Application of a Proteolysis/Mass Spectrometry Method for Investigating the Effects of Inhibitors on Hydroxylase Structure

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Limited proteolysis coupled to matrix-assisted laser desorption/ionization (MALDI) mass spectrometric analyses can be used to screen for compounds that alter protein structure by monitoring stabilizing/destabilizing effects with respect to the rate and nature of proteolysis. When applied to prolyl hydroxylase 2, a key enzyme involved in human oxygen sensing, the method efficiently revealed differential effects on proteolytic stability for structurally similar compounds and for different substrates.

Introduction

Both limited proteolysis and mass spectrometry (MS[a]) are techniques that are used extensively in proteomic analyses. Coupling of these techniques can be useful for studies on protein dynamics, where proteolysis often occurs preferentially in flexible regions (for review, see ref 1). Such studies can involve time-consuming HPLC analyses coupled to high resolution electrospray ionization (ESI) MS. MALDI-MS is an efficient technique that has also been widely used in proteomic analyses, often to obtain qualitative information.[2] Limited proteolysis has been coupled to MALDI-MS to look for the effects of protein−protein and protein−ligand interactions on patterns and rates of proteolysis.[3−7] We have optimized a limited proteolysis/mass spectrometry technique to study the differential effects of a range of small molecule inhibitors on enzyme stability with respect to proteases and exemplified its utility by using a biomedicinally important oxygen-sensing enzyme as a model system.

All animals must be able to sense and respond to changes in oxygen availability. The prolyl hydroxylase domain enzymes (PHDs), and in particular PHD2, are key components of the human oxygen sensing system (for reviews see refs 8, 9). PHD2 catalyzes the oxygen-dependent post-translational hydroxylation of two prolyl residues, Pro402 and Pro564 (Figure 1, inset), in the α-subunit of the hypoxia inducible transcription factor (HIF-α). These modifications signal for the degradation of HIF-α via the ubiquitin proteasome system.[10,11] Under low oxygen conditions (hypoxia), PHD activity slows, resulting in upregulation of HIF-α and increased transcription of HIF target genes. These include those encoding for erythropoietin and vascular endothelial growth factor among many others, which are involved in the coordinated response to low oxygen levels.[12] PHD2 is a current pharmaceutical target for inhibition, with a view to developing new treatments for anemia and ischemic disease.[13]

PHD2 is a member of the Fe(II)/2-oxoglutarate (2OG)-dependent oxygenase superfamily. All known 2OG oxygenases contain a core domain with a double-stranded β-helix fold, as revealed by crystallographic analyses,[14] and catalyze the two electron oxidation of their target substrates with the concomitant oxidation of 2OG to succinate and carbon dioxide.[15] Recent NMR studies have implied that binding of 2OG significantly stabilizes the core domain of PHD2 and some other 2OG oxygenases;[16] both the core domain and mobile regions surrounding the active site can be involved in substrate binding to 2OG oxygenases, which for PHD2 likely involves a flexible loop region.[17,18]

We optimized our limited proteolysis/MALDI-MS method and applied it to study the effects of a set of inhibitors, as well as two peptide substrates (fragments of HIF-α), on the stability of the catalytic domain of PHD2 (residues 181−426, hereafter PHD2) with respect to proteolysis. The results are consistent with previous biophysical and inhibition studies[16,18,19] but reveal distinctive modes of binding for structurally related inhibitors, as manifested by variable susceptibilities of PHD2 toward proteolysis. Such information is often not readily accessible to medicinal chemists. The two HIF-α peptide fragment substrates of PHD2 were also shown to bind differently to the enzyme, revealing structural information about these interactions not currently available through other biophysical analyses.

Results and Discussion

A limited proteolysis/MALDI-MS method was optimized whereby the molecule of interest (inhibitor or (co)substrate) was added to the catalytic domain of PHD2 complexed to Fe(II) (PHD2·Fe(II)), followed by proteolysis with trypsin (24 h at 37 °C), during which time samples were removed at regular intervals, quenched, and analyzed by MALDI-MS (see Experimental Details for full methodology). Results from NMR and ESI-MS indicate that the PHD2·Fe(II)·2OG complex is thermodynamically stabilized relative to the PHD2·Fe(II) complex.[16,20] The unusually (for studied 2OG oxygenases) tight binding of PHD2·Fe(II) to 2OG is a property proposed to relate to the oxygen sensing role of PHD2.[20] We therefore initially used our method to investigate the relative stabilities to

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[a] Abbreviations: MS, mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; ESI, electrospray ionization; PHD, prolyl hydroxylase domain; HIF, hypoxia-inducible factor; 2OG, 2-oxoglutarate; NOG, *N*-oxalyl glycine; BIC, bicyclic compound; FIH, factor inhibiting HIF.

