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Real-time fluorescence monitoring of GSK3β-catalyzed phosphoryation by use of a BODIPY-based Zn(II)–Dpa chemosensor

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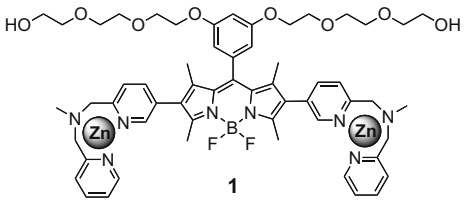
ABSTRACT

We developed a new fluorescence assay system for GSK3β-catalyzed kinase reaction using the BODIPY-based fluorescent chemosensor. This system exploits the selective sensing property of the chemosensor for a (*i*, *i*+4) bis-phosphorylated peptide, which allows us to conveniently detect the phosphorylation reaction with a fluorescence increase in a real-time fashion.

Small molecular-based chemosensors that are able to detect kinase catalyzed phosphorylations with a fluorescence signal change offer a direct method to evaluate kinase actions, and therefore should be a valuable tool for screening kinase inhibitors. Several fluorescent chemosensors or synthetic receptors have been recently developed for detection of phosphorylated proteins and peptides, in which they are commonly designed as the Zn(II) complex to utilize coordination interaction in the binding with the phosphate group(s).[1] Although these chemosensors are useful in simple biological assays such as phosphoprotein detection in SDS–PAGE,[2] application use for kinase catalyzed protein phosphorylation is still strictly limited. This is mainly due to their intrinsic strong binding affinities for polyphosphate species such as ATP, a common phosphate donor in kinase reactions, which competitively prevents the binding of the chemosensors to a target phosphorylated protein/peptide.[1a]

Glycogen synthase kinase 3β (GSK3β) is a unique protein kinase in its substrate specificity, in which GSK3β phosphorylates a serine or threonine residue located at *i*+4 position of N-terminal away from another phosphoserine (Fig. 2).[3] GSK3β was initially identified as an enzyme involved in the control of glycogen metabolism. In recent years, GSK3β has been unveiled to play critical roles in diverse range of cellular events such as insulin signal transduction and formation of abnormal neurofibrillary tangles in brain tissue, which prompt considerable efforts to develop GSK3β inhibitors as promising therapeutics for diabetes and Alzheimer's disease.[4] We describe herein a convenient fluorescence assay system for a phosphorylation reaction catalyzed by GSK3β using the BODIPY-based chemosensor **1** (Fig. 1). The unique binding selectivity of **1** for a (*i*, *i*+4) bis-phosphorylated peptide allowed us to detect GSK3β-catalyzed reaction in a real-time manner with a large fluorescence enhancement (Fig. 2).

Recently, we revealed that the binuclear Zn(II)–Dpa (2,2′-dipicolylamine) complex **1** serves as a selective fluorescent chemosensor for (*i*, *i*+4) bis-phosphorylated peptides.[5] For example, **1**



227 238

tau(227-238)-2P: YAVVR **pT**PPK**pS**PSS

tau(227-238)2E-2P: YAVVE **pT**PPE**pS**PSS

tau(227-238)2E-1P: YAVVE **T**PPE**pS**PSS

Figure 1. Structure of chemosensor **1** and sequences of the peptides. N-Terminal tyrosine (*) was introduced for determination of the peptide concentration by UV absorbance.

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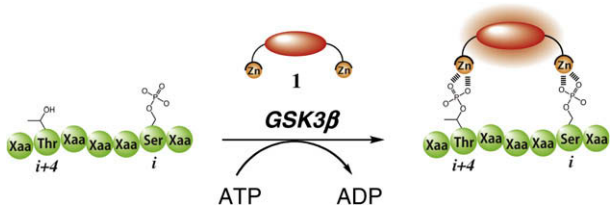

Figure 2. Schematic illustration of the fluorescent assay system using chemosensor **1** reported herein.

bound to tau(227–238)-2P peptide (Fig. 1), a fragment of the hyperphosphorylated tau protein,[6] through coordination interaction with a dissociation constant (K_d) of 43 μM, and maximally increased its fluorescence by up to ca. 1.5-fold.[5] Since the interaction of **1** with a mono-phosphorylated peptide is negligible (K_d >100 μM), we expected that the formation of a (*i*, *i*+4) bis-phosphorylated peptide from the corresponding mono-phosphorylated peptide catalyzed by GSK3β could be monitored by a fluorescence increase of **1**.

