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Effects of polynitrogen compounds on the activity of recombinant human HIF-1α prolyl hydroxylase 3 in *E. coli*

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ABSTRACT

Hypoxia inducible factor 1α (HIF-1α) becomes an important regulation factor within the histiocyte when it is under the hypoxia condition. Recently, prolyl hydroxylases (PHDs) have been identified to inactivation HIF-lα by hydroxylation. In this study, polynitrogen compounds were screened as HIF-1α PHD3 inhibitors. The coding region of human PHD3 DNA was optimized by using synonymous codons according to the code bias of *Escherichia coli*. Soluble and active human PHD3 was expressed in the *E. coli* with a Trx fusion tag under a lower induction temperature of 25 °C. Mass spectrometry analysis of the resultant peptide product indicated a mass increase of 16 daltons, consistent with hydroxylation of the proline residue in the HIF-1α (556–574) peptide substrate. Polynitrogen compounds (1–4) inhibited the enzymatic hydroxylation of HIF-1α peptide in a concentration-dependent manner, and the apparent IC_{50} values were 29.5, 16.0, 12.8 and 60.4 μM respectively. Double reciprocal (1/V versus 1/[HIF-1α peptide]) plots showed that these compounds are noncompetitive inhibitors of the hydroxylation by recombinant human PHD3 with K_i values of 67.0, 25.3, 67.3, and 82.1 μM respectively. On the other hand, the metal complexes of these polynitrogen compounds (1–4) cannot inhibit the catalytical activity of PHD3. We hypothesized that the inhibitory mechanism of PHD3 activity by polynitrogen compounds is due to their binding to iron to form stable coordination complexes. Our results in this study indicated that polynitrogen compounds (1–4) could be potential inhibitors of PHD3 to regulate the transcriptional activity of HIF-1α.

1. Introduction

As a transcriptional activator, hypoxia inducible factor 1 (HIF-1) mediates the expression of many genes, e.g. vascular endothelial growth factor (*VEGF*), erythropoietion (*EPO*), glucose transporters et al., which play crucial roles in the acute and chronic adaptation to oxygen deficiency [1–3]. In tumor biopsies, HIF-1 overexpression is correlated with vascular density in brain tumors [4,5] and ovarian carcinoma [6], as well as in ductal carcinoma *in situ* (the early preinvasive stage of breast cancer) [7], indicating that HIF-1 activity contributes to the angiogenic switch. HIF-1 is a heterodimer consisting of an alpha subunit (HIF-1α) and a beta subunit (HIF-1β). Under normal oxygen pressure, prolyl hydroxylases (PHDs) hydroxylate proline residue 564 on the HIF-1α oxygen-dependent degradation domain by using molecular oxygen and thereby increase ubiquitin-proteosome-dependent degradation of HIF-1α [8–17]. Under hypoxic conditions, HIF-1α modification by the PHDs is greatly decreased, resulting in its stabilization and accumulation [16].

Since the PHD isozymes play an integral role in oxygen homeostasis, inhibition of the HIF-1α prolyl hydroxylases is attractive from the perspective of developing pharmaceuticals for diseases, such as myocardial infarction, stroke, peripheral vascular disease, heart failure, diabetes, and anemia [18–20]. So far there are few reports which describe small molecule as an inhibitor of human HIF-1a prolyl hydroxylase [19–26]. Thus it is desirable to explore the hydroxylation mechanism of prolyl hydroxylases and select small molecule inhibitors of prolyl hydroxylase to regulate the homeostasis of HIF-1α. To date, small molecular inhibitors of HIF-1α prolyl hydroxylases were most 2OG analogues, such as pyruvate [27], oxaloacetate [27], N-oxalylglycine [28], dimethyloxalylglycine (DMOG) [29], and ethyl-3,4-dihydroxybenzoate (EDHB) [29]. Pyruvate and oxaloacetate are glycolytic and tricarboxylic acid cycle metabolites, respectively, belonging to endogenous 2-oxoacids. Pyruvate and oxaloacetate can prominently increase mRNA and protein levels of PHD3 and thus regulate a feedback loop featuring HIF and PHD homologues independent of hypoxia [27]. These analogues acted as competitive inhibitors of PHDs which can effectively stabilize HIF-1α and activate HIF mediated gene expression.