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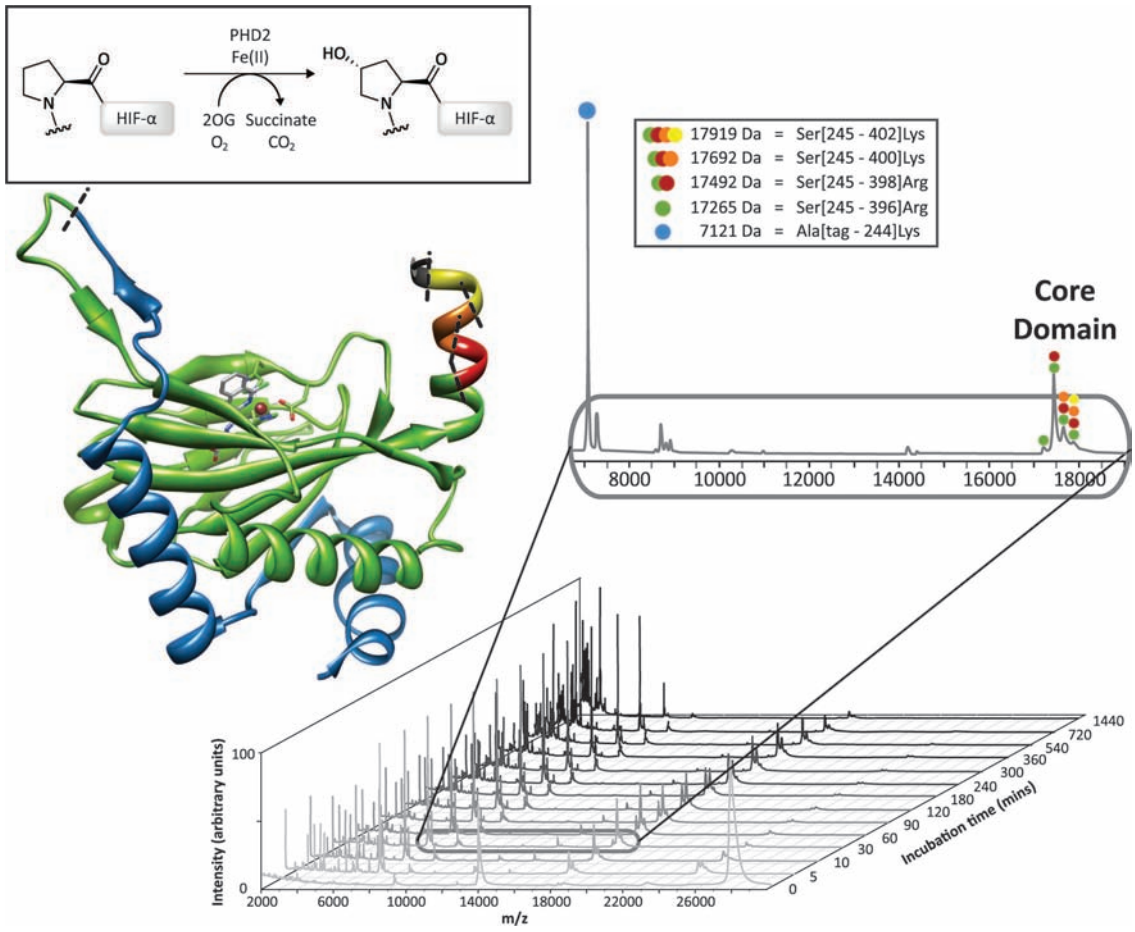


