$. B) Effect of varying mole fractions x_{ATP}, in which $x_{ATP}=c[ATP]/(c[ATP]+c[PP])$and $c[cAMP]=c[PP]$, on the relative luminescence intensity (arbitrary units) of EuTC at a) $c=42\ \mu mol\,L^{-1}$, b) $c=63\ \mu mol\,L^{-1}$, and c) $c=84\ \mu mol\ L^{-1}$ (MOPS buffer, pH 7.4; $5\ \mu mol\,L^{-1}\ Ca^{2+}$; $5\ mmol\,L^{-1}\,Mg^{2+}$; reaction time: 10 min at 30 °C). The error bars represent the standard deviations as calculated from four wells containing equal compositions.

Again, calibration plots were recorded for different mole fractions of ATP, cAMP, and PP, thereby mimicking the conversion of the adenylate cyclase reaction (Figure 5B). The experiments were performed under conditions that are required for high adenylate cyclase activities (pH 7.4, $c[Ca^{2+}]=5\ \mu mol\,L^{-1}$, $c[Mg^{2+}]=5\ mmol\,L^{-1}$, $T=30$ °C). The response is linear for decreasing concentrations (at simultaneously increasing concentrations of cAMP and PP) from 25–19 $\mu mol\,L^{-1}$ of ATP. This corresponds to a consumption of 25% of initial ATP and is associated with a 2.3-fold increase in fluorescence emission. The slope of the linear response (and hence, the sensitivity of the assay) is governed by the concentration of EuTC. The maximal slope is obtained at an excess of EuTC over ATP of 3:1, to give an LOD of $0.25\ \mu mol\,L^{-1}$. This corresponds to a 1% conversion of ATP to cAMP. Thus, enzymatic conversion of ATP to cAMP and also to ADP should be detectable by means of the EuTC probe.

Model system for a kinase activity assay: The applicability of the EuTC-based fluorescent ATP assay for the determination of enzymatic activities was proved with creatine kinase MM isoenzyme. This enzyme serves as a model for non-membrane-bound kinases. Under the experimental conditions selected in this study (pH 7.4, $c[Ca^{2+}]=5\ \mu mol\,L^{-1}$, $c[Mg^{2+}]=0.5\ mmol\,L^{-1}$, $T=30$ °C), creatine kinase (CK) can be activated by *N*-acetylcysteine (NAC) to catalyze the following dephosphorylation reaction [Eq. (5)]:

$$\text{creatine phosphate} + \text{ADP} \xrightarrow{(CK)} \text{creatine} + \text{ATP} \quad (5)$$

In standardized diagnostic assays for CK determination in serum recommended by the International Federation of Clinical Chemistry (IFCC), the ATP formation is detected photometrically in a two-step reaction [Eqs. (6, 7)]:[24]

$$\text{ATP} + \text{glucose} \xrightarrow{(\text{hexokinase})} \text{ADP} + \text{glucose-6-phosphate} \quad (6)$$

$$\text{glucose-6-phosphate} + \text{NADP}^{+} \xrightarrow{(\text{phosphate dehydrogenase})} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^{+} \quad (7)$$

Formation of NADPH (the reduced form of NADP) is detected by means of its UV absorption. In our model system, the enzymatic activity can be monitored directly by means of the ATP synthesis, because it causes a strong quenching of EuTC luminescence as predicted in the calibration plot in Figure 5A. It should be noted that the cosubstrate creatine phosphate (CPO) induces only a negligible quenching of the fluorescence of EuTC. The response is of the same magnitude as in the case of the monophosphate AMP shown in Figure 2, top. The presence of creatine does not affect the fluorescence emission either. The activator *N*-acetylcysteine can be added only in a maximum concentration of $1\ mmol\,L^{-1}$ (with $c[EuTC]=21\ \mu mol\,L^{-1}$), because in higher concentrations ($>2\ mmol\,L^{-1}$) it has a very strong quenching effect (results not shown). The presence of Ca^{2+} and Mg^{2+} is essential for kinase activity; however, both cations also induce weak quenching of EuTC fluorescence. Hence, their concentrations must be exactly balanced and kept constant throughout all assays.

