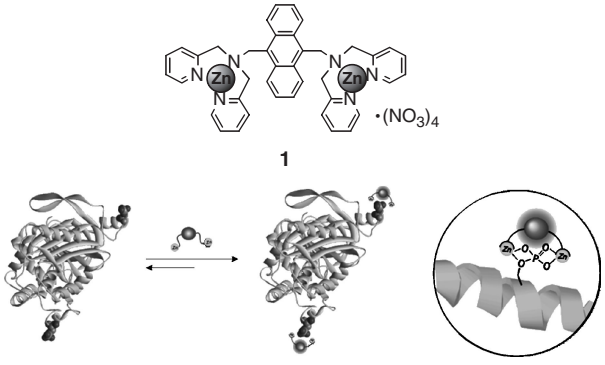
Phosphoprotein-Selective Recognition and Staining in SDS-PAGE by Bis-Zn(II)-dipycolylamine-Appended Anthracene

Akio Ojida,[†††] Takahiro Kohira,[†††] and Itaru Hamachi*[†,††,†††]
[†]*PRESTO (Organization and Function, JST)*
[††]*Institute for Materials Chemistry and Engineering (IMCE)*
[†††]*Department of Chemistry and Biochemistry, Graduate School of Engineering, Kyushu University, Fukuoka, 812-8581*

(Received June 2, 2004; CL-040620)

A novel fluorescence detection system using a chemosensor for phosphoprotein in gel electrophoresis analysis has been developed. The system employed bis-Zn(II)-dipycolylamine (Dpa)-appended anthracene as a fluorescent staining dye to carry out convenient and selective detection of phosphoproteins in SDS-PAGE.

Protein phosphorylation is one of the major chemical reactions in post-translational modification of naturally occurring proteins. In particular, phosphorylation or dephosphorylation of hydroxy groups of amino acid residues located on a protein surface plays crucial roles in intracellular signal transduction cascades of living cells, such as cell growth, differentiation and apoptosis.[1] Thus, it is now important to detect and analyse phosphorylation of proteins among a protein mixture. We have recently developed the first artificial receptors to a phosphorylated peptide in aqueous solution.[2–4] In addition to the selective binding, some of them act as a fluorescent chemosensor which can selectively detect a phosphorylated peptide with fluorescence increase. In this paper, we describe that bis-Zn(II)-dipycolylamine (Dpa)-appended anthracene can be used to fluorescently sense phosphorylated proteins in aqueous solution and it can also stain them selectively in gel electrophoresis analysis such as SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis).



1

Figure 1. Schematic representation of phosphoprotein recognition with fluorescent chemosensor **1**.

To test phosphoprotein selectivity of 9,10-bisZn(Dpa)-anthracene (**1**) as a staining reagent in SDS-PAGE analysis, a protein mixture was used as an analyte sample including 6 distinct proteins (β-galactosidase, bovine serum albumin, phospho-ovalbumin, phospho-α-casein, avidin, and lysozyme). After electrophoresis of 13%T polyacrylamide gel, **1** was used in staining process instead of CBB (Coomassie Brilliant Blue), a conventional staining reagent.[5] Figure 2 shows a typical example of the SDS-PAGE stained by CBB or **1**. Apparently, 6 blue bands corresponding to the molecular weight of 6 proteins are detected in Lane 1 stained by CBB, whereas only two distinct bands are observed in the Lanes 2 and 3 stained by **1** under the photoirradiation using a transilluminator (UV-lamp, λ_{max} at 360 nm). By comparing the molecular weight (MW), these two bands are ascribed to phospho-ovalbumin (MW = 45.0 kDa) and phospho-α-casein (MW = 23.6 kDa) from the top. Brighter emission are observed in the band of α-casein than ovalbumin, which may be due to the higher phosphorylation number (8–9P)[6] of α-casein than that of ovalbumin (2P).[7] Although a weak emission band is observed at a hydrophobic BSA (bovine serum albumin) due to a slight nonspecific absorption in Lane 2 (5 μg of each protein), the nonphosphorylated 4 proteins are not detected in Lane 3 (2.5 μg of each protein).[8] Thus it is clear that **1** can selectively stain the phosphoproteins in SDS-PAGE among the protein mixture.

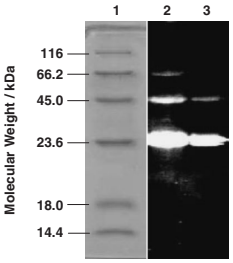


Figure 2. Phosphoprotein selective detection in SDS–polyacrylamide gels using **1**. Each Lane includes 2 phosphorylated proteins (ovalbumin (45.0 kDa) and α-casein (23.6 kDa)), and 4 nonphosphorylated proteins (β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), avidin (18.0 kDa), and lysozyme (14.4 kDa)). Lane 1; CBB staining of the 6 proteins. Lanes 2, 3; Detection of the phosphoproteins with UV transilluminator after staining with **1**. The analysed amounts of each protein in Lanes 2 and 3 are 5 and 2.5 μg, respectively.