Figure 1. MALDI-MS spectra showing limited trypsinolysis of the PHD2·Fe(II)·2OG complex. The 30 min spectrum is expanded. Vertically aligned spots over peaks in the spectrum indicate fragments of digested PHD2 and are color-coded to PHD2 regions based on crystallographic analyses.[18] Other peaks include intact PHD2 +1, +2, and +3 charge states (~27500, ~14000, and ~9200 Da), Ala[tag-398/396]Arg +1 and +2 charge states (~24000 and ~12000 Da), the "core domain" +2 charge state (~8750 Da) and peptide digestion products (<7000 Da). The inset scheme outlines the reaction catalyzed by PHD2. *m*/*z* = mass/charge (Da).

proteolysis of the PHD2·Fe(II) and PHD2·Fe(II)·2OG complexes (Figure 1). The data revealed rapid cleavage of ~3 kDa and ~7 kDa fragments from the C- and N-termini of PHD2, respectively. The resulting ~17 kDa fragment (Ser245−Lys402, termed the "core domain") was assigned (by ESI-MS) to a region containing the double-stranded β-helix core motif.[18] This overall pattern of fragmentation was observed both in the presence and absence of 2OG. However, in the presence of 2OG, proteolysis of the core domain proceeded much more slowly, taking 24 h as compared to 60 min in the absence of 2OG (Figure 2A,B). This observation suggests that 2OG markedly stabilizes the core domain, consistent with its stabilizing effect on PHD2 observed by other more demanding biophysical methods.[16,20] At the C-terminus of the ~17 kDa core domain are four potential trypsin cleavage sites (Lys402, Lys400, Arg398, and Arg396). The core domain therefore consists of four clustered peaks in the MALDI spectra (Figure 1); successive cleavage at the four sites was observed to occur as a function of time (Figure 3).

PHD2 inhibitors are of pharmaceutical interest for the treatment of ischemic diseases.[21−23] We therefore used the method to study the effects of different inhibitors on the rate and pattern of limited proteolysis of PHD2. *N*-Oxalylglycine (NOG) is an unreactive 2OG analog inhibitor of PHD2[24] (Table 1, IC_{50} = 18.5 μM). In the presence of NOG, rapid cleavage of the C- and N-terminal ~3 kDa and ~7 kDa fragments was observed, leaving the intact core domain as observed in the presence of 2OG; however, complete degradation of the core domain fragments was observed (i.e., no core domain fragments were observed) within 360 min as compared to >1440 min with 2OG (Figure 2C). This difference reveals a reduced ability of NOG to stabilize the PHD2 core domain with respect to trypsinolysis as compared with 2OG. These results demonstrate the utility of the method in elucidating subtle differences in binding: 2OG and NOG have very similar structures (Table 1), and X-ray diffraction studies have shown them to bind similarly to different 2OG oxygenases in crystal structures (see, e.g., 14, 25−27).

We then used the method to study the effects of succinate and fumarate on PHD2 stability. Succinate, a coproduct of PHD2 catalysis, has been reported along with another tricarboxylic acid cycle intermediate, fumarate, to inhibit PHD2, rationalizing the association with cancer of mutations in the genes encoding fumarate and succinate dehydrogenases.[28−31] PHD2 was rapidly digested to the core domain, as observed with 2OG, in the presence of both succinate and fumarate. In comparison with 2OG, however, the degree of stabilization to trypsinolysis of the core domain was reduced significantly with complete digestion of the core domain observed within 180 min with succinate and 720 min with fumarate (Figure 2D,E). In the case of succinate and fumarate, reduced stabilization to trypsinolysis of the core domain may be related to monodentate, or otherwise weakened, coordination to the active site Fe(II), as opposed to the bidentate coordination observed for 2OG and NOG.[14,18,28]