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PHDs are non-heme, Fe^{2+}-dependent redox enzymes, belonging to the 2-oxoglutarate (2OG) -dependent-oxygenase superfamily [10,16]. Fe^{2+} is bound to the enzyme to form a Fe^{2+}–enzyme complex, which sequentially binds 2OG and then the protein substrate (HIFα subunit) [16]. Substitution of a Fe-bound water molecular by dioxygen and subsequent decarboxylation of 2OG produce carbon dioxide, succinate and a ferryl ($Fe^{IV}=O$) species that mediate substrate hydroxylation [16,30–32]. Since the enzyme activity was dependent on exogenous Fe^{2+}, desferrioxamine (DFO, iron chelator) can influence the hydroxylation by Fe chelation [27]. Considering that a series of polynitrogen compounds have some biological functions and act as iron chelators, some kinds of polynitrogen compounds were selected as small molecule inhibitors of PHD3 to mediate the transcription of HIF-1α. In the hydroxylation mechanism, iron binding by the two-histidine, one carboxylate motif is relatively labile [16]. Fe^{2+} plays an important role in the hydroxylation of PHD3, forming stable complexes with polynitrogen compounds which leads to a decrease in the concentration of Fe^{2+} gradually. Thus, PHD3 is readily inhibited by these polynitrogen ligands (Table 1). These small molecules may be used as pharmaceuticals to treat many diseases induced by HIF-1α with great therapeutic effectiveness.

Treatment of human cardiac myocytes, smooth muscle cells, and endothelial cells with hypoxia or $CoCl_2$, a hypoxia mimic, resulted in a significant time-dependent increase in PHD3, but not PHD1 or PHD2, mRNA levels, which correlated with an increase in HIF-1a protein expression [33]. The same study revealed that PHD3 levels influence HIF-1a stability in both normoxic and hypoxic conditions, suggesting that PHD3 may participate in a feedback loop controlling HIF activity. PHD3 was proved inactive in fusion with maltose binding protein, after purification from *Escherichia coli* (*E. coli*) [34]. In addition, PHD3 has been found to aggregate when overexpressed in mammalian cells [35]. PHD3 has been the least studied isoenzyme in the PHD family because of its unstable activity [36]. In this study, we studied the expression and purification of soluble and active human PHD3 fused with a Trx·Tag in *E. coli* and selected four polynitrogen compounds as PHD3 inhibitors. The results showed that polynitrogen compounds (1–4) were the potent inhibitors of hydroxylation of HIF-1α peptide by recombinant human PHD3 in a noncompetitive manner. Their effects on the activity of recombinant human PHD3 were concentration-dependent. Combined with the apparent stability constants of these polynitrogen compounds with iron, the inhibition of enzyme activity was attributed to the forming stable coordination complexes.

2. Materials and methods

2.1. Materials

Expression host, *E. coli* BL21 (DE3) pLysS, and pET32α(+) vector were obtained from Novagen. Isopropyl β-D-thiogalactopyranoside (IPTG), 2-oxoglutarate, ascorbate, bovine serum albumin (BSA), dithiothreitol (DTT) and catalase were from Sigma. All other reagents were of analytical grade and all solutions were prepared using Milli-Q deionized water.

2.2. DNA manipulations

In order to increase the expression amount of protein, the *PHD3* gene was modified using synonymous codons according to the code bias of *E. coli*. The optimized *PHD3* gene was synthesized and inserted into the plasmid pUC57 by Jinsite biotechnology company. The optimized and original human PHD3 DNA sequences were aligned as shown in Fig. S1. The gene has a *Eco*RV restriction site at the 5′ end immediately before the start codon ATG and a sequence to encode the 6×His-tag and a *Sal*I site at the 3′ end immediately before and after the stop codon respectively, to facilitate the cloning of the *PHD3* gene into the expression vector, pET-32a(+). The modified human *PHD3* gene was inserted between the *Eco*RV and *Sal*I sites of the pET-32a vector to produce the expression plasmid pET-32a-*PHD3*. The expression host, *E. coli* BL21 (DE3) pLysS (Novagen), was transformed by the ligated plasmid by heat shock (42 °C, 30 s) and the colonies were selected on standard ampicillin-containing agar plates and confirmed by restriction enzyme analysis. The modified human *PHD3* gene was sequenced using the double-stranded dideoxy method (described by Sanger et al. [37]).

Table 1
Biological activity of polynitrogen ligands against PHD3.

