1
Real-Time and Continuous Sensors of Protein Kinase Activity Utilizing Chelation-Enhanced Fluorescence

Laura B. Peterson and Barbara Imperiali

1.1
Introduction

Protein kinases, the enzymes responsible for phosphoryl transfer from a chemical donor such as adenosine triphosphate (ATP) to a peptide or a protein acceptor, are integral enzymes in signaling cascades, play crucial roles in numerous cellular processes, and are of fundamental importance in systems biology. In addition, aberrant kinase activities are commonly associated with disease states, making kinases important therapeutic targets in current drug development initiatives. Therefore, understanding kinase activation dynamics is of utmost biological and clinical importance. Accurate and physiologically relevant methods to quantify kinase activities are needed to understand the intricate dynamics of kinase activation and inactivation. This chapter describes the design, evolution, and application of fluorescent-based Ser/Thr/Tyr kinase activity sensors that take advantage of chelation-enhanced fluorescence (CHEF). These sensors are compatible with physiological conditions, are selective for specific protein kinases, and provide real-time kinetic information regarding kinase activity.

1.2
The Biological Problem

Phosphorylation, or the attachment of a phosphate group to amino acid side chains, is one of the most abundant posttranslational modifications (PTMs) of proteins. Phosphorylation reactions are mediated by phosphotransferase enzymes, termed *kinases*, with ATP as the typical source of the transferred phosphoryl group. Ser, Thr, and Tyr are the most commonly phosphorylated residues in eukaryotes, while His and Asp phosphorylation has also been observed, predominantly in prokaryotes. Protein activity, localization, and structure as well as protein–protein interactions are all affected by protein phosphorylation [1, 2]. As kinases play integral roles in cellular signaling, dysregulated kinase function has emerged as a driver for many different disease states, including

Concepts and Case Studies in Chemical Biology, First Edition. Edited by Herbert Waldmann and Petra Janning.

cancer, neurodegenerative diseases, and metabolic disorders [3, 4]. Accordingly, much effort has been put forth toward understanding kinase structure, function, and activity as well as toward the clinical development of kinase inhibitors for the treatment of human disease.

Of considerable value to the scientific community are methods to study kinase activity, providing a means to evaluate kinase activity dynamics, inhibitor activities, and roles in cell signaling. Traditional assays for monitoring kinase activity utilize antibodies specific for the phosphorylated (activated) kinase, which is a common proxy for kinase activation, or rely on radioactivity-based measurements by monitoring the transfer of the radioactive γ-phosphoryl group from [γ-^{32}P]ATP to a substrate protein or peptide. Although the use of phosphopeptide/protein-specific antibodies is widely accepted as a useful detection method of kinase activity, antibodies may not take into account other factors affecting kinase activity, including kinase or substrate localization or additional PTMs that may also modulate activity. Radioactivity-based assays are limited in throughput, are inherently noncontinuous, and radioactive reagents require special handling. Mass spectrometry-based methods have also been developed and rely on the detection of phosphopeptides after enzymatic degradation. Fluorescence-based approaches represent valuable alternative methods for monitoring kinase activity.

Many strategies using fluorescence have been employed to detect kinase activity. Generally, these kinase sensors manifest increased fluorescence emission upon phosphorylation, while both dual fluorophore (fluorescence resonance energy transfer, FRET, Box 1.1) sensors and single fluorophore-containing sensors have been developed.

Box 1.1 Förster Resonance Energy Transfer (FRET)

FRET is the process by which one fluorophore, "the donor," transfers energy to a second fluorophore, "the acceptor." When both chromophores are fluorescent, FRET occurs. In the case of FRET between fluorophores, the emission spectrum of the donor fluorophore must overlap with the absorption spectrum of the acceptor. In this case, the emission from the donor excites the acceptor causing it to emit light (fluoresce). The efficiency of FRET depends on the distance between the two fluorophores, the spectral overlap, and the relative orientations between the donor emission dipole and the acceptor absorption dipole.

FRET-based sensors rely on conformational changes that often accompany phosphorylation, which alter the FRET efficiency. Many FRET sensors are plagued by small changes in fluorescence upon phosphorylation, rely on the bulky *Aequorea victoria* fluorescent proteins (AFP), are not compatible with high-throughput methods, and/or require genetic manipulation to incorporate the sensor into the system of choice [5]. Therefore, peptide-based fluorescent sensors provide an alternative approach to kinase sensing. Ideal kinase activity sensors should manifest high fluorescence changes upon phosphorylation,

should provide a quantitative measure of catalytic activity, and be amenable to the establishment of continuous assays, ideally in a high-throughput format. They should also be selective for the kinase of interest, readily prepared, and the design should be generalizable to the diverse families of kinases that comprise the kinome. In addition, the sensors should be operationally compatible with endogenous concentrations of the ATP cosubstrate. This chapter describes the design, development, and application of fluorescent sensors for kinase activity that are based on the principle of CHEF using 8-hydroxyquinoline fluorophores.

1.3 The Chemical Approach

Kinase substrate peptides represent ideal platforms for sensor design. Peptides are readily prepared by solid-phase peptide synthesis (SPPS), can be chemically modified with fluorescent probes or other small molecules, and retain recognition elements contained within endogenous kinase substrates. Commonly, kinases recognize a consensus sequence of four or five amino acids flanking the phosphorylated residue. Each kinase recognizes a unique consensus sequence and uses this molecular interaction as one level of substrate selectivity. Several methods for consensus sequence determination exist and have allowed for the generation of kinase-specific substrate peptides. Further modifications of substrate peptides with fluorophores have inspired new methods for kinase activity sensing that offer many advantages over conventional methods.

Fluorophores capable of CHEF and other types of environment-sensitive (or solvatochromic) fluorophores have been utilized in sensors for kinase activity. Chelation-sensitive fluorophores manifest altered fluorescent properties upon chelation of various metal ions, while environment-sensitive fluorophores exhibit altered excitation and emission properties with changing environment, such as solvent polarity (Figure 1.1).