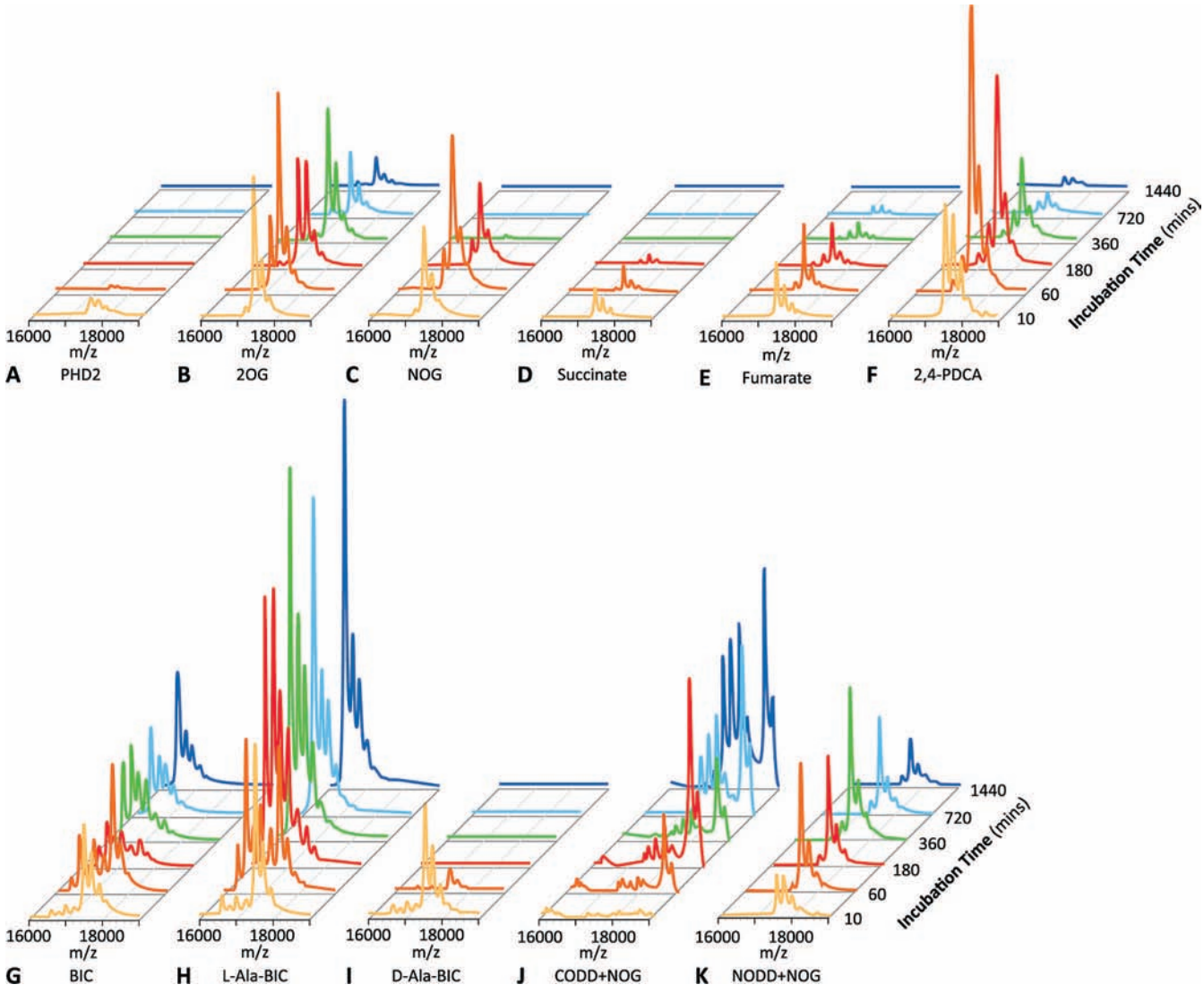


Figure 2. Stability of the PHD2 core domain with different combinations of inhibitors and (co)substrates. Representative MALDI-MS spectra revealing the stability to proteolysis of the PHD2 core domain with, (A) no added (co)substrates or inhibitors, (B) 2OG, (C) NOG, (D) succinate, (E) fumarate, (F) 2,4-PDCA, (G) BIC, (H) L-Ala-BIC, (I) D-Ala-BIC, (J) CODD + NOG, and (K) NODD + NOG. CODD = HIF-1$\alpha_{556-574}$; NODD = HIF-1$\alpha_{395-413}$. Structures of 2OG and inhibitors are shown in Table 1. *m*/*z* = mass/charge (Da). Note differences in maximum peak height of the analyses under different conditions are normalized to the base (most intense) peak in the full spectrum, therefore, although indicative of the ratio of the core domain relative to smaller tryptic fragments in the same assay, quantitative comparisons between different assays cannot be made.