As specified by the producer, one enzyme unit U of CK transfers 1.0 μmol of phosphate per minute from creatine phosphate to ADP, at 37 °C and a pH of 7.5. The activity of CK under the experimental conditions optimized for the EuTC probe (see Experimental Section) was qualitatively tested by means of a commercially available ATP assay based on the bioluminescent luciferin/luciferase system.[25] A kinetic response for the ATP-induced bioluminescence was found for enzyme activities within a dynamic range of 0.1–$2\ U\,mL^{-1}$ of CK (results not shown). These results suggested that the creatine kinase activity remains sufficiently high under the selected conditions. It should be considered, however, that the true activities are lower than the calculated values given in the case of the dilution series.

In a reference experiment, the interference of CK on the fluorescence of EuTC was investigated. It has been reported that the fluorescence of europium complexes responds to several proteins.[14,15] In the course of this study it was found

that bovine serum albumin (BSA) induces a significant enhancement of the EuTC fluorescence (about 30% in the presence of 0.1% BSA). Thus, it is important to reveal the unspecific effect of the activated enzyme in the absence of the (co)substrates ADP and CPO. However, no significant changes in the fluorescence of EuTC were obtained in the presence of activated CK within a concentration range of 1–20 mU mL^{-1}, if no ADP and CPO were added.

After addition of CK to a substrate solution containing EuTC, the fluorescence intensity decreases due to the enzymatic conversion of ADP to ATP. Figure 6 shows the kinetic response of the EuTC fluorescence to increasing activities of CK. The CK activity can be monitored under these specific conditions within a dynamic range of 0.05–2.0 U mL^{-1}. This would cover the range of increased total CK activity in the case of a myocardial infarction patient (0.08–0.60 U mL^{-1}, reference values for healthy adults are 0.01–0.08 U mL^{-1}[26]). As outlined above, the dynamic range and the limit of detection of the assay can be controlled by the added EuTC concentration. Therefore, a further decrease in the LOD and dynamic range is achievable. The plots in Figure 6 show the normalized intensity I/I_0. I_0 represents the fluorescence intensity at $t=0$ immediately after addition of the enzyme. The simultaneously recorded time trace of the blank sample I_{ref} (in the absence of CK) is subtracted from every response curve. This referenced data evaluation is favorable because the blank sample shows a slight signal decrease due to temperature effects and the long equilibration time of the EuTC–ADP system. The CK activity can be determined from the slope of the linear range of the kinetic response. The data can be fitted linearly or by a single exponential-decay function corresponding to the CK activity. As EuTC responds with a time delay (approximately 10 min) to changing analyte concentrations, the curves do not represent the real-time kinetics of the enzyme. Alternatively, the enzyme activity can be determined after a fixed reaction time. Figure 7 shows the corresponding calibration plot after a 10-min exposure to CK. In this case, two linear fits can be applied.

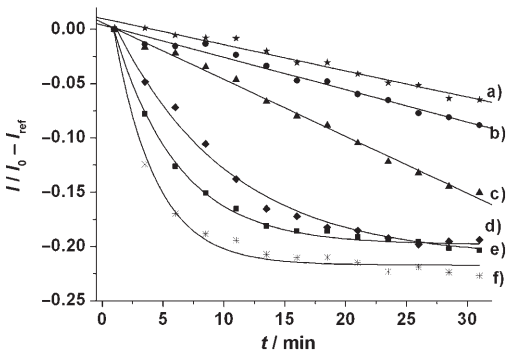

Figure 6. Kinetic analysis of the fluorescence response of EuTC following the enzymatic production of ATP after addition of a) 0.075, b) 0.1, c) 0.2, d) 0.5, e) 1, and f) 2 U mL^{-1} of CK at 30 °C. The time trace of the blank sample (without CK) has been subtracted, respectively. CK is activated by 1 mmol L^{-1} NAC, the MOPS-buffered (pH 7.4) substrate solutions contain ADP (50 μmol L^{-1}), CPO (750 μmol L^{-1}), $CaCl_2$ (5 μmol L^{-1}), and $MgCl_2$ (0.5 mmol L^{-1}).