To confirm the distinguishing capability of **1**, we next used phosphorylated (8–9P) and dephosphorylated (1–2P)[9] α-casein, both of which are commercially available, as analysed samples. As shown in Figure 3, bright emission is observed in the phospho-α-casein band, whereas weak emission is observed in the dephospho-α-casein band. Although quantitative analysis of band emission was not carried out, it is apparent that **1** can distinguish the difference of the phosphorylation state of an identical protein in SDS-PAGE. In the Lanes 3 to 7 of Figure 3, we examined the minimum concentration to detect the phospho-α-casein using **1** as a staining reagent. From 1 to 5 μg of the applied sample, the emission is clearly detected and the band becomes

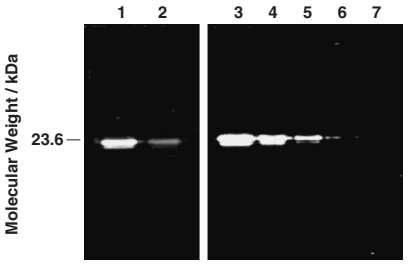


Figure 3. Staining selectivity between phospho- and dephosphoprotein and its sensitivity in SDS–polyacrylamide gels using **1**. Lanes 1, 2; Comparison of detection sensitivity against equal amount (2.5 μg) of phospho- (8–9P, Lane 1) and dephospho- (1–2P, Lane 2) α-casein. Lanes 3–7; Detection of serially diluted phospho-α-casein. Each Lane contains 5, 2.5, 1, 0.5, 0.25 μg of phospho-α-casein from Lanes 3 to 7, respectively.

smear at 0.5 μg (0.02 nmol). Thus, it is apparent that **1** can fluorescently stain phospho-α-casein with 0.02 nmol sensitivity in SDS-PAGE.

Figure 4 shows the fluorescence spectral changes of **1** induced by phospho-α-casein (8–9P) and dephospho-α-casein (1–2P) under aqueous conditions (50 mM HEPES buffer, pH 7.2). As observed for phosphorylated peptide,[2,4] the chemosensor **1** enhances its fluorescence intensity upon binding to the phosphoprotein. The emission of **1** largely increases in the presence of 0.15 equiv of the phospho-α-casein, whereas the emission slightly increases by addition of dephospho-α-casein. In addition to the emission enhancement, its emission maximum shifts from 438 to 470 nm in the case of phospho-α-casein. This may be due to the excimer formation of **1** upon binding to the phospho-α-casein because it possesses the adjacently aligned phosphoserine residues, i.e., a hyperphosphorylated site (α_{S1}-casein, pSer^{66}-pSer^{67}-pSer^{68}) on its surface.[10] On the other hand, such emission shift is not observed for the dephospho-α-casein addition. The gentle increase of the emission by addition of dephospho-α-casein may be ascribed to the remained phosphorylated sites of dephospho-α-casein. Figure 4b shows the titration curves of the integrated fluorescence intensity from 400 to 600 nm. Apparently, phospho-α-casein induces larger fluorescence enhancement of **1** compared to dephospho-α-casein, especially in the presence of low equivalent of the protein.[11] These fluorescence data in aqueous solution are reasonably consistent

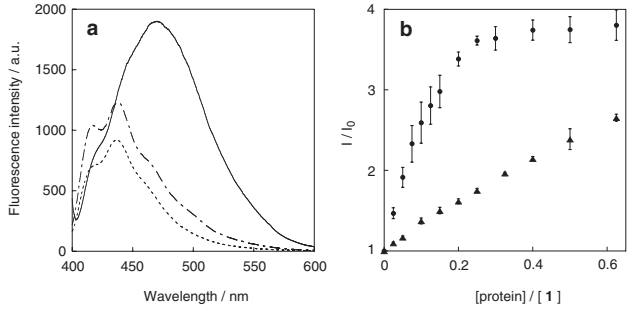


Figure 4. (a) Fluorescence intensity of **1** (1 μM, - - - - -) in the presence of 0.15 μM of phospho-α-casein (—) or dephospho-α-casein (– – – -) in 50 mM HEPES buffer, pH 7.2 at 20 °C, $\lambda_{ex} = 380$ nm. (b) The titration curves of the integrated fluorescence intensity of **1** (from 400 to 600 nm) with phospho-α-casein (●) or dephospho-α-casein (▲).

with the SDS-PAGE results mentioned above, and thus suggest that the clear contrast between phospho- and dephospho-α-casein in SDS-PAGE is ascribed to the binding-induced fluorescence enhancement of **1**, as well as the difference of the phosphorylation number of the proteins.