The lower stabilization observed for succinate compared to fumarate may be consistent with its reduced potency as an inhibitor (succinate IC_{50} = 85.3 μM compared with fumarate IC_{50} = 19.1 μM under the present assay conditions).

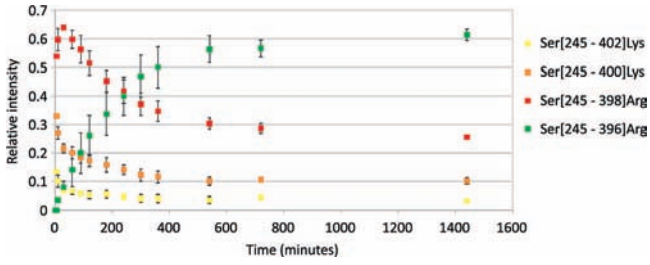
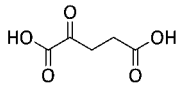
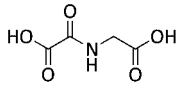
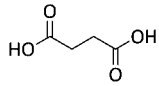
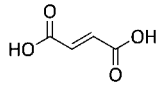
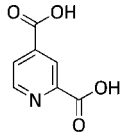
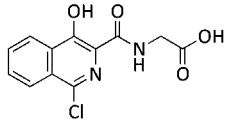
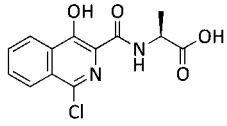
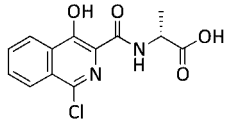


Figure 3. Progression of the core domain digestion of the PHD2·Fe(II)·2OG complex. Intensities of each of the core domain fragments (Ser245−Arg396, Ser245−Arg398, Ser245−Lys400, and Ser245−Lys402) were measured relative to the total intensity of all the core domain fragments at each time point. Data represent mean intensities of all digestion repeats (n = 3), error bars show ±1 standard deviation. The data support the observation of sequential C-terminal cleavage from the core domain (larger fragments are precursors of smaller fragments).

We then examined a cyclic 2OG analogue inhibitor of PHD2: 2,4-pyridine dicarboxylate (2,4-PDCA), initially identified as an inhibitor of a collagen prolyl hydroxylase,[32] has also been shown to inhibit PHD2[33] (Table 1, IC_{50} = 1.91 μM). With the limited proteolysis/MALDI-MS method, 2,4-PDCA caused a similar degree of stabilization of PHD2 with respect to proteolysis to that observed with 2OG, i.e., the core domain remained detectable by MALDI-MS after 24 h. The extent of core domain stabilization by 2,4-PDCA was greater than that seen with NOG, succinate, and fumarate, suggesting that stabilization of the enzyme with respect to proteolysis may correlate with inhibitor potency.

Crystallographic analyses have revealed that bicyclic isoquinolinyl inhibitors of PHD2, e.g., BIC (Table 1, IC_{50} = 0.073 μM), form multiple interactions at the PHD2 active site in the crystalline state (PDB ID: 2HBT).[18,34] BIC binds in a competitive manner with respect to 2OG with its glycine appendage occupying the position of the 2OG backbone within the active

Table 1. Half-Lives of the PHD2 "Core Domain" in the Presence of Various Inhibitors, Indicating Their (De)stabilizing Effects with Respect to Proteolysis[a]

Inhibitor	Structure	PHD2 'core domain' half life (mins)	IC_{50} (μM)
-	-	15	N/A
2OG		>720	N/A
NOG		150	18.5
Succinate		120	85.3
Fumarate		360	19.09
2,4-PDCA		>720	1.91
BIC		>720	0.073
L-Ala-BIC		>720	0.512
D-Ala-BIC		45	230

[a] Half life and IC_{50} values for each inhibitor were determined to compare the stabilization effect and potency of each inhibitor. Recombinant PHD2 purified from *E. coli*[20] was used to measure IC_{50} values for all inhibitors using a time-resolved fluorescence-based assay[38] (Supporting Information Figure 3). Variations between the IC_{50} values reported here and those previously reported[28,30,33] probably reflect differences in assay conditions.