One of the first reported examples of a fluorescence-based kinase activity sensor exploits the native fluorescence of Trp and a change in the local environment of the indole fluorophore upon phosphorylation. A peptide-substrate-based sensor for cyclic adenosine monophosphate (cAMP)-dependent kinase, containing a Trp-Ser motif, was prepared and manifested a 20% increase in fluorescence upon phosphorylation (**1**, Figure 1.2a) [6]. Additional examples include peptide- and protein-based sensors with appended environment-sensitive fluorophores, although these sensors are generally plagued by small signal changes resulting in low sensitivity [7–9].

1.3.1 Chelation-Enhanced Fluorescence

CHEF was originally exploited for the detection and quantification of metal ions, such as those developed for the detection and quantification of Ca^{2+} [10]. Given

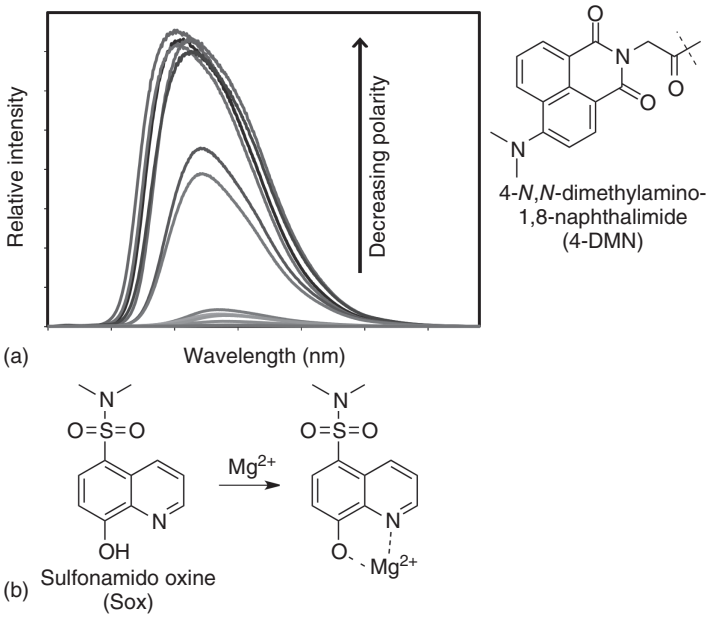

Figure 1.1 *Environment-sensitive fluorescence.* (a) Fluorescence emission spectrum of the environment-sensitive fluorophore, 4-DMN, in solvents of various polarities and (b) structure of the Sox fluorophore demonstrating chelation-enhanced fluorescence.

the ability of alkyl and aryl monophosphate esters to also chelate metal ions, it was soon after realized that chelation-sensitive fluorophores could be incorporated proximal to the phosphorylatable Ser/Thr/Tyr residue into peptide substrates to provide robust fluorescence readout upon phosphorylation.

One early example, modeled on the aforementioned Ca^{2+} sensors, included a carboxylate-containing fluorophore proximal to a Ser residue in a substrate peptide for protein kinase C (PKC) (**2**, Figure 1.2b). Upon phosphorylation, the Ser-phosphate and fluorophore carboxyl groups chelate Ca^{2+} resulting in a twofold enhancement of fluorescence [11]. This sensor relied on chemical modification of the peptide substrate following peptide synthesis. An advantageous alternative is the use of modified synthetic amino acid building blocks, wherein the fluorescent reporting moiety can be directly incorporated during peptide synthesis.

A class of zinc ion (Zn^{2+}) sensors, which utilized an unnatural amino acid that included the chelation-sensitive fluorophore, 8-hydroxy-4-(*N*,*N*-dimethylsulfonamido)-2-methylquinoline (sulfonamido oxine (Sox), Figure 1.3a), provided inspiration for a second class of kinase activity sensors [12–14]. The Sox amino acid was prepared via asymmetric synthesis and converted to the fluorenylmethyloxycarbonyl (Fmoc)-protected derivative and incorporated via SPPS into a peptide containing a proline-mediated β-turn sequence [13]. The β-turn was flanked by both Zn-chelating amino acids and the Sox fluorophore (Figure 1.3b). In this case, the β-turn was included to provide preorganization

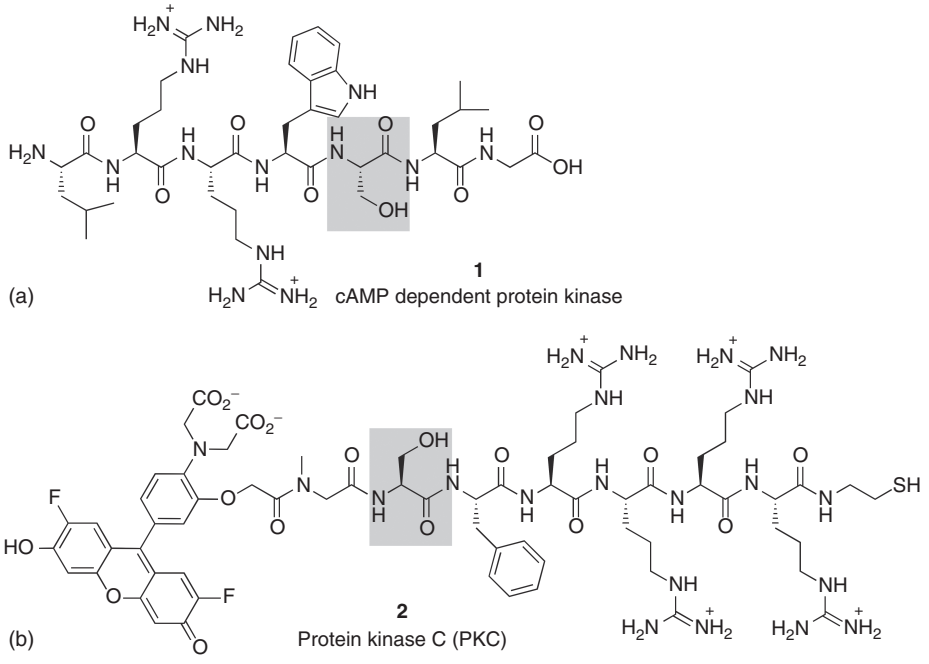

Figure 1.2 *Structures of (a) tryptophan-based and (b) fluorescein-based kinase activity sensors.* Phosphorylated residue (Ser) highlighted for clarity.

for the Zn^{2+} chelation event. In the presence of Zn^{2+}, the flanking residue (for example, His, Cys, Glu, or Asp) and the Sox fluorophore chelate Zn^{2+}, while metal ion binding to the Sox moiety results in an increased fluorescence signal due to CHEF (Figure 1.3c,d, Box 1.2).