In conclusion, Zn(II)-Dpa-based fluorophore (**1**) can be successfully applied to selective staining of phosphoprotein in SDS-PAGE.[12] This system enable one to detect phosphate groups attached to tyrosine, serine or threonine residues of protein at one time, which is difficult in the case of standard Western blotting procedure using phosphoprotein specific antibody. Further structural modification of **1** would be anticipated to realize highly sensitive detection of phosphoprotein in SDS-PAGE. In the research field of proteomics, phosphoproteome that involves the identification of phosphoprotein and the comprehensive analysis of phosphorylation state of proteins in living cell is one of the most significant topics.[12,13] Though 1D- and 2D-PAGE are fundamental methods to detect phosphoprotein so far, the rapid and convenient phosphoprotein detection system described above should be useful and thus contributes to the progress of this research area.

References and Notes

1 a) B. M. Sefton and T. Hunter, "Protein Phosphorylation," Academic Press, New York (1998). b) L. N. Johnson and R. J. Lewis, *Chem. Rev.*, **101**, 2209 (2001). c) T. Pawson, M. Raina, and P. Nash, *FEBS Lett.*, **513**, 2 (2002).
2 A. Ojida, Y. Mito-oka, M. Inoue, and I. Hamachi, *J. Am. Chem. Soc.*, **124**, 6256 (2002).
3 A. Ojida, M. Inoue, and I. Hamachi, *J. Am. Chem. Soc.*, **125**, 10184 (2003).
4 A. Ojida, Y. Mito-oka, K. Sada, and I. Hamachi, *J. Am. Chem. Soc.*, **126**, 2454 (2004).
5 Experimental procedure for phosphoprotein staining; SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed by a standard method using 13%T gel with protein samples which were denatured by heating for 3 min at 95 °C. The gel was fixed repeatedly (x2) by incubation for 30 min in 10%AcOH–50%MeOH–40%H_2O followed by washing with water for 10 min (x2). The fixed gel was incubated for 30 min with a solution of 50 μM of **1** in 50% MeOH–50% H_2O and subsequently washed with aqueous 500 μM $Zn(NO_3)_2$ solution for 10 min. All incubation and washing steps were conducted at room temperature with gentle agitation. Fluorescent band was detected with UV transilluminator ($\lambda_{max} = 360$ nm) equipped with CCD camera.
6 J. Tauzin, L. Miclo, S. Roth, D. Mollé, and J.-L. Gaillard, *Int. Dairy J.*, **13**, 15 (2003).
7 P. E. Stein, A. G. W. Leslie, J. T. Finch, and R. W. Carrell, *J. Mo. Biol.*, **221**, 941 (1991).
8 Washing with aqueous $Zn(NO_3)_2$ solution (500 μM) instead of distilled water after staining improved the detection selectivity. This is probably due to that the excess of Zn(II) may prevent the decomplexation of Zn(II) from **1** so as to suppress the nonspecific hydrophobic adsorption of the Zn(II)-unbounded **1** with protein during the washing process.
9 Dephospho-α-casein purchased from Sigma–Aldrich is at least 80% dephosphorylated, meaning that 1–2 phosphate residues are remained in dephospho-α-casein.
10 H. E. Swaissgood, "Developments in Dairy Chemistry-1 Proteins," Applied Science, London (1982).
11 The saturation of fluorescence enhancement is observed nearly at the point of [protein] / [**1**] = 2.5 in the case of the titration of **1** with dephospho-α-casein (1–2P).
12 a) T. H. Steinberg, B. J. Agnew, K. R. Gee, W.-Y. Leung, T. Goodman, B. Schulenberg, J. Hendrickson, J. M. Beechem, R. P. Haugland, and W. F. Patton, *Proteomics*, **3**, 1128 (2003). b) B. Schulenberg, R. Aggeler, J. M. Beechem, R. A. Capaldi, and W. F. Patton, *J. Biol. Chem.*, **278**, 27251 (2003). c) K. Martin, T. H. Steinberg, T. Goodman, B. Schulenberg, J. A. Kilgore, K. R. Gee, J. M. Beechem, and W. F. Patton, *Comb. Chem. High Throughput Screening*, **6**, 331 (2003).
13 a) D. E. Kalume, H. Molina, and A. Pandey, *Curr. Opin. Chem. Biol.*, **7**, 64 (2003). b) M. Mann, S.-E. Ong, M. Gronborg, H. Steen, O. N. Jensen, and A. Pandey, *Trends Biotechnol.*, **20**, 261 (2002).

Published on the web (Advance View) July 12, 2004; DOI 10.1246/cl.2004.1024