Ligands	IC50 (μM)	K_i (μM)
Compound 1	29.5	67.0
Compound 2	16.0	25.3
Compound 3	12.8	67.3
Compound 4	60.4	82.1

2.3. Protein expression and purification

A starter culture of 4 mL Luria–Bertani broth (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter) supplemented with 4 μL 100 mg/mL ampicillin was incubated with a single colony of pET32α-*PHD3*, grown overnight in a shaker (37 °C 220 rpm). Later, the main culture volume of 200 mL Luria–Bertani broth, supplemented with 2 mL transformed cells and 200 μL ampicillin (100 mg/mL) was incubated at 25 °C in a shaker (220 rpm) until the optical density at 600 nm was 0.6–0.8, then was induced with 200 μL IPTG to a final concentration of 1 mM, grown for additional 4 h at 25 °C in a shaker (180 rpm). PHD3 was overexpressed in *E. coli* as a histidine-tagged fusion protein, and purified by nickel-affinity chromatography.

The cultured cells were centrifuged at 10,000 *g* for 10 min at 4 °C, resuspended in 8 mL of ice-cold 1× binding buffer (20 mM Tris–HCl buffer at pH 7.9 containing 0.5 M NaCl and 5 mM imidazole), sonicated on ice, and centrifuged at 14,000 g for 20 min. The postcentrifugation supernatant was filtered through a 0.45-μm membrane to prevent clogging of resins, and loaded onto a 2.5 mL nickel nitrilotriacetic acid (Ni-NTA) agarose column (Novagen) at a flow rate of about 20 mL/h. The Ni-NTA agarose column was pre-equilibrated with three column volumes of 1× binding buffer. After adsorption of recombinant human PHD3, the column was washed with ten column volumes of 1× binding buffer. Then the contaminated proteins were eluted with six column volumes of 1× wash buffer (20 mM Tris–HCl buffer at pH 7.9 containing 0.5 M NaCl and 60 mM imidazole), and the protein (recombinant human PHD3) was eluted with 6 column volumes of 1× elution buffer (20 mM Tris–HCl buffer at pH 7.9 containing 0.5 M NaCl and 1 M imidazole). The recombinant human PHD3 was dialyzed twice against sodium phosphate buffer (20 mM, pH 7.0) containing 2 M NaCl, 5 mM DTT to remove imidazole. The purified recombinant human PHD3 yielded a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the recombinant PHD3 was purified with affinity chromatography to approximately 95% purity. Protein concentration was measured by a Bradford assay based on a bovine serum albumin standard curve.

2.4. Analysis of recombinant human PHD3 by western blot

The mouse monoclonal anti-His-tag antibody (Tiangen Biotechnology) was used for the western-blot analysis of recombinant human PHD3. Purified recombinant human PHD3 was dissolved in 2×SDS-PAGE sample buffer and boiled for 10 min. Protein was resolved on 12% SDS-PAGE gel and electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in TBST buffer (10 mM Tris–HCl at pH 7.5, 150 mM NaCl and 0.05% Tween 20) for 1 h at room temperature, and was probed for 1 h at room temperature with mouse monoclonal anti-His-tag

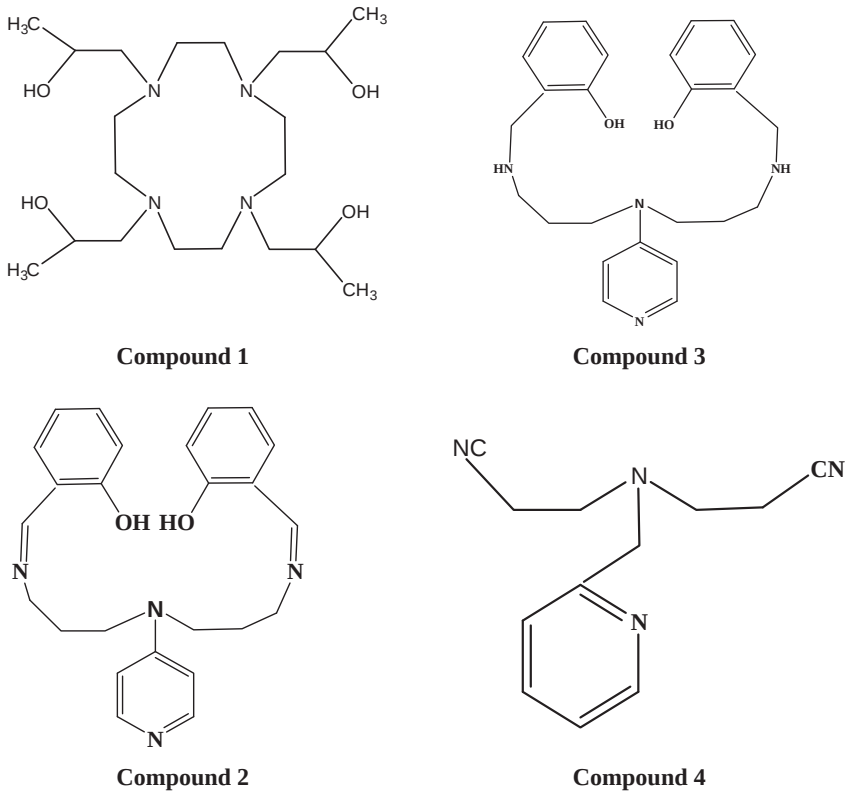


Fig. 1. Structure formulae of compounds 1–4.