Box 1.2 Chelation-Enhanced Fluorescence of 8-hydroxyquinolines

The 8-hydroxyquinoline chromophore manifests weak fluorescence in the absence of metal ions in aqueous solution. However, in the presence of various metal ions (e.g., Zn^{2+} and Mg^{2+}), 8-hydroxyquinoline becomes strongly fluorescent through CHEF. One plausible mechanism to describe the observed CHEF of 8-hydroxyquinolines involves the change in the lowest energy excited state. The lowest energy transition of unbound 8-hydroxyquinoline is the *n* to π^* transition, rapid intersystem crossing prevents this transition from producing fluorescence. However, upon metal chelation, the lowest energy transition becomes the π to π^* transition, which does not undergo intersystem crossing and is fluorescent [15]. A second theory to describe CHEF involves a photoinduced proton transfer from the phenol to the quinoline ring nitrogen, which upon excitation quenches fluorescence. Metal chelation promotes deprotonation of the phenol, preventing fluorescence quenching upon excitation [16].

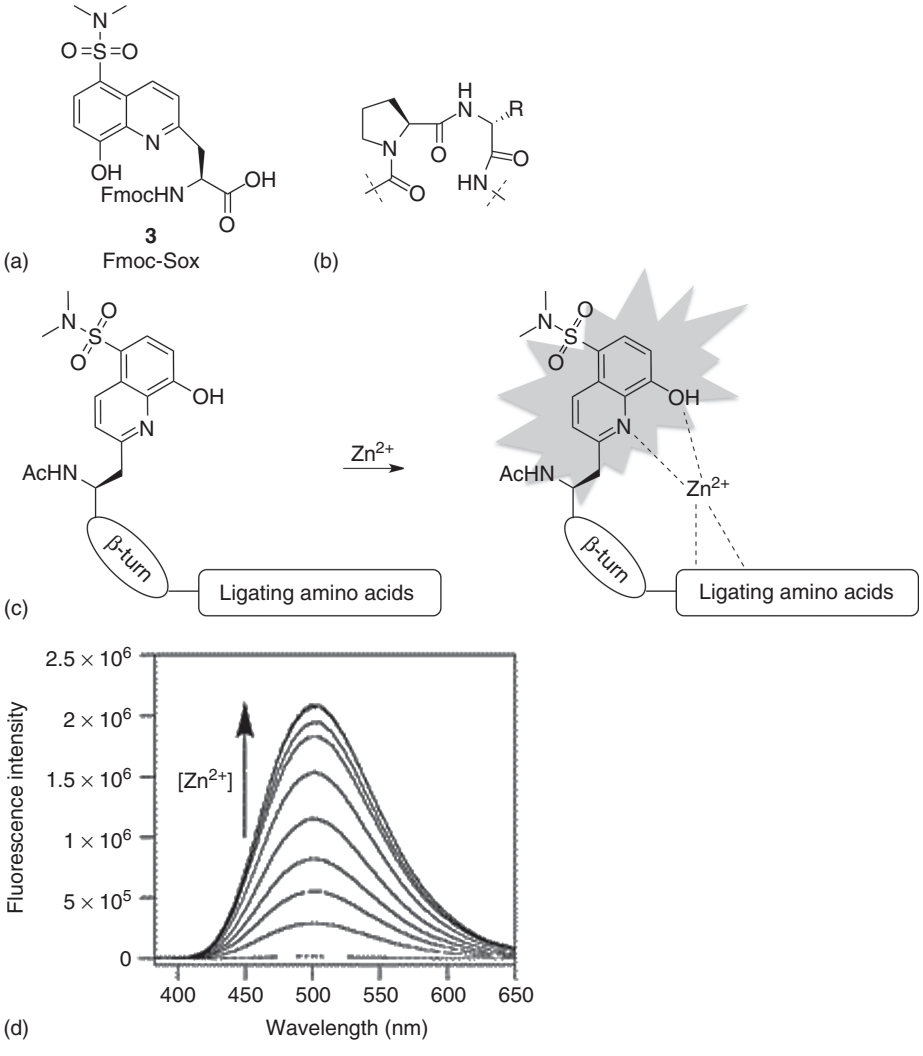

Figure 1.3 *CHEF-based sensors for divalent zinc*. (a) Structure of Fmoc-Sox used in SPPS synthesis of Zn^{2+} sensors; (b) depiction of the β-turn motif; (c) schematic of a peptidic CHEF-based Zn^{2+}; and (d) fluorescence emission spectrum of a peptide-based Zn^{2+} sensor with increasing concentrations of Zn^{2+} ($\lambda_{ex} = 360$ nm). (Reprinted with permission from Ref. [13]. Copyright 2003 American Chemical Society.)

Altering the flanking amino acids provides a means to fine-tune the binding affinity for Zn^{2+}, and thus the capacity to detect the divalent ion at different target concentrations.

Although this strategy was originally employed for the detection of Zn^{2+}, it was quickly realized that there would potentially be greater impact in the application of the quinolone fluorophore and CHEF for detecting kinase activity, as the

phosphate transferred to Ser/Thr/Tyr could serve as the flanking group capable of metal chelation (Figure 1.4a).

