Analytical Biochemistry 450 (2014) 63–69

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

A continuous and direct assay to monitor leucine-rich repeat kinase 2 activity

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ARTICLE INFO

Article history:
Received 19 November 2013
Received in revised form 7 January 2014
Accepted 13 January 2014
Available online 23 January 2014

Keywords:
Leucine-rich repeat kinase 2
Chelation-enhanced fluorescence
Parkinson's disease
LRRK2 mutations
LRRK2 kinetics

ABSTRACT

Leucine-rich repeat kinase 2 (LRRK2) is a multi-domain enzyme displaying activities of GTP hydrolase and protein threonine/serine kinase in separate domains. Mutations in both catalytic domains have been linked to the onset of Parkinson's disease, which triggered high interest in this enzyme as a potential target for drug development, particularly focusing on inhibition of the kinase activity. However, available activity assays are discontinuous, involving either radioactivity detection or coupling with antibodies. Here we describe a continuous and direct assay for LRRK2 kinase activity, combining a reported peptide sequence optimized for LRRK2 binding and an established strategy for fluorescence emission on magnesium ion chelation by phosphorylated peptides carrying an artificial amino acid. The assay was employed to evaluate apparent steady-state parameters for the wild type and two mutant forms of LRRK2 associated with Parkinson's disease as well as to probe the effects of GTP, GDP, and autophosphorylation on the kinase activity of the enzyme. Staurosporine was evaluated as an inhibitor of the wild-type enzyme. It is expected that this assay will aid in mechanistic investigations of LRRK2.

Leucine-rich repeat kinase 2 (LRRK2)[1] is a large multi-domain protein of 2527 amino acids and approximately 288 kDa with two distinct enzymatic activities, namely GTP hydrolase (GTPase) and protein threonine/serine kinase, in different domains [1]. Mutations in both the kinase and GTPase domains have been shown to be statistically prevalent in Parkinson's disease patients, which elicited interest in LRRK2 as a potential target for drug discovery and design with a special focus on inhibition of its kinase activity [2]. The most common LRRK2 mutations associated with Parkinson's disease are G2019S and I2020T [3,4], proposed to increase kinase activity, and R1441C, R1441G, and Y1699C, thought to disrupt GTP hydrolysis [5,6]. In spite of intense research on this enzyme, its physiological protein substrate has not yet been identified, and few detailed kinetic and mechanistic studies have been reported, employing discontinuous enzymatic assays that require either radiolabel or antibody detection of phosphorylated synthetic peptides [7–9].

A valuable advance for investigation of LRRK2 activity was the development of Nictide, a synthetic peptide optimized for binding to the enzyme showing an apparent K_m value of 10 μM, approximately 10-fold lower than the value for LRRKtide, the most common peptide substrate used with LRRK2 [10]. Another powerful chemical tool for monitoring protein kinase activity is the chelation-enhanced fluorescence of sulfonamide-oxine (Sox) chromophore-containing peptides, in which Mg^{2+} ion normally present in kinase assays is rapidly chelated by the chromophore and the phosphate group of the phosphorylated peptide, leading to increased fluorescent signal and continuous monitoring of kinase activity [11,12]. To accommodate the fluorescent moiety, removal of residues located to either the N or C terminus of the phosphorylated residue of the peptide containing the specific kinase recognition sequence is part of the original design of Sox-based peptides for chelation-enhanced fluorescence [11]. Because this has in some cases led to significant loss of affinity of the kinase for the modified peptide, a second generation of Sox-containing sensor peptides was designed in which a Sox-modified cysteine (C-Sox) residue replaces one of the residues near the phosphorylated residue, thereby keeping most of the recognition sequence of the substrate intact and preserving affinity for the kinase [13].

