Analytical Biochemistry 384 (2009) 213–223

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Kinetic characterization and identification of a novel inhibitor of hypoxia-inducible factor prolyl hydroxylase 2 using a time-resolved fluorescence resonance energy transfer-based assay technology

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ARTICLE INFO

Article history:
Received 10 June 2008
Available online 14 October 2008

Keywords:
2-Oxoglutarate
HIF
Non-heme iron
Oxygen
PHD2
Prolyl hydroxylase
Von Hippel–Lindau protein
vHL

ABSTRACT

The human hypoxia-inducible factor prolyl hydroxylases 1, 2, and 3 (HIF–PHD1, -2, and -3) are thought to act as proximal sensors of cellular hypoxia by virtue of their mechanism-based dependence on molecular oxygen. These 2-oxoglutarate (2-OG) and non-heme iron-dependent oxygenases constitutively hydroxylate HIF, resulting in high-affinity binding to Von Hippel–Lindau protein (pVHL). Some reported affinities for the HIF–PHDs for 2-OG and iron approach the estimated physiological concentrations for these cofactors, suggesting that the system as described is not catalytically optimal. Here we report the enzymatic characterization of full-length recombinant human HIF–PHD2 using a novel and sensitive catalytic assay. We demonstrated submicromolar affinities for 2-OG and ferrous iron and HIF–PHD2 K_m values for oxygen that are greater than atmospheric oxygen levels, suggesting that molecular oxygen is indeed the key regulator of this pathway. In addition, we observed enhancement of HIF–PHD2 catalytic activity in the presence of ascorbic acid with only minor modifications of HIF–PHD2 requirements for 2-OG, and a detailed pH study demonstrated optimal HIF–PHD2 catalytic activity at pH 6.0. Lastly, we used this sensitive and facile assay to rapidly perform a large high-throughput screen of a chemical library to successfully identify and characterize novel 2-OG competitive inhibitors of HIF–PHD2.

The prolyl hydroxylase domain (PHD)[1] enzymes are a conserved family of cellular oxygen sensing proteins belonging to the extensive family of 2-oxoglutarate (2-OG) and non-heme iron-dependent oxygenases. The non-heme iron oxygenases catalyze a multitude of diverse oxidative reactions. They share in common the requirement for oxygen, iron, and 2-OG for oxidative decarboxylation to catalyze protein substrates into product with the generation of succinic acid and carbon dioxide [1].

PHDs play a key role in cellular oxygen homeostasis via the regulation of hypoxia-inducible factors (HIFs). HIF is an α/ß-heterodimeric transcription factor that binds to hypoxia response elements (HREs) of DNA to regulate a variety of cellular functions, including upregulation of a variety of growth factors such as erythropoietin (EPO) for hematopoiesis and vascular endothelial growth factor (VEGF) for angiogenesis [2]. Oxygen regulation is mediated by the alpha subunit of HIF, for which there are three human isoforms: HIF-1α, HIF-2α, and HIF-3α. All are substrates for at least one of the three known PHDs (PHD1, PHD2, and PHD3) [3,4], although there is supporting evidence that PHD2 plays a key role in HIF-1α regulation for a variety of cell lines as determined by small interfering RNA (siRNA) suppression of the PHDs [5,6].

Although both HIF-1α and HIF-1ß are continuously synthesized, protein levels for HIF-1α are low due to rapid protein degradation during normoxia. Under normal oxygen levels, PHD2 hydroxylates two conserved proline residues on HIF-1α, allowing recognition and binding by the Von Hippel–Lindau protein (pVHL). pVHL forms a complex with elongin B, elongin C, Rbx1, and Cul2 to function as an E3 ubiquitin ligase, targeting HIF-1α for polyubiquitination and subsequent proteasomic degradation [7–9] (Fig. 1). The oxygen sensing mechanism is tied to PHD's high oxygen requirement for enzyme catalysis and regulation of HIF-1α. The HIF–PHD2 K_m for