1.3.2 β-Turn-Focused Kinase Activity Sensors

The first generation of Sox-containing kinase activity sensors, the β-turn-focused sensors (BTF), utilized a β-turn motif flanked by the Sox amino acid and either an N- or C-terminal kinase recognition motif, which includes the phosphorylatable residue (Figure 1.4b) [17–19]. The Sox amino acid is an ideal fluorophore in this context as it is relatively small in size, which prevents perturbation of native kinase–substrate interactions. In addition, the Sox fluorophore is relatively stable and resistant to photobleaching. Finally, Sox undergoes CHEF upon Mg^{2+} chelation, resulting in a robust increase in fluorescence ($\lambda_{ex} = 360$ nm; $\lambda_{em} = 485$ nm).

In this kinase sensor design, the role of the β-turn, made up of two amino acids, namely, XaaPro or ProXaa, is to preorganize the incipient Mg^{2+}-binding site comprised of Sox and the transferred phosphoryl group. The kinase recognition motif, typically based on an optimum peptide substrate or substrate consensus sequence, can be placed at either the C- or N-terminus of the peptide, relative to the β-turn/Sox motif. This modular design allows one to empirically determine the contribution of either the N- or C-terminal recognition elements and establish which may contribute optimally to kinase selectivity and/or enzyme turnover.

An essential feature of Sox-containing sensors is the differential binding affinity for Mg^{2+} between the substrate and product (phosphorylated) peptides. A 10- to 25-fold enhancement of binding affinity for Mg^{2+} (as measured by dissociation constant, K_D) is observed upon phosphorylation. Therefore, substrate peptides manifest low background fluorescence, while phosphorylation results in robust fluorescence increases (three- to eightfold) in the presence of Mg^{2+}. In the presence of Mg^{2+} and ATP, these sensors accurately report kinase activity, while providing kinetic detail. The kinetic parameters (K_M and V_{max}) of the BTF sensors are in agreement with the corresponding non-Sox-containing substrate peptides as determined by ^{32}P incorporation from radiolabeled ATP and scintillation counting [17]. Further application of these sensors is discussed in Section 1.4. Although the BTF sensors provided a reliable method to quantify kinase activity, second-generation Sox sensors addressed one shortcoming of the first-generation design and provided a means to incorporate both N- and C-terminal kinase recognition elements.

1.3.3 Recognition-Domain-Focused Kinase Activity Sensors

Given the multitude of kinases encoded in the human genome, substrate selectivity and specificity is of paramount importance when designing kinase activity sensors for application in complex unfractionated samples. For this reason, one

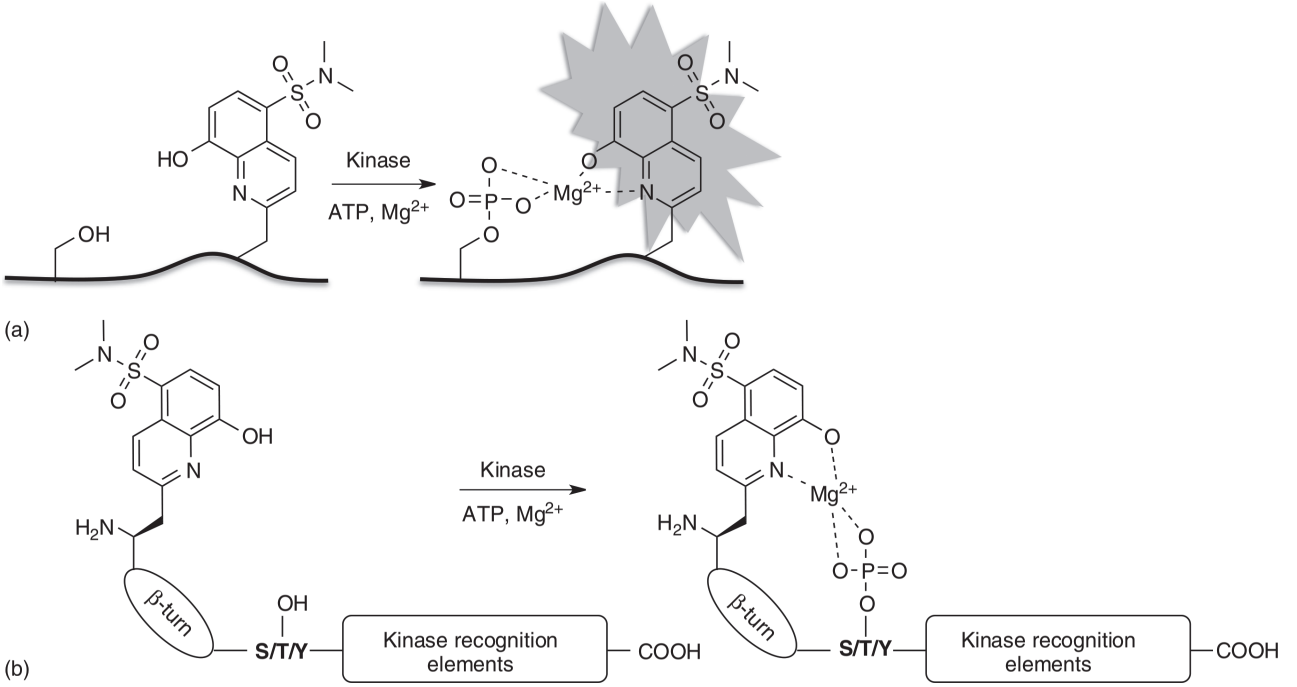

Figure 1.4 *Sox-containing kinase activity sensors.* (a) Schematic of Sox-based peptide sensors of kinase activity and (b) β-turn-focused kinase sensors including C-terminal recognition elements.

major disadvantage of the BTF sensors was the loss of either the N- or C-terminal substrate recognition determinants. Accordingly, strategies to mitigate this limitation were investigated, leading to the development of a recognition-domain focused (RDF) sensor design that utilized the more flexible Sox-containing unnatural amino acid, cysteine-Sox (C-Sox, Figure 1.5). Given the increased flexibility, the preorganizing β-turn motif became unnecessary.

