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Design, synthesis, and evaluation of a selective chemosensor for leucine-rich repeat kinase 2

David A. Szalewski, Jon R. Beck, Cliff I. Stains *

Department of Chemistry, University of Nebraska—Lincoln, Lincoln, NE 68588, United States

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ABSTRACT

We describe the design, synthesis, and evaluation of a selective activity probe for leucine-rich repeat kinase 2 (LRRK2), a possible molecular target for the treatment of Parkinson's disease. Our optimal chemosensor design, termed Nictide-S2, incorporates a phosphorylation-sensitive sulfonamido-oxine fluorophore at an engineered cysteine within the substrate sequence. This design allows for the direct, real-time analysis of LRRK2 kinase activity with a detection limit of 2.5 nM. Under optimized conditions, we measured a Z′ factor of 0.7 demonstrating the potential utility of this assay for inhibitor screening. Off-target kinases capable of phosphorylating Nictide-S2 are identified and an optimized inhibitor cocktail for suppressing background signal is provided. The resulting chemosensor could be utilized to identify LRRK2 inhibitors as well as selectively report on LRRK2 activity in the presence of off-target kinases.

Mutations that increase the catalytic activity of the serine/threonine kinase LRRK2 have been identified in both familial and sporadic forms of Parkinson's disease (PD).[1–4] Inhibition of LRRK2 kinase activity has been demonstrated to reverse the neurodegenerative phenotype observed in PD models,[5] indicating that LRRK2 is a potential molecular target for the treatment of PD. Current approaches for assessing inhibition of LRRK2 kinase activity in heterogeneous samples, such as cell lysates, rely on phosphorylation of S910, S935, or S1292 as a proxy for LRRK2 activity.[6–8] As predicted, exposure of cells to LRRK2 inhibitors causes a decrease in the phosphorylation of S910 and S935. This strategy has been utilized with great success to characterize LRRK2 inhibitor efficacy in biological systems.[9,10] However, S910 and S935 are not directly phosphorylated by LRRK2 and the regulatory enzymes that control phosphorylation at these sites have not been identified. Consequently, selective activity assays for LRRK2 could provide a starting point to develop approaches to directly monitor LRRK2 activity in heterogeneous samples, avoiding potential difficulties with currently available LRRK2 proxies.[11]

Towards the goal of developing a selective LRRK2 activity assay, we set out to design and evaluate the selectivity of potential chemosensors for LRRK2 in vitro. Recently, a variety of discontinuous LRRK2 activity assays have been described that utilize radioactivity,[12] time-resolved fluorescence resonance energy transfer,[13] or amplified luminescent proximity homogeneous assay[14] formats. In contrast, fluorescence-based activity sensors for protein kinases are capable of providing a real-time measure of enzyme function.[15–17] Importantly, fluorescence-based activity assays can also be utilized to quantify kinase activity in biological samples, such as cell lysates or tissue homogenates.[18–20] Accordingly, we chose to employ a phosphorylation-sensitive sulfonamido–oxine fluorophore, known as Sox,[21] to directly report on LRRK2 kinase activity in real-time. First generation Sox-based kinase activity sensors require the removal of either N- or C-terminal recognition sequences to afford efficient activity probes. Using this approach, a first generation Sox-based activity sensor for LRRK2 was recently developed by Silva et al.[22] This sensor was capable of detecting 10.5 nM LRRK2 and was employed to assess several biochemical properties of LRRK2 including the effects of GTP, GDP, and autophosphorylation on kinase enzymatic activity. However, the ability of this first generation Sox-based probe to selectively report on LRRK2 activity in presence of closely related enzymes was not demonstrated. To improve upon the selectivity of Sox-based chemosensors, the Imperiali laboratory has described a second generation Sox-based probe design that relies on the alkylation of a single engineered cysteine residue within a kinase substrate.[23] These so-called CSox-based substrates allow for the incorporation of both N- and C-terminal recognition sequences into a peptide substrate and can increase chemosensor selectivity (Fig. 1a).

We hypothesized that a LRRK2 substrate containing both N- and C-terminal recognition elements may afford a selective activity sensor. Accordingly, we synthesized two potential CSox-based substrates using the sequence of an efficient LRRK2 peptide substrate

* Corresponding author. Tel.: +1 402 472 2617; fax: +1 402 472 9402.
E-mail address: cstains2@unl.edu (C.I. Stains).

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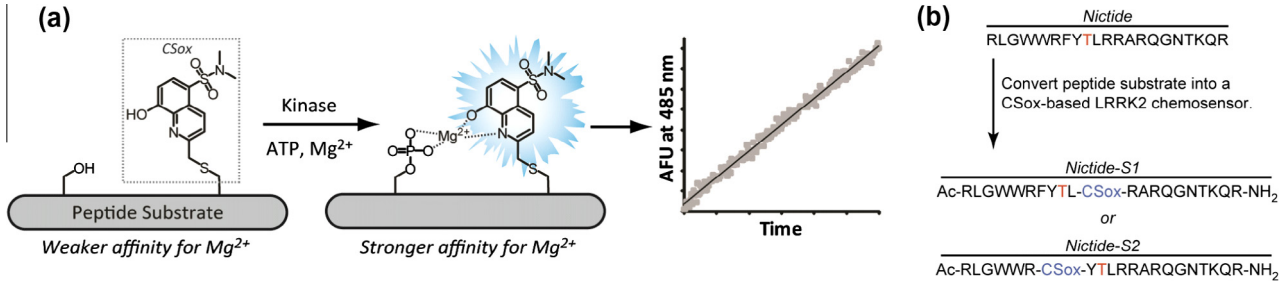