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[1] *Abbreviations used:* PHD, prolyl hydroxylase domain; 2-OG, 2-oxoglutarate; HIF, hypoxia-inducible factor; HRE, hypoxia response element; EPO, erythropoietin; VEGF, vascular endothelial growth factor; siRNA, small interfering RNA; pVHL, Von Hippel–Lindau protein; DFO, desferroxamine mesylate; ECL, electrochemiluminescence; TR–FRET, time-resolved fluorescence resonance energy transfer; HTS, high-throughput screen; VCB, pVHL–elongin B–elongin C complex; IPTG, isopropyl-β-D-thiogalactopyranoside; HSL, homoserine lactone; Ni-NTA, nickel–nitrilotriacetic acid; SEC, size exclusion chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Eu, europium; Ru, ruthenium; NHS, *N*-hydroxysuccinimide ester; MOI, multiplicity of infection; TCEP, Tris-(2-carboxyethyl) phosphine; MALDI, matrix-assisted laser desorption/ionization; TPA, tripropylamine; APC, allophycocyanin; POC, percentage of control; DMSO, dimethyl sulfoxide; ITC, isothermal titration calorimetry; FP, fluorescence polarization; FIH, factor-inhibiting HIF; PAHX, phytanoyl–coenzyme A 2-hydroxylase; NOG, *N*-oxalylglycine.

0003-2697/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2008.09.052

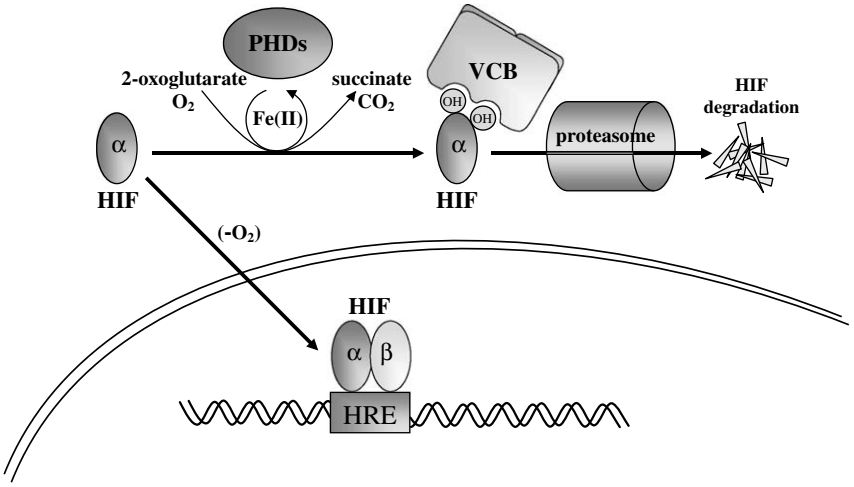

Fig. 1. Schematic diagram of HIF-1α regulation by HIF–PHDs. During normoxia, PHDs hydroxylate two proline residues on HIF-1α (P402 and P564) in an O_2-, 2-OG-, and iron-dependent manner. VCB (pVHL in a complex with elongin B and elongin C) binds to the hydroxylated HIF-1α proteins, resulting in HIF-1α polyubiquitination and proteasomic degradation. In hypoxia, the activities of HIF–PHDs are inhibited due to lack of O_2. Stabilized HIF-1α proteins translocate to the nucleus and dimerize with HIF-1ß to bind the HREs of DNA, resulting in upregulation of a variety of growth factors in response to the low oxygen levels.

oxygen was determined to be that of atmospheric oxygen levels (230–250 μM) [10,11], warranting much greater oxygen sensitivity than other oxygenases such as collagen PHD where collagen PHD retained activity under hypoxia with a significantly lower measured K_m for oxygen (40 μM) [11]. Under conditions of hypoxia, HIF–PHD2 is inactive; hence, HIF-1α is not targeted for proteasomic destruction by hydroxylation. The accumulating HIF-1α dimerizes with HIF-1ß and binds to HRE in the cell's nucleus and induces a cellular response to ameliorate the low oxygen conditions by increasing circulatory oxygen-carrying capabilities, thereby restoring the affected tissue's oxygen homeostasis.