C-Sox was able to coordinate the phosphate-bound Mg^{2+} without the predisposed β-turn-mediated conformational bias. Synthesis of both Fmoc-protected C-Sox (**4**) and Sox-Br (**5**) allowed for facile incorporation into peptides via either SPPS or cysteine-selective alkylation, respectively, (Figure 1.5) [20]. This increasingly versatile approach allowed recognition elements on both sides of the phosphorylatable residue to be included in the peptidic sensor, generally resulting in superior kinase specificity and selectivity.

The optimal C-Sox location was empirically determined to be at the +2/−2 location, relative to the phosphorylatable Ser/Thr/Tyr, in most cases. One notable exception being for the mitogen-activated protein kinases (MAPKs), which recognize either SerPro or ThrPro as the minimal consensus sequence. MAPK activity sensors included C-Sox juxtaposed to Pro at the −3 position. Using the RDF approach, sensors were prepared for a variety of different kinases (Table 1.1) that generally exhibited good fluorescence increases upon phosphorylation (3- to 10-fold) and showed significant improvements in kinetic parameters as compared to the first-generation BTF sensors.

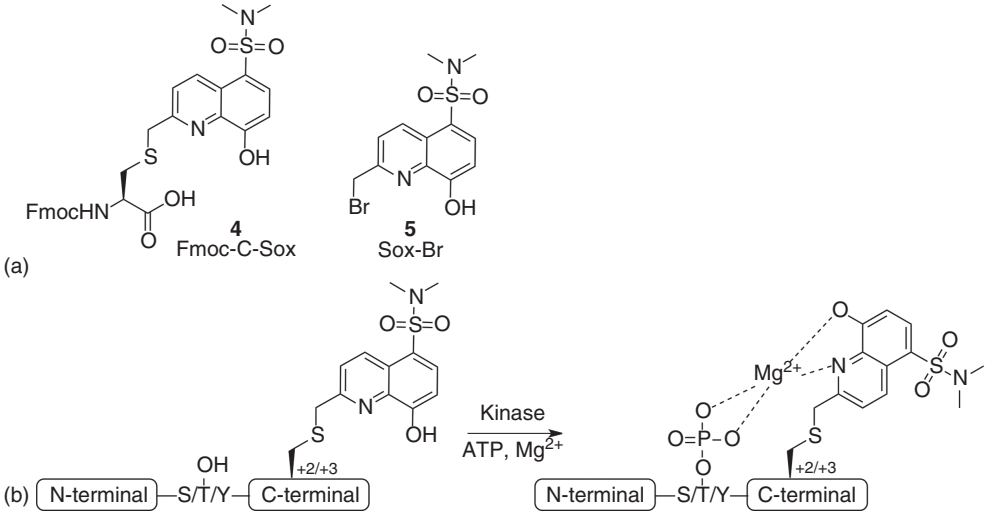

Figure 1.5 *Recognition-domain-focused kinase activity sensors*. (a) Structures of Fmoc-C-Sox and Sox-Br used in sensor synthesis and (b) RDF sensors with the C-Sox moiety placed in the +2/+3 position relative to the phosphorylated residue.

Table 1.1 C-Sox-containing kinase activity sensors.

Kinase	Substrate sequence	Fluorescence increase	K_M (μM)	Vmax (μmol mg^{-1} min^{-1})
PKC	Ac-RRR-CSox-GS*FRRR-$CONH_2$	3.5	0.1	1.8
Akt1	Ac-ARKRERAYS*F-CSox-HHA-$CONH_2$	3.9	0.69	2.5
MK2	Ac-AHLQRQLS*I-CSox-HH-$CONH_2$	4.4	1.2	1.3
Src	Ac-AEE-CSox-IY*GEFEAKKKK-$CONH_2$	2.2	7	3.4
Pim2	Ac-ARKRRRHPS*G-CSox-PTA-$CONH_2$	3.2	1.4	0.67
PKA	Ac-ALRRAS*L-CSox-AA-$CONH_2$	5	2.6	17.9
Abl	Ac-E-CSox-IY*AAPFAKKK-$CONH_2$	5.2	10.5	19.1
IRK	Ac-R-CSox-DY*-Nle-TMQIGKK-$CONH_2$	4.2	25.9	8.7

Fluorescent properties and kinetic parameters for RDF-based kinase activity sensors. Asterisk (*) denotes the phosphorylated residue, while underlined residues are those important for kinase recognition, Nle = norleucine.
Adapted with permission from Ref. [20]. Copyright (2008) American Chemical Society.

1.3.4 Chimeric Kinase Activity Sensors

Although RDF-based sensors provided improvement over BTF sensors, some kinase targets were particularly challenging targets for sensor design, in particular, those with short or ubiquitous consensus sequences. Although many kinases have linear consensus sequences comprising 8–10 amino acids, the MAPKs require only the presence of SerPro or ThrPro at the phosphorylation site. The MAPKs are involved in many important signaling pathways, making the development of activity sensors for individual MAPKs, such as epithelial growth factor-related kinase (ERK), c-jun N-terminal kinase (JNK), and p38 an important endeavor. In nature, MAPKs achieve high target specificity by interactions with a secondary recognition element proximal to the kinase active site where ATP and substrate peptides bind (Figure 1.6b).

This "docking" site is typified by an acidic cleft adjacent to a hydrophobic pocket. Kinases that are upstream in signaling pathways and substrate proteins include a complementary basic-hydrophobic motif that docks into this groove. This docking interaction provides a second layer of specificity for the MAPKs. Accordingly, incorporation of a docking motif into an MAPK activity sensor was proposed to enhance the selectivity for a given MAPK (Figure 1.6a).