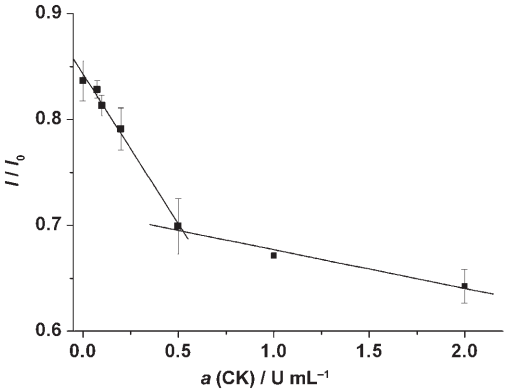

Figure 7. Plot of the normalized fluorescence intensity I/I_0 vs. the activity (a) of CK after 10-min reaction time indicated by EuTC, as deduced from the kinetic time traces (Figure 6). The error bars represent the standard deviations as calculated from four wells of equal CK activity.

Nitrate is reported to be a competitive inhibitor for CK, with a K_i value of 20 μM.[27] As it has no effect on the fluorescence of EuTC, it was used to give advanced proof of applicability of this method in the form of an inhibition assay. Magnesium nitrate was added to the enzyme solution in different concentrations to give final concentrations of 1–10 mmol L^{-1} of NO_3^{2-}. Figure 8 shows the kinetic responses at a CK concentration of 1 U mL^{-1}. Inhibition of enzyme activity in the presence of NO_3^{2-} is clearly demonstrated by the decreasing slope of the fluorescence response after addition of CK. At nitrate concentrations greater than 8 mmol L^{-1}, no quenching can be observed, indicating that at this level complete deactivation of the enzyme occurs and no ATP is formed.

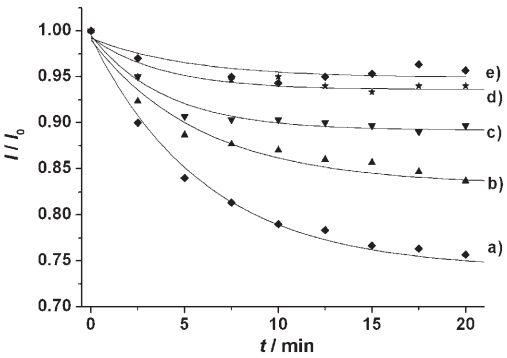

Figure 8. Kinetic analysis of the inhibition of CK by NO_3^{2-} indicated by the fluorescence response of EuTC. The CK activity was 1 U mL^{-1}. NO_3^{2-} was added simultaneously in final concentrations of a) 0, b) 1, c) 2, d) 4, and e) 8 mmol L^{-1} after 30-min equilibration time of the EuTC/ADP/CPO/NAC system.

Conclusion

This study introduces the first direct fluorimetric method for monitoring conversions of ATP to its enzymatic reaction products ADP, cAMP, or PP, respectively. It can also be used for monitoring guanosine phosphate conversions. It is based on the strong quenching effect of ATP (and GTP) on the luminescence of the europium–tetracycline (1:1) complex. The advantages of this novel method over the existing assays for ATP are manifold:

1) it is affordable, straightforward, and shows a versatile applicability;
2) no additional enzymes (such as luciferase in bioluminescent ATP assays or hexokinase/glucose-6-phosphatedehydrogenase in diagnostic assays for kinases) have to be applied;
3) no fluorescently labeled antibodies (such as anti-phosphotyrosine or anti-cAMP) are required for the screening of protein kinase or adenylate cyclase activities;
4) radioactively labeled substrates (such as [γ-^{32}P]ATP) become redundant.

The high non-specificity of the EuTC probe is a drawback of this method. Thus, applications will be conceivable in the field of pharmaceutical screening of enzyme regulators rather than in cellular assays or medical diagnosis. The robustness of the assay needs to be supported by a thorough control of temperature, pH, and the concentrations of ions and other quenching agents. We assume that this approach can lead to an easily accomplishable alternative to existing assays such as immunoassays and radiometric methods, or for secondary enzymatic reactions that are applied for determination of the activities of (protein) kinases, adenylate cyclases, or GTPase. It can be employed in the high-throughput screening of enzyme inhibitors (or activators) in microwell-plate formats. The applicability was confirmed by means of the determination of creatine kinase activities as a model system for enzymatic ADP/ATP conversions. A simple inhibition assay performed with nitrate anions demonstrates that this fluorescent method is applicable for the screening of enzyme regulators.