Here we report a continuous and direct assay of LRRK2 kinase activity using a Sox-containing version of Nictide that displays increased fluorescence on phosphorylation (Fig. 1). Versions of Sox-containing Nictide were designed and tested in which residues either N or C terminal of the phosphorylated threonine were removed. Alternatively, a C-Sox residue replaced a phenylalanine

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[1] *Abbreviations used:* LRRK2, leucine-rich repeat kinase 2; GTPase, GTP hydrolase; Sox, sulfonamide-oxine; C-Sox, Sox-modified cysteine; WT, wild-type; EDTA, ethylenediaminetetraacetic acid; ON, Omnia–Nictide; LC–MS, liquid chromatography–mass spectrometry; NTA, nitrilotriacetate.

http://dx.doi.org/10.1016/j.ab.2014.01.007
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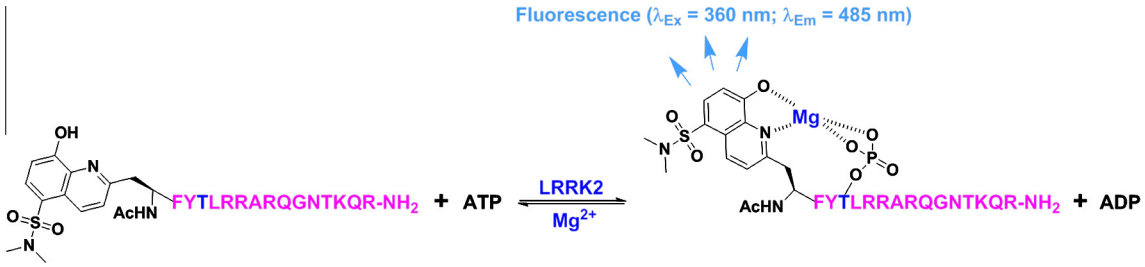

Fig.1. Schematic representation of ON-4 and its phosphorylation, which leads to increased fluorescence emission.

in the otherwise complete Nictide sequence. Steady-state apparent parameters and the effects of GTP and GDP on kinase activity are assessed by this assay for wild-type (WT) and Parkinson's disease-linked mutants G2019S–LRRK2 and R1441C–LRRK2 located in the kinase and GTPase domains, respectively. The effect of LRRK2 autophosphorylation on kinase activity of WT and G2019S isoforms was also investigated, and the presence of autophosphorylation was confirmed by mass spectrometry. Inhibition of WT–LRRK2 by staurosporine is evaluated.

Materials and methods

Materials

Recombinant full-length WT–, G2019S–, R1441C–, and D1994A–LRRK2 were purchased from Life Technologies. ATP, ADP, GTP, GDP, Hepes, $MgCl_2$, Triton X-100, staurosporine from *Streptomyces sp.*, and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma–Aldrich. The acetylated (Ac-) peptides Ac-Arg-Leu-Gly-Trp-Trp-Arg-Phe-Tyr-Thr-Leu-(d-Pro)-Sox-Gly, Ac-Sox-Phe-Tyr-Thr-Leu-Arg-Arg-Ala-Arg-Gln-Gly-Asn-Thr-Lys-Gln-Arg-NH_2, and Ac-Arg-Leu-Gly-Trp-Trp-Arg-(C-Sox)-Tyr-Thr-Leu-Arg-Arg-Ala-Arg-Gln-Gly-Asn-Thr-Lys-Gln-Arg-NH_2—dubbed Omnia–Nictide-3 (ON-3), Omnia–Nictide-4 (ON-4), and Omnia–Nictide-6 (ON-6), respectively—were custom-synthesized by Life Technologies.

Enzymatic assay

LRRK2 kinase activity was assayed at 22 °C by monitoring the continuous increase in fluorescence ($\lambda_{excitation}$ = 360 nm and $\lambda_{emission}$ = 485 nm) on phosphorylation of ON-3, ON-4, or ON-6 in 384-well ProxiPlate-384 Plus plates (PerkinElmer) and an Infinite M1000 (Tecan) plate reader. Typical reaction mixtures (25 µl) contained 20 mM Hepes (pH 7.5), 10 mM $MgCl_2$ (pH 7.5), 1 mM EDTA (pH 8.0), 0.01% Triton X-100, 400 µM ATP, 160 µM peptide substrate, and varying concentrations of WT–LRRK2 (10.8–84.0 nM). Alternatively, G2019S–, R1441C–, or D1994A–LRRK2 replaced WT–LRRK2. Reactions were started by the addition of ON-4. Control experiments contained no enzyme. A dose–response factor for product formation was calculated by completely converting 5, 10, and 20 µM ON-4 to product in the presence of 1 mM ATP and either 21 nM WT–LRRK2 (10 and 20 µM ON-4) or 42 nM WT–LRRK2 (5 µM ON-4). Time courses of fluorescence increase were measured and fitted to Eq. (1), where k is the first-order rate constant, t is time, F is fluorescence, and A is the total fluorescence amplitude change. Measurements were carried out in duplicate.