In pursuit of an ERK activity sensor, a chimeric sensor was envisioned taking advantage of this secondary docking interaction [21]. The chimeric sensor comprised a docking motif (the N-terminal pointed domain (PNT) from an ERK substrate) [22] fused to a C-Sox-containing ERK substrate peptide (Figure 1.6c). The two parts were independently prepared, the substrate peptide via SPPS and the PNT domain via homologous expression, and joined via native chemical ligation (Figure 1.6c). The resulting ERK sensor displayed vastly improved kinetic

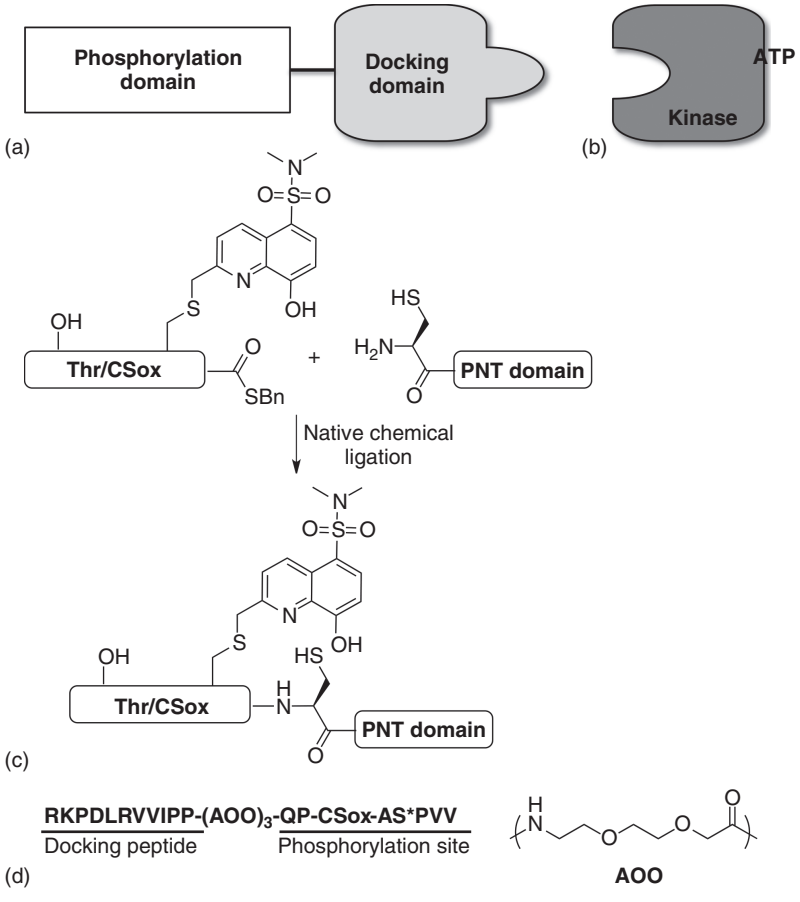

Figure 1.6 *Chimeric kinase activity sensors*. (a) Schematic of a chimeric sensor; (b) schematic of kinase illustrating docking groove relative to ATP binding site; (c) preparation of ERK activity sensor; and (d) sequence of p38 activity sensor.

parameters and selectivity as compared to the phosphorylation motif alone. This example demonstrates the utility and adaptability of C-Sox-containing kinase activity sensors. A similar approach facilitated the design of a p38α chemosensor [23]. One advantage of the p38α sensor is that the docking motif used was only 11 amino acids; its incorporation could be achieved through SPPS. The docking motif and C-Sox phosphorylation motif were connected via a flexible polyethyleneglycol (PEG) linker, which was also installed via SPPS using commercially available Fmoc-$(PEG)_X$-CO_2H units. The development of C-Sox-containing sensors illustrates how the CHEF principle can be combined with unnatural amino acids and exploited to generate valuable protein kinase sensors. These sensors accurately and robustly report kinase activity in recombinant systems, in cell lysates, and even in tissue homogenates. The next section addresses the evaluation and application of these sensors.

1.4 Chemical Biological Research/Evaluation

Sox-based kinase probes report activity in continuous assays, are compatible with cell lysates and tissue homogenates, and can be used for small-molecule inhibitor screening. Upon design and synthesis, kinetic parameters for each Sox-peptide are determined and kinase selectivity is addressed. Subsequently, sensors are evaluated in cell-lysate-based systems in the presence and absence of selective inhibitors to establish selectivity in complex target samples.

1.4.1 Kinetic Parameters

It is straightforward to determine the kinetic parameters, namely, K_M and V_{max}, for each kinase sensor as fluorescence emission (F_{em}) is monitored over time in a continuous format (Figure 1.7a). Reaction volumes are relatively small, while fluorescence can be monitored in microcuvettes or in multiwell plates (96-, 384-, or even 1536-well format). Initial slopes taken directly from the F_{em} versus time plots can be used to determine Michaelis constants. In order to determine V_{max}, one must have a way to convert F_{em} to units of product formation, which can be achieved by chemically synthesizing the corresponding product (phosphorylated) peptide assuming that total F_{em} is the sum of the product and substrate intensities. In general, both BTF and RDF sensors manifest kinetic parameters very similar to those determined by other means, such as [γ-^{32}P]ATP-based assays.

1.4.2 Assessing Kinase Selectivity

In order to determine the selectivity of each peptide sensor substrate, assays with a panel of recombinantly expressed, activated kinases can be performed (Figure 1.7b). In this case, the concentration of substrate peptide is held constant at two to three times the determined K_M. For example, the p38 sensor depicted in Figure 1.6 was incubated with various MAP (mitogen-activated protein) and non-MAP kinases and fluorescence emission was monitored. Figure 1.7b demonstrates the selectivity of the p38 sensor for the target kinase [23]. Following this initial screen, kinase selectivity can be addressed directly in cell lysates. Cells can be stimulated to activate the kinase of interest and kinase activity can be determined in the presence and absence of a selective inhibitor of the kinase of interest. Residual activity in the presence of inhibitor would indicate sensor cross talk with other kinases.

Alternatively, following stimulation, the kinase of interest can be immunodepleted using an antibody specific for the desired kinase (Figure 1.7c). In this case, residual kinase activity in the depleted lysate can be attributed to sensor cross talk. The chimeric ERK sensor was found to be selective for ERK in this manner.