site. For BIC, the limited proteolysis/MALDI-MS method again revealed rapid degradation of the N- and C-termini of intact PHD2. However, the core domain was modified differently compared to previous analyses, with additional cleavage sites (Lys249 and Arg252) being observed at its N-terminus (the cleavage sites were assigned by ESI-MS, Figure 4). The data imply that binding of BIC to PHD2 induces a conformation that enables access of trypsin to these additional cleavage sites: crystal structures of PHD2 complexed with bicyclic inhibitors reveal that they prevent a flexible loop from closing over the active site (see ref 18 and PDB ID: 2HBT). We propose that BIC binding causes increased flexibility in this loop region, permitting more facile cleavage at Lys249 and Arg252. Interestingly, in contrast to the other inhibitors studied, the (modified) core domain was apparently more stable with BIC compared to 2OG (Figure 2G).

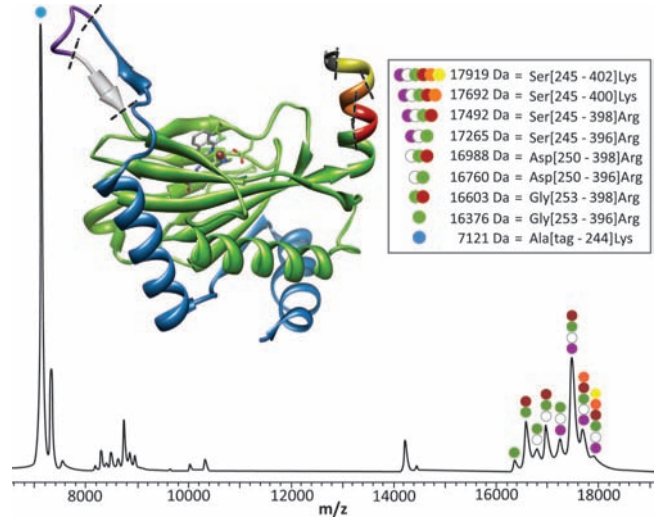


Figure 4. Limited trypsinolysis of PHD2·Fe(II)·BIC. MALDI-MS spectrum showing the result of limited trypsinolysis of PHD2·Fe(II)·BIC after 60 min. Vertically aligned spots over peaks in the spectrum indicate fragments of digested PHD2 and are color-coded to PHD2 regions based on crystallographic analyses (PDB ID: 2HBT and ref 18). Further cleavage of the "core domain" was observed C-terminal to Lys249 and Arg252 in the presence (but not absence) of BIC, indicating that the BIC perturbs the structure in this region. *m/z* = mass/charge (Da).

Work on PHD2 and factor inhibiting HIF (FIH), a HIF asparaginyl hydroxylase,[9,35−37] has shown that selectivity between these HIF hydroxylases can be achieved by varying the amino acid derived side chain of *N*-oxalyl amino acid derivatives, with D-amino acid derivatives preferentially inhibiting FIH and L-amino acid derivatives preferentially inhibiting PHD2.[19] We therefore analyzed L- and D-alanine derivatives of BIC (Table 1, Figure 2H,I) for their effect on PHD2 trypsinolysis. Inhibition studies employing a fluorescence-based assay[38,39] showed that D-Ala-BIC is a weak inhibitor of PHD2 (IC_{50} = 230 μM), whereas L-Ala-BIC and BIC are stronger inhibitors (IC_{50} values = 0.512 and 0.073 μM, respectively, Table 1). These results are consistent with those for the *N*-oxalyl amino acid inhibitors.[19] The stability and fragmentation pattern of the core domain in the presence of L-Ala-BIC suggests that this inhibitor binds in a very similar manner to BIC itself. In contrast, the D-Ala-BIC inhibitor appeared to have little stabilizing effect relative to PHD2 alone. Consistent with kinetic work and the proteolysis assays results, nondenaturing ESI-MS analyses[40] (Supporting Information Figure 1) revealed that D-Ala-BIC forms a weaker complex with PHD2·Fe(II) than either BIC or L-Ala-BIC, although some binding of D-Ala-BIC was still observed. The relative lack of inhibition/stabilization to proteolysis with D-Ala-BIC may be due to the steric clashes with the side chain of Val376 in the 2OG binding pocket at the PHD2 active site.[18] Thus, the lifetime of a fragment (or where appropriate, intact protein) could be used for inhibitor screening, at least when comparing inhibitors from the same class.