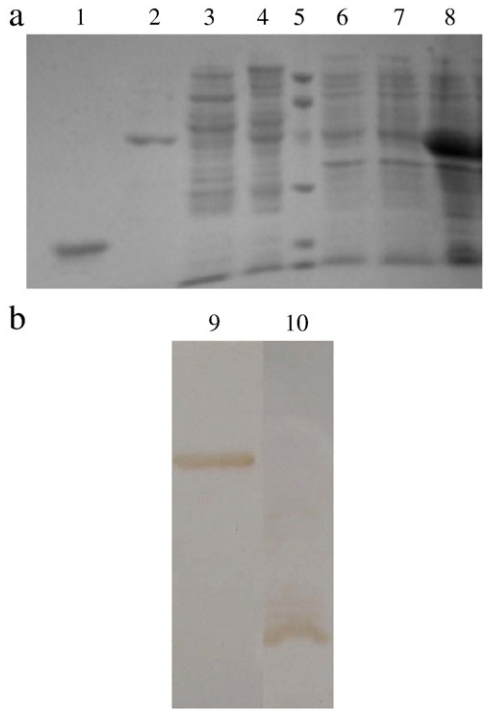


Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western-blot analysis of *E. coli* expressed human PHD3. a Coomassie blue stained 12% SDS-PAGE gel of fractions generated in the purification of recombinant human PHD3. b Western-blot analysis performed using a monoclonal antibody against the 6×His-tag at 1:1000 dilution. Lanes 1 and 10 the purified tag (Trx-HIS-S), Lanes 2 and 9 purified recombinant human PHD3, Lane 3 flow-through fraction, Lanes 4, 6, and 7 soluble fraction, Lane 5 marker, and Lane 8 total proteins of induced cells.

antibody (1:1000) that was diluted with 5% non-fat milk. Following incubation with horseradish peroxidase conjugated goat-antimouse IgG antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature, recombinant human PHD3 protein was visualized with diaminobenzidine as a substrate.

2.5. Analysis of recombinant human PHD3 and hydroxylation peptide by matrix-assisted laser desorption ionization, time-of-flight mass spectrometry (MALDI-TOF-MS)

Buffers and salts were removed of protein samples and reaction mixtures in accordance with the guide of ZipTip C_4 and C_{18} (Millipore), respectively. The tips were prewetted twice with wetting buffer (50% acetonitrile in water), and equilibrated twice with equilibration buffer (0.1% trifluoroacetic acid in water). The samples could then be applied

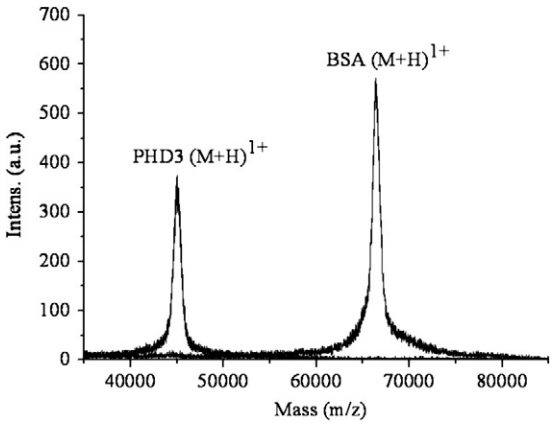


Fig. 3. Matrix-assisted laser desorption/ionization time of flight mass spectral analysis of purified recombinant human PHD3. The results are the average of three determinations. Bovine serum albumin (BSA) was used as a standard protein.

and the flow-through fraction could be disposed of. After they had been washed with two cycles of 0.1% trifluoroacetic acid and 5% methanol in a 0.1% trifluoroacetic acid/H_2O mixture, the samples were eluted into a new Eppendorf tube with 4 μL elution buffer (50% acetonitrile in water). During the process air pressure generated by the 10-μL pipette was used to drive the liquids through the resin bed. For MALDI-TOF-MS analysis the samples were prepared according to a dried droplet preparation protocol. The desalted reaction mixtures were mixed with an equal volume of a saturated a-cyano-4-hydroxycinnamic acid solution in 40% acetonitrile/60% H_2O containing 0.1% trifluoroacetic acid [23]. The desalted protein samples were mixed with an equal volume of a saturated sinnapinic acid solution in 40% acetonitrile/60% H_2O containing 0.1% trifluoroacetic acid [38]. One microliter was spotted on a polished steel target plate and allowed to air-dry prior to mass analysis. MS was performed using an autoflex II TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in reflector positive mode with a 19-kV acceleration voltage for peptides or in linear positive mode with a 20-kV acceleration voltage for proteins. Reported masses are the average of three separate determinations.