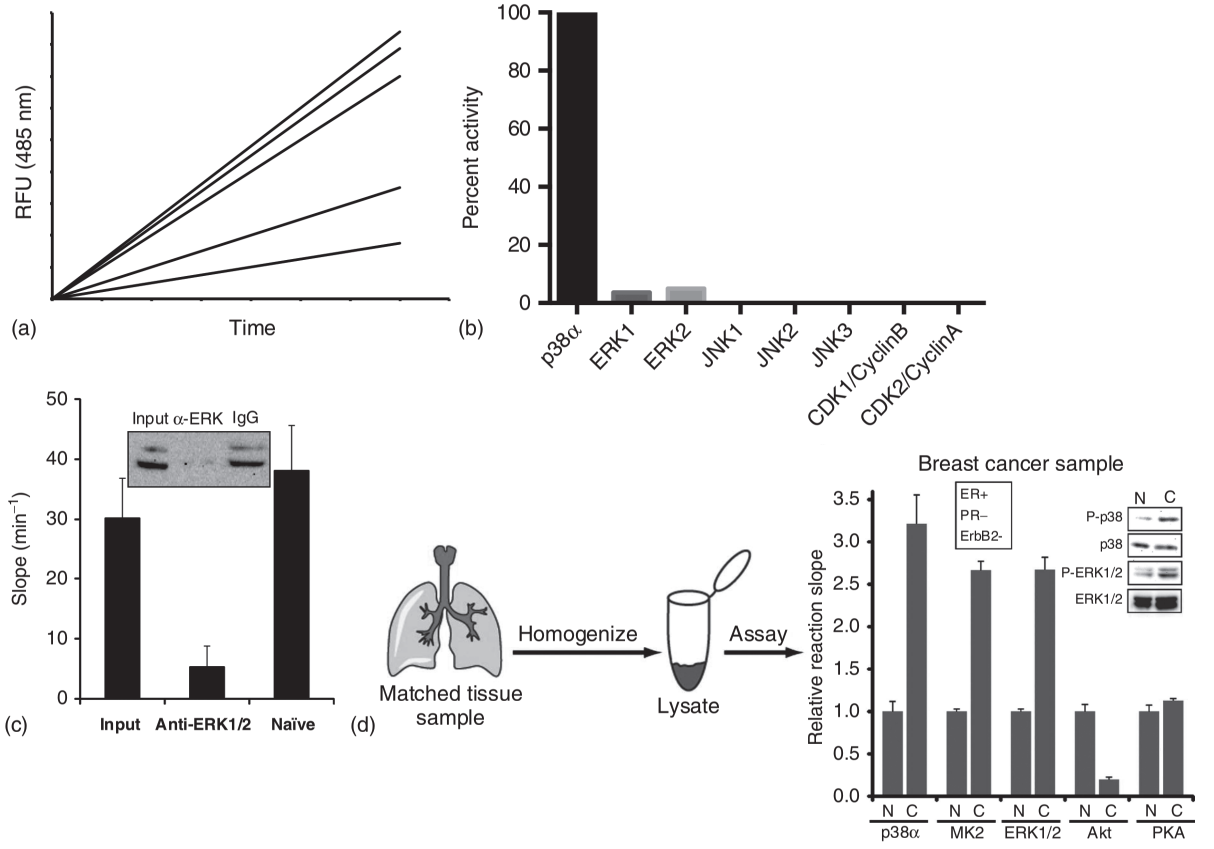

Figure 1.7 *Characterization and application of Sox-containing kinase activity sensors*. (a) Example data for a typical kinetic experiment; (b) kinase selectivity profile for p38 sensor shown in Figure 1.6d; and (c) ERK sensor activity in ERK-stimulated lysates following immunodepletion. (Reprinted with permission from Ref. [21]. Copyright (2009) Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.) (d) Diagram of kinase profiling in human samples and kinase activity profile comparing a normal "N" and breast cancer "C" sample. (Reprinted with permission from Ref. [24]. Copyright (2012) Cell Press.)

ERK activity was stimulated with EGF (epithelial growth factor) in HeLa cells. The cells were lysed and either incubated with no antibody, an antibody for ERK, or a control-naïve antibody. Following immunodepletion, ERK activity was assessed. As evident in Figure 1.7c, most of the ERK activity is absent following depletion of ERK, providing evidence that the sensor is selective for ERK. In the event that a kinase activity sensor is not completely selective, adding specific kinase inhibitors to the assay buffer can minimize activity resulting from "off-target" kinases. Once the selectivity of a kinase sensor has been determined, these sensors can be used to monitor kinase activity in cell lysates and/or tissue homogenates under a variety of different contexts.

1.4.3
Kinase Profiling in Cell Lysates and Tissue Homogenates

Sox-containing kinase activity sensors provide a means to directly quantify enzymatic activity in unfractionated cell lysates and tissue homogenates. Assays with lysates can be performed in multiwell plates, allowing one to monitor the activity of multiple kinases simultaneously. In one example, a panel of five activity sensors (MK2 ((MAPK)-activated protein kinase 2), p38α, ERK, Akt, PKA (protein kinase A)) was used to monitor kinase activation dynamics in a model of skeletal muscle differentiation [24]. For Sox-based assays, the amount of lysate required is comparable to the alternative method of Western blotting (10–40 μg total protein/replicate in a 96-well format). In another example, the same kinase sensors were used to quantitatively determine kinase activity in human cancer tissue samples and were compared to matched healthy tissue controls (Figure 1.7d). Experiments were validated by comparing results to traditional Western blot analysis. These types of experiments highlight the utility of this method of detecting kinase activity and provide the means to profile kinase activities under a variety of conditions.