Finally, we applied our method to study the stability of two PHD2·Fe(II)·substrate complexes with the two prolyl hydroxylation sites from human HIF-1α: PHD2·Fe(II)·NOG·HIF-$1\alpha_{556-574}$ and PHD2·Fe(II)·NOG·HIF-$1\alpha_{395-413}$. NOG was used instead of 2OG to prevent HIF hydroxylation. The presence of both peptide substrates appeared to have a stabilizing effect on PHD2. Interestingly, the cleavage pattern was markedly different between the two substrates (Figure 2J,K). With HIF-$1\alpha_{556-574}$, initial cleavage of the C-terminal fragment was observed at Lys408 (Ser[245−408]Lys = 18610 Da) instead of at Lys402 as in the absence of peptide substrate. This result reveals that

HIF-1$\alpha_{556-574}$ binding imposes a structure that reduces the susceptibility of Lys402 to trypsinolysis. The standard core domain fragments were only apparent after 60 min, with very little cleavage at Arg396. Cleavage of the N-terminus of the core domain at Ser245 occurred similarly to that seen in the absence of HIF-1$\alpha_{556-574}$. The composition and stability of the core domain in the presence of HIF-1$\alpha_{556-574}$ therefore indicates regions of PHD2 that are involved in the binding of this substrate. In contrast, limited proteolysis of a PHD2·Fe(II)·NOG·HIF-1$\alpha_{395-413}$ substrate complex led to formation of the standard core domain (Figure 2K), suggesting that in this case Lys402 and Arg396 are more amenable to trypsin digestion. Thus, Lys402 and Arg396 may be less important in HIF-1$\alpha_{395-413}$ binding than they are in HIF-1$\alpha_{556-574}$ binding (although of course with intact HIF-1α the situation may be different). These results may be related to the differences observed in both recombinant protein and cell-based studies with respect to PHD catalyzed hydroxylation of HIF-1$\alpha_{395-413}$ and HIF-1α_{556-57}.[17,41] Overall, these results demonstrate the ability of our method to efficiently identify differences in substrate binding modes.

Conclusion

By applying our limited proteolysis/MALDI-MS method to the study of PHD2 and the proteolytic (de)stabilizing effects of inhibitors and (co)substrates, we have demonstrated that the method can be used to readily obtain structural and functional information about the binding mode of small molecules to proteins. The results are consistent with and extend other reports on PHD2 structure and inhibition, thus verifying the method, but also clearly reveal different effects of different types of inhibitors on enzyme stability. This method could therefore be used to probe the effects of other inhibitors on the proteolytic stability of the enzyme and may provide new insights into their mode of inhibition. In addition, because the assay takes place in aqueous solution, it can be used to provide useful functional data complementary to structural techniques that employ nonaqueous conditions. The method might also be applied to specifically screen for compounds that both bind tightly to proteins and (de)stabilize proteins to proteolysis, a mechanism for therapeutic intervention that has not been extensively exploited to date. (It is important to note that proteolytic stability does not necessarily correlate with thermodynamic stability.) Although the method is limited by the requirement that intact proteins/domains can be observed by MS, it is likely that this will be the case for many globular enzymes and can therefore be applied to other members of the Fe(II)/2OG-dependent oxygenase family and beyond.

Experimental Details

Materials. All chemicals were from Sigma-Aldrich, with the exception of the peptide substrates HIF-1$\alpha_{556-574}$ and HIF-1$\alpha_{395-413}$ (Peptide Protein Research Ltd.), MALDI-MS matrices, matrix buffers and calibrants, (LaserBioLabs) and sequencing grade modified porcine trypsin (Promega). L-Alanine and D-alanine derivatives of BIC were synthesized (Supporting Information Experimental Details).

Expression and Purification of PHD2$_{181-426}$. Human PHD2$_{181-426}$ (termed PHD2 throughout) was cloned, expressed in *E. coli* BL21(DE3), and purified by cation exchange and size exclusion chromatography, as described previously.[20] Purified PHD2 was buffer exchanged into 15 mM NH_4OAc (pH 7.5) and >95% purity verified by SDS-PAGE.