Experimental Section

Microwell-plate-based fluorescence assays: Fluorescence intensities (time-resolved) were acquired by using a Tecan GENios+ microplate reader (Tecan, Groedig, Austria). The excitation/emission filters were set to 405 and 612 nm, respectively. All dilution series and calibration plots were obtained in 96-microwell plates with four replicates applying time-gated detection at a time lag of 60 μs after the excitation pulse and a signal-integration time of 40 μs. All experiments were performed at 30 °C, unless otherwise stated. The 96-microwell plates were obtained from Greiner Bio-One GmbH (Frickenhausen, Germany). Specifications of fluorescence-lifetime determination by TCSPC and ratiometric imaging (rapid lifetime determination) were given in previous publications.[9,14]

Reagents: All inorganic salts were obtained in analytical purity from Merck (Darmstadt, Germany). Europium(III) trichloride hexahydrate was from Alfa Products (Danvers, USA), tetracycline hydrochloride was from Serva (Heidelberg, Germany). A stock solution of EuTC with $c = 0.63\ \mathrm{mmol\,L^{-1}}$ was prepared by mixing 10 mL of a 6.3-mM solution of $EuCl_3$ hexahydrate with 10 mL of a 6.3-mM solution of tetracycline hydrochloride, and then diluting this to 100 mL with a 10-mM MOPS (3-(*N*-morpholino)propanesulfonic acid) buffer of pH 7.4. Adenosine-5′-triphosphate disodium salt, adenosine-5′-diphosphate sodium salt, adenosine-5′-monophosphate sodium salt, adenosine-3′,5′-cyclic monophosphate, guanosine-5′-triphosphate sodium salt hydrate and guanosine-5′-diphosphate sodium salt were from Sigma, pyrophosphoric acid and *N*-acetylcysteine (NAC) were from Fluka. Recombinant creatine kinase (MM fraction, from human heart) was obtained from Sigma.

Enzyme-activity assays

The ATP determination kit based on luciferin/luciferase bioluminescence was obtained from Molecular Probes. The procedure was applied according to the manufacturer's instructions. Bioluminescence was recorded by using the Tecan GENios+ microplate reader.

Creatine kinase assay using [Eu(TC)] as indicator: Table 2 lists the reagents (all dissolved in 10-mM MOPS buffer, pH 7.4) that were pipetted into one well of a 96-well microplate. After an incubation time of 20–30 min at 30 °C, CK was added in different volumes (0.5–20 μL) to yield final CK activities of 0.05–5 $\mathrm{U\,mL^{-1}}$. MOPS buffer was then added to give a final volume of 100 μL per well. The kinetic response was recorded at 30 °C over a period of 30 min.

Table 2.

Reagent solution (conc.)	Volume	Final concentration
$CaCl_2$ (50 μM)	10 μL	5 $\mu\mathrm{mol\,L^{-1}}$
$MgCl_2$ (5 mM)	10 μL	500 $\mu\mathrm{mol\,L^{-1}}$
N-acetylcysteine (10 mM)	10 μL	1 $\mathrm{mmol\,L^{-1}}$
ADP (500 μM)	10 μL	50 $\mu\mathrm{mol\,L^{-1}}$
CPO (5 mM)	15 μL	750 $\mu\mathrm{mol\,L^{-1}}$
EuTC (84 μM)	25 μL	21 $\mu\mathrm{mol\,L^{-1}}$

The resulting kinetic data can be fitted linearly in the case of low CK activities and by a single exponential-decay function for higher activities (in this case, from 0.05–0.2, and from 0.4–2 $\mathrm{U\,mL^{-1}}$, respectively).

Acknowledgements

The authors acknowledge the assistance of N. Hinterreiter, and of S. Nagl and Dr. C. Cano-Raya for performing the fluorescence-lifetime measurements. M.S. thanks the Deutsche Forschungsgemeinschaft (DFG) for financial support.

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Received: October 23, 2006
Published online: February 26, 2007