1.5
Conclusions

This chapter details the development and application of kinase activity probes that utilize CHEF manifested by 8-hydroxyquinoline derivatives. These sensors provide significant advantages over traditional kinase-sensing protocols. Namely, Sox-based sensors provide a quantitative readout of kinase activity in a sensitive and continuous format. The approach is generalizable and has been applied to Ser, Thr, and Tyr kinases representing many diverse families of kinases. Owing to the importance of kinases in different diseases, the need for additional probes of this type is clear, as these probes provide valuable insight into kinase (in)activation dynamics as well as kinase inhibitor activities. The potential for these sensors in a systems biology platform is significant, as many kinases can be

profiled in high throughput. Subsequent generations of sensors should address other difficult-to-target kinases, should expand the application of these probes to live-cell imaging, and should provide a means to multiplex the assay with modified Sox fluorophores.

References

1. Endicott, J.A., Noble, M.E.M., and Johnson, L.N. (2012) The structural basis for control of eukaryotic protein kinases. *Annu. Rev. Biochem.*, **81**, 587–613.
2. Manning, G., Whyte, D.B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) The protein kinase complement of the human genome. *Science*, **298**, 1912–1934.
3. Lahiry, P., Torkamani, A., Schork, N.J., and Hegele, R.A. (2010) Kinase mutations in human disease: interpreting genotype-phenotype relationships. *Nat. Rev. Genet.*, **11**, 60–74.
4. Blume-Jensen, P. and Hunter, T. (2001) Oncogenic kinase signalling. *Nature*, **411**, 355–365.
5. Rothman, D.M., Shults, M.D., and Imperiali, B. (2005) Chemical approaches for investigating phosphorylation in signal transduction networks. *Trends Cell Biol.*, **15**, 502–510.
6. Wright, D.E., Noiman, E.S., Chock, P.B., and Chau, V. (1981) Fluorometric assay for adenosine 3′,5′-cyclic monophosphate-dependent protein kinase and phosphoprotein phosphatase activities. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 6048–6050.
7. Bowman, B.F., Peterson, J.A., and Stull, J.T. (1992) Pre-steady-state kinetics of the activation of rabbit skeletal muscle myosin light chain kinase by Ca2+/calmodulin. *J. Biol. Chem.*, **267**, 5346–5354.
8. McIlroy, B.K., Walters, J.D., and Johnson, J.D. (1991) A continuous fluorescence assay for protein kinase C. *Anal. Biochem.*, **195**, 148–152.
9. Post, P.L., Trybus, K.M., and Taylor, D.L. (1994) A genetically engineered, protein-based optical biosensor of myosin II regulatory light chain phosphorylation. *J. Biol. Chem.*, **269**, 12880–12887.
10. Minta, A., Kao, J.P., and Tsien, R.Y. (1989) Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J. Biol. Chem.*, **264**, 8171–8178.
11. Chen, C.-A., Yeh, R.-H., and Lawrence, D.S. (2002) Design and synthesis of a fluorescent reporter of protein kinase activity. *J. Am. Chem. Soc.*, **124**, 3840–3841.
12. Pearce, D.A., Jotterand, N., Carrico, I.S., and Imperiali, B. (2001) Derivatives of 8-hydroxy-2-methylquinoline are powerful prototypes for zinc sensors in biological systems. *J. Am. Chem. Soc.*, **123**, 5160–5161.
13. Shults, M.D., Pearce, D.A., and Imperiali, B. (2003) Modular and tunable chemosensor scaffold for divalent zinc. *J. Am. Chem. Soc.*, **125**, 10591–10597.
14. Walkup, G.K. and Imperiali, B. (1998) Stereoselective synthesis of fluorescent α-amino acids containing oxine (8-hydroxyquinoline) and their peptide incorporation in chemosensors for divalent zinc. *J. Org. Chem.*, **63**, 6727–6731.
15. Seitz, W.R. (1980) *CRC Critical Reviews in Analytical Chemistry: Fluorescence Derivatization*, Vol. 8, CRC Press, Boca Raton, FL.
16. Bronson, R.T., Montalti, M., Prodi, L., Zaccheroni, N., Lamb, R.D., Dalley, N.K., Izatt, R.M., Bradshaw, J.S., and Savage, P.B. (2004) Origins of "on-off" fluorescent behavior of 8-hydroxyquinoline containing chemosensors. *Tetrahedron*, **60**, 11139–11144.
17. Shults, M.D., Carrico-Moniz, D., and Imperiali, B. (2006) Optimal Sox-based fluorescent chemosensor design for serine/threonine protein kinases. *Anal. Biochem.*, **352**, 198–207.
18. Shults, M.D. and Imperiali, B. (2003) Versatile fluorescence probes of protein

kinase activity. *J. Am. Chem. Soc.*, **125**, 14248–14249.
19. Shults, M.D., Janes, K.A., Lauffenburger, D.A., and Imperiali, B. (2005) A multiplexed homogeneous fluorescence-based assay for protein kinase activity in cell lysates. *Nat. Methods*, **2**, 277–284.
20. Luković, E., González-Vera, J.A., and Imperiali, B. (2008) Recognition-domain focused chemosensors: versatile and efficient reporters of protein kinase activity. *J. Am. Chem. Soc.*, **130**, 12821–12827.
21. Luković, E., Vogel Taylor, E., and Imperiali, B. (2009) Monitoring protein kinases in cellular media with highly selective chimeric reporters. *Angew. Chem. Int. Ed.*, **121**, 6960–6963.
22. Rainey, M.A., Callaway, K., Barnes, R., Wilson, B., and Dalby, K.N. (2005) Proximity-induced catalysis by the protein kinase ERK2. *J. Am. Chem. Soc.*, **127**, 10494–10495.
23. Stains, C.I., Luković, E., and Imperiali, B. (2010) A p38α-Selective chemosensor for use in unfractionated cell lysates. *ACS Chem. Biol.*, **6**, 101–105.
24. Stains, C.I., Tedford, N.C., Walkup, T.C., Luković, E., Goguen, B.N., Griffith, L.G., Lauffenburger, D.A., and Imperiali, B. (2012) Interrogating signaling nodes involved in cellular transformations using kinase activity probes. *Chem. Biol.*, **19**, 210–217.