Therapeutic interests have been generated in the development of specific inhibitors that target the PHD enzymes for the treatment of a wide array of medical conditions, including inflammation, ischemia, stroke, and anemia [12]. Desferroxamine mesylate (DFO), a potent iron chelator, was found to upregulate EPO and hemoglobin levels in patients afflicted with chronic disease anemia and rheumatoid arthritis [13]. Cobalt, shown to inactivate PHD2 by competing with iron, was found to upregulate renal HIF-1α protein expression as well as reduce ischemic injury in the kidney of rats [14]. Although such data support therapeutic implications of PHDs, inhibition by iron chelation or substitution would lack specificity.

Despite the important physiological roles that PHDs play, these proteins are largely uncharacterized in terms of enzymatic activity, in part owing to both a lack of facile and sensitive assay methodology and a dearth of potent nonchelating inhibitors. The lack of high-capacity PHD assays has also hindered the discovery and development of inhibitors for potential therapeutic implications. A commonly used 2-OG-dependent oxygenase assay involves capture and detection of $^{14}CO_2$ gas resulting from oxidative decarboxylation of [1-14 C]2-OG [15]. This assay has the advantage of being a generic 2-OG oxygenase assay but is relatively difficult and time-consuming to execute and requires the use of radioactive reagents. As such, we developed a novel and high-throughput nonradioactive electrochemiluminescence (ECL) assay as well as a homogeneous time-resolved fluorescence resonance energy transfer (TR–FRET) assay to elucidate PHD2 enzymatic reaction by characterizing and exploiting the biological affinity of pVHL to the catalytic product. We used both assay technologies to quantify PHD2 kinetic activity and to characterize the enzyme requirements for iron, 2-OG, oxygen, ascorbic acid, and the HIF-1α peptide substrate as well as to determine catalytic activity in various pH levels. The TR–FRET assay was ultimately used to aid drug discovery by performing a large-scale high-throughput screen (HTS) to find small molecule inhibitors of PHD2. Here we report the novel PHD2 inhibitor that surfaced from the screening campaign and the subsequent kinetic characterization of the active compound discovered.

Materials and methods

Expression and purification of recombinant VCB

The pVHL–elongin B–elongin C heterotrimeric complex (VCB) was coexpressed in *Escherichia coli* and purified from the soluble fraction as described previously [16]. The pVHL amino acid sequence (54–213) containing an N-terminal 6-histidine affinity tag was cloned into pAMG21 (pLux promoter) between the *Nde*I and *Xho*I sites. Immediately downstream of this was the elongin C gene cloned into the *Xho*I site to *Sac*II with a 13-bp spacer between the stop codon of VHL and the initiating codon of elongin C. Elongin B was cloned into pTA2 (pACYC184.1-based vector) under the control of a Lac promoter. Induction of the system was initiated with the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) and homoserine lactone (HSL) at 30 °C.

Bacterial cells were lysed by a microfluidizer in aqueous buffer of pH 8.0, and the soluble fraction was separated by centrifugation and subjected to nickel–nitrilotriacetic acid (Ni-NTA) chelating chromatography to bind the 6-histidine affinity tag of the pVHL construct. The pooled fractions from the nickel column were applied to a Superdex 200 size exclusion chromatography (SEC) column. The protein eluted as a single fraction on SEC, indicating that the three protein components formed a complex in solution. The fractions from the SEC column were pooled and applied to a Q Sepharose anion exchange column for the final purification step. The composition of the purified complex was visualized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the identities of the three protein components were confirmed by N-terminal amino acid sequencing.

Europylation of the VCB complex

Purified VCB was exchanged into 50 mM sodium carbonate buffer (pH 9.2) and labeled with LANCE europium (Eu)-W1024 ITC