An important prerequisite for the fluorescence sensing of GSK3β-catalyzed phosphorylation is that the interaction between **1** and the bis-phosphorylated peptide is not competitively inhibited by ATP. Unfortunately, the dissociation constant (K_d) of **1** with tau(227–238)-2P peptide largely decreased from 43 to 150 μM in the presence of 100 μM of ATP, the value of which is apparently too weak for efficient fluorescence monitoring of the kinase reaction. In order to circumvent the unfavorable effect of ATP, we modified the sequence of the substrate peptide to increase the binding affinity with **1**. We have previously reported that the Zn(II)–Dpa complexes shows a strong binding affinity for the phosphorylated peptide having a negative net charge.[1b] Thus, we designed the new substrate tau(227–238)2E-2P peptide (Fig. 1), in which both of the positively charged Lys and Arg were replaced with Glu.

The fluorescence titration revealed that **1** increased its fluorescence by up to ca. 1.5-fold upon binding to tau(227–238)2E-2P (Fig. 3b). The dissociation constant (K_d) was estimated to be 2.0 μM, the value of which is more than 20-fold stronger than the original tau(227–238)-2P peptide. More significantly, the sensing and binding properties of **1** to tau(227–238)2E-2P peptide were not largely disturbed by ATP, that is, a substantial fluorescence enhancement (ca. up to 2-fold) and a strong binding affinity (K_d = 8.5 μM) are maintained even in the presence of 100 μM of ATP (Fig. 3a and b). In contrast, the mono-phosphorylated tau(227–238)2E-1P did not induce a fluorescence change of **1** (Fig. 3b). These results encouraged us to apply **1** for the real-time

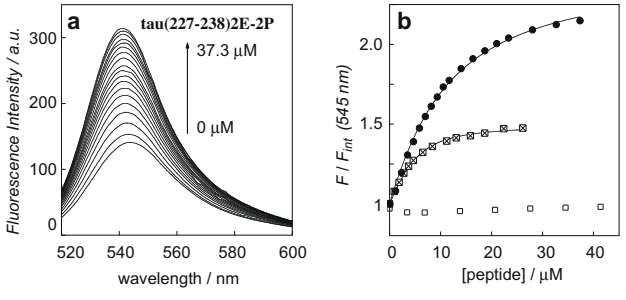

Figure 3. (a) Fluorescence response of **1** upon addition of tau(227–238)2E-2P peptide (0~37.3 μM) in the presence of 100 μM ATP. (b) fluorescence titration profiles of **1** for tau(227–238)2E-2P peptide in the presence (●) and absence (⊠) of 100 μM ATP, and for tau(227–238)2E-1P peptide (□) in the presence of 100 μM ATP. Measurement conditions: 5 μM of **1**, 50 mM HEPES, 10 mM $MgCl_2$, pH 7.2, 25 °C, λ_{ex} = 520 nm.

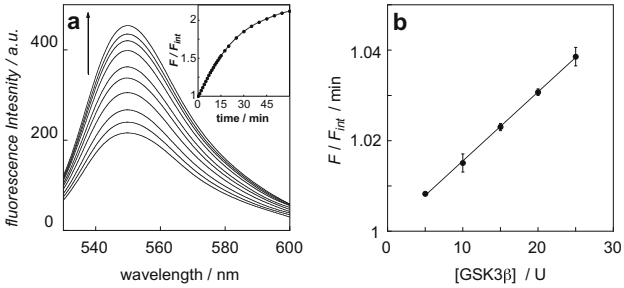

Figure 4. Fluorescence spectral change of **1** during the GSK3β-catalyzed reaction from 0 to 60 min. (inset) Time-trace plot of the fluorescence intensity (λ_{em} = 545 nm) of **1**. Assay conditions; 20 μM of **1**, 20 μM tau(227–238)1E-2P peptide, 100 μM ATP, 25 U GSK3β, 50 mM HEPES, 10 mM $MgCl_2$, 2 μg/μL BSA, pH 7.2, 30 °C, λ_{ex} = 520 nm. (b) Plot of the initial rate of the fluorescence increase (F/F_{int}/min) as a function of the amount of GSK3β.

fluorescence monitoring of GSK3β-catalyzed phosphorylation using tau(227–238)2E-1P as a substrate.