Limited Trypsinolysis. Sequencing grade modified trypsin was prepared in 15 mM NH_4OAc (pH 6.5) at 100 μg mL^{-1}. PHD2, peptide substrates HIF-1$\alpha_{556-574}$ and HIF-1$\alpha_{395-413}$, 2OG, fumarate, and succinate were prepared in 15 mM NH_4OAc (pH 7.5), whereas all other inhibitors were prepared to 100 mM in DMSO prior to dilution in 15 mM NH_4OAc (pH 7.5). Fe(II) was prepared by dissolving diammonium iron(II) sulfate to 250 mM in 20 mM HCl(aq) prior to dilution with water.

The trypsinolysis procedure was carried out by preincubating PHD2 and Fe(II) on ice for 30 min, before adding 2OG/inhibitor/peptide substrate, followed by addition of trypsin. The final assay mixture composition typically contained 50 μM PHD2, 50 μM Fe(II), 500 μM 2OG/inhibitor, and/or 250 μM peptide substrate and trypsin (trypsin/PHD2 1:200 w/w). Nondenaturing mass spectrometry confirmed that stable enzyme complexes formed under these conditions (Supporting Information Figure 2). The digest mixture was then incubated in a 37 °C water bath. At regular intervals over a 24 h period, 2 μL samples were removed and quenched by adding to 18 μL of quenching buffer (H_2O/MeOH/HCO_2H, 90:10:0.1) and then immediately stored at −80 °C until analysis.

MALDI-MS Analyses. Prior to analysis, quenched digest samples were thawed and kept on ice. A 10 mg mL^{-1} solution of recrystallized sinapinic acid was prepared in MeCN/H_2O/CF_3CO_2H (30:70:0.1) as supplied by the manufacturer. The digest sample (3 μL) and sinapinic acid solution (3 μL) were mixed and then 2 μL was pipetted onto a MALDI plate spot and allowed to air-dry. MALDI-MS analyses were performed using a MALDI micro MX mass spectrometer (Waters) in linear positive ion mode with laser energy 180%, pulse voltage 1250 V, detector voltage 2350 V, and mass suppression 2000 Da. Calibration was performed using a mixture of Protein Mix 2 and 3 (Laser BioLabs). MALDI-MS data were acquired for 30 s per spot, sampling a minimum of 10 different laser positions during this time. Data were analyzed using MassLynx v4.1. Spectra were smoothed using the Savitzky−Golay method (smooth windows: 10; number of smooths: 2).

ESI-MS Analyses. ESI-MS analyses were performed on a Q-ToF micro (Waters) with a Nanomate chip-based nano-ESI source (Advion Biosciences) and standard Z-spray source block. Calibration was performed using clusters of $Na_{n+1}I_n$ (1 mg mL^{-1} NAI in H_2O/isopropanol 50:50). Five μL of the samples were loaded into a 96-well plate and infused into the spectrometer with collisional cooling. Acquisition and scan times were 30 and 1 s, respectively. The instrument settings were as follows: spraying voltage 1.60 kV; sample cone voltage 50, 80, and 200 V; source temperature 40 °C; backing pirani 6.6 mbar; data acquisition range 500−5000 Da. ESI-MS data were analyzed using MassLynx. Raw data were processed by smoothing (Savitzky−Golay method; smooth windows: 10; number of smooths: 2), subtracting the background (polynomial order: 15; % below curve: 40; tolerance: 0.01) and centering peaks (minimum peak width at half-height: 2; % centroid top: 80).

Inhibition assays. IC_{50} values for all inhibitors were determined using a time-resolved fluorescence resonance energy transfer (FRET)-based assay, essentially as described.[38] Further details can be found in Supporting Information Figure 3.

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Supporting Information Available: Nondenaturing mass spectrometry verifying conditions for formation of enzyme·(co)substrate/inhibitor complexes. Nondenaturing mass spectrometry comparing binding of D- and L-alanine derivatives of BIC to PHD2. Dose−response curves showing inhibition of PHD2 by NOG,

succinate, fumarate, 2,4-PDCA, BIC and its L-Ala and D-Ala derivatives. Details for the synthesis of L-Ala and D-Ala derivatives of BIC. This material is available free of charge via the Internet at http://pubs.acs.org.

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