Figure 1. Design of a CSox-based activity sensor for LRRK2. (a) A single amino acid in a peptide substrate is replaced with the CSox unnatural amino acid. Addition of the target kinase, in the presence of ATP and Mg^{2+}, leads to phosphorylation of the substrate and a concurrent increase in fluorescence due to chelation of Mg^{2+} (ex. = 360 nm, em. = 485 nm). This increase in fluorescence can be monitored in real-time and is directly proportional to kinase enzymatic activity. (b) Conversion of Nictide into a CSox-based chemosensor by replacement of the +2 (Nictide-S1) or −2 (Nictide-S2) residues, relative to the site of phosphorylation (red) with CSox (blue). Peptides are capped with an acetyl group (Ac) at the N-terminus and an amide (NH_2) at the C-terminus.

known as Nictide[24] as a template. These chemosensors contain CSox at the +2 or −2 position relative to the site of phosphorylation, termed Nictide-S1 and Nictide-S2 respectively (Fig. 1b). In addition, we synthesized the corresponding phosphorylated peptides as controls, Nictide-P1 and Nictide-P2, respectively, which contain a phospho–threonine at the site of phosphorylation. Since the signal generation of CSox-based activity sensors is a result of chelation-enhanced fluorescence induced by binding of Mg^{2+}, it is important to measure the affinities of corresponding pairs of nonphosphorylated and phosphorylated peptides for Mg^{2+}. This information allows one to tune the concentration of Mg^{2+} in the assay in order to obtain maximal fluorescence enhancements upon phosphorylation. In general, the affinities of nonphosphorylated peptides for Mg^{2+} are >100 mM while phosphorylated peptides bind Mg^{2+} with a K_D of ~10 mM. However, the affinities of each newly designed CSox-based sensor for Mg^{2+} should be investigated as the sequence context can influence Mg^{2+} affinities for both nonphosphorylated and phosphorylated constructs. To investigate this parameter with our Nictide-based chemosensors, we measured the affinity of each substrate, and corresponding product, peptide for Mg^{2+} (Fig. 2). As expected each substrate peptide displayed a decreased affinity for Mg^{2+}, relative to the corresponding product. However, these data also indicated that Nictide-P2 had a relatively weak affinity for Mg^{2+} (K_D = 69 mM) compared to Nictide-P1 (K_D = 10 mM). Based on these results we reasoned that higher concentrations of Mg^{2+} than are typically employed with CSox-based activity sensors may be required to effectively discriminate between the phosphorylated and nonphosphorylated version of

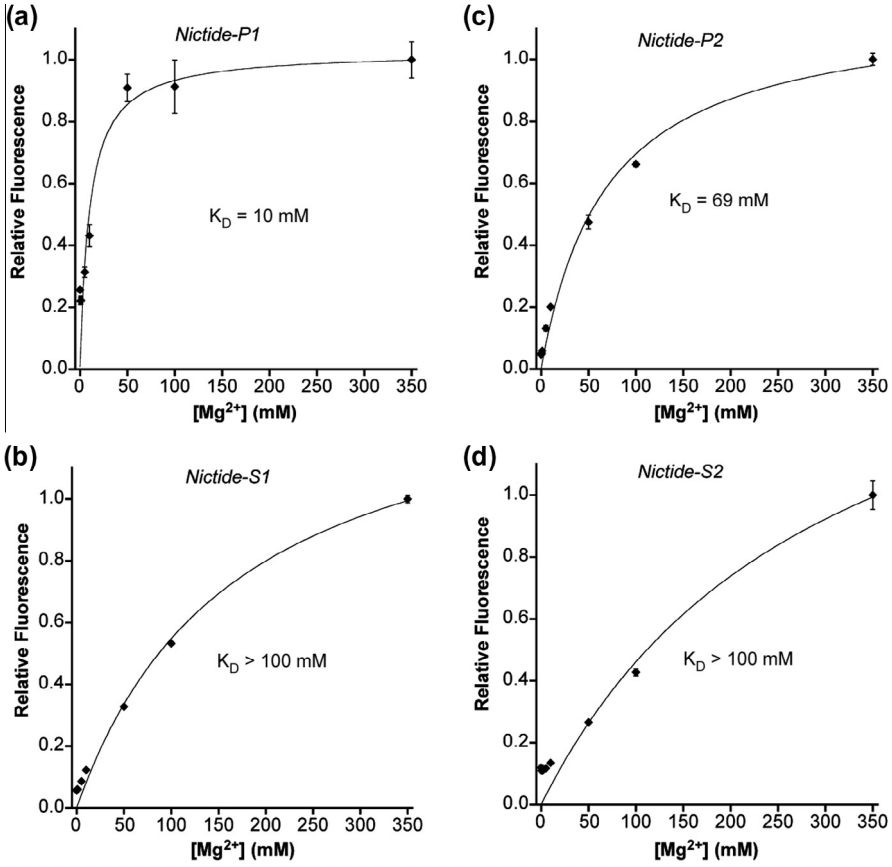

Figure 2. Binding affinities of phosphorylated (a and c) versus nonphosphorylated (b and d) CSox-based Nictide constructs. Phosphorylated peptides (a and c) display tighter affinities for Mg^{2+} than the corresponding nonphosphorylated peptides (b and d).