$$F = A\left(1 - e^{-kt}\right) \tag{1}$$

Saturation curves

Apparent kinetic parameters for ATP and ON-4 with WT–, G2019S–, and R1441C–LRRK2 were determined by measuring initial velocities at varying concentrations of one substrate and a fixed saturating concentration of the other at 22 °C. The concentrations of WT–, G2019S–, and R1441C–LRRK2 were 29, 26, and 37 nM, respectively. Reactions were started by the addition of ON-4. Data were fitted to Eq. (2), where S is the concentration of the varied substrate, v/E_T is the observed catalytic constant (E_T refers to the concentration of kinase active sites), k_{cat} is the limiting steady-state catalytic constant, and K_m is the Michaelis constant for S. Rates were averaged from duplicate measurements.

$$v/E_T = k_{cat} \times S/(K_m + S) \tag{2}$$

Effects of GTP and GDP

The effect of GTP or GDP on LRRK2 kinase activity was assessed by measuring initial rates at 22 °C in the presence of 800 µM of either GTP or GDP and 45, 39, and 56 nM of WT–, G2919S–, and R1441C–LRRK2, respectively. The concentrations of ON-4 were 30 µM with WT– and G2019S–LRRK2 and 50 µM with R1441C–LRRK2, whereas the concentrations of ATP were 30, 90, and 60 µM with WT–, G2919S–, and R1441C–LRRK2, respectively. Reactions were started by the addition of ON-4. Positive controls contained no GTP or GDP, and negative controls contained either no enzyme or no ATP. Each rate is the average of duplicate measurements.

LRRK2 autophosphorylation

The effect of autophosphorylation of WT– or G2019S–LRRK2 on kinase activity toward ON-4 was tested by measuring initial velocities with varying concentrations of ATP at 22 °C. Reactions were started by the addition of ATP. The concentration of ON-4 was 120 µM, and the concentrations of WT–LRRK2 and G2019S–LRRK2 were 45 and 39 nM, respectively.

To confirm autophosphorylation, 2.9 µg of either WT– or G2019S–LRRK2 was incubated for 40 min at 22 °C in the presence of either 800 µM ATP or 800 µM ADP in 25 µl. Each sample was treated with 225 µl of ethanol, incubated at −80 °C for 2 h, and centrifuged at 16,500*g* for 10 min. Supernatants were discarded, and pellets were vacuum-dried under centrifugation and treated with 10 µl of 8 M urea, 0.4 M NH_4HCO_3, and 4.5 mM dithiothreitol for 20 min at 50 °C. Samples were allowed to cool, treated with 1.2 µl of 0.1 M iodoacetamide at 22 °C for 25 min, diluted by the addition of 30 µl of water, and incubated with 1 µg of trypsin at 37 °C overnight. Then 12 µl of each digest was reserved for direct liquid chromatography–mass spectrometry (LC–MS) analysis, and the remainder was subjected to affinity capture of phosphopeptides using spin columns from the Pierce Fe–NTA (nitrilotriacetate) Phosphopeptide Enrichment Kit (Thermo Scientific). Product fractions were evaporated to a final volume of 20 µl each and analyzed by LC–MS. LC–MS was conducted using 8-µl injections of either the whole digest or the product of Fe–NTA affinity capture. LC–MS was carried out by capillary flow reversed-phase high-performance liquid chromatography (HPLC) and an LTQ Orbitrap XL mass