When a phosphorylation reaction was carried out with tau(227–238)2E-1P peptide in the presence of GSK3β (rabbit skeletal muscle), the fluorescence intensity of **1** gradually increased by ca. 2-fold for 60 min (Fig. 4a). The formation of the bis-phosphorylated tau(227–238)2E-2P peptide was confirmed by MALDI-TOF mass analysis, in which the peak of tau(227–238)2E-1P at 1440 disappeared and the new peak of tau(227–238)2E-2P at 1548 concomitantly formed in a time-dependent manner. The HPLC analysis of the reaction sample revealed that the yield of tau(227–238)2E-2P reached to 90% after 60 min. The MALDI-TOF mass analysis also showed that almost the same amount of tau(227–238)2E-2P was produced regardless of the presence or absence of **1** in the assay solution, indicating that **1** did not inhibit the GSK3β-catalyzed phosphorylation reaction. The fluorescence enhancement became faster with increasing the amount of GSK3β, and the plot between the initial rate of the fluorescence increase (F/F_{int}/min) and the enzyme concentration (0–25 U) showed a linear relationship (Fig. 4b). These results suggest that the fluorescence increase evidently represents the progress of the enzyme reaction. Kinetic analysis based on a Lineweaver-Burk plot was also conducted in a successful manner, which estimated the K_m and k_{cat} value of tau(227–238)2E-1P peptide to be 20 μM and 4.8 s^{-1}, respectively. This enzyme assay system would be potentially useful for screening GSK3β inhibitors, a promising therapeutic for the treatment of diabetes and Alzheimer's disease.[4] In a proof-of-principle experiment, the enzyme reaction was carried out in the presence of inhibitor XI, a potent indolylmaleimide-type inhibitor for GSK3β.[7] As shown in Figure 5, the fluorescence enhancement rate apparently decreased by the presence of inhibitor XI, the extent of which

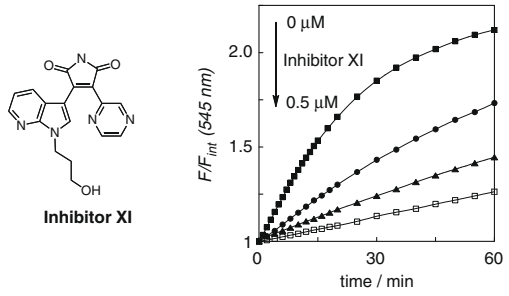

Figure 5. Time-trace plot of the fluorescence intensity (λ_{em} = 545 nm) of **1** during the GSK3β-catalyzed reaction in the absence (■) and the presence of 0.125 (●), 0.25 (▲), and 0.50 (□) μM of inhibitor XI. Assay conditions were identical with those shown in Figure 3.

positively correlated with the concentration of the inhibitor. The HPLC analysis confirmed that the formation of tau(227–238)2E-2P peptide was actually suppressed to 13% yield at 60 min upon addition of 0.5 μM of inhibitor XI. The K_i of inhibitor XI was estimated to be 45 nM based on Cheng–Prusoff equation,[8] which is comparable to the reported value (K_i = 25 nM).[7] These results demonstrate that the present assay system is sufficiently sensitive to examine the screening of GSK3β inhibitors.

In conclusion, we have demonstrated that **1** is a useful chemosensor for the real-time fluorescence monitoring of GSK3β-catalyzed phosphorylation. The unique two point binding mode of **1** with the bis-phosphorylated sequence allowed us to fluorescently detect the phosphorylation reaction without being significantly affected by ATP. To our knowledge, **1** is the first small molecule-based chemosensor that is applicable to a real-time monitoring of protein kinase reaction.[9] Application of this assay system for screening of new GSK3β inhibitors will be forthcoming.

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