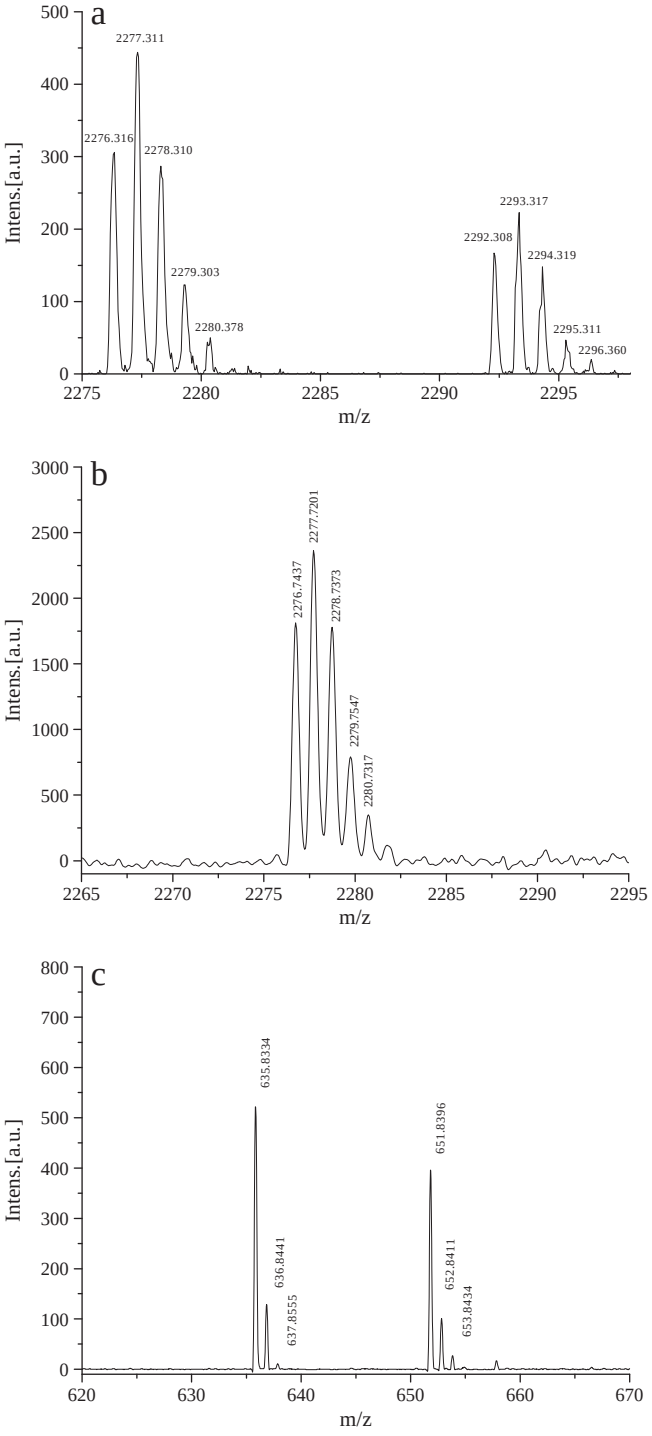


Fig. 4. Matrix-assisted laser desorption/ionization time of flight mass spectral analysis of the native and hydroxylated HIF-1α peptides and LEMLAP motif mutants (M to A). a MALDI-TOF-MS analysis of HIF-1α peptide catalyzed by recombinant human PHD3. The mass of native HIF-1α peptide was 2276.316 Da ($M + Na^+$). The mass of the resultant peptide product was 2292.308 Da, illustrating a mass increase of 16 Da corresponding to hydroxylation of the proline residue 564. b MALDI-TOF-MS analysis of HIF-1α peptide catalyzed by the tag (Trx-HIS-S). The proline residue 564 of HIF-1α peptide could not be hydroxylated by the tag (Trx-HIS-S). c MALDI-TOF-MS analysis of the peptide of LEALAP catalyzed by recombinant human PHD3. The mass of native peptide was 635 Da (LEALAP + Na^+), and the mass of the hydroxylated peptide was 651 Da (LEALAP-OH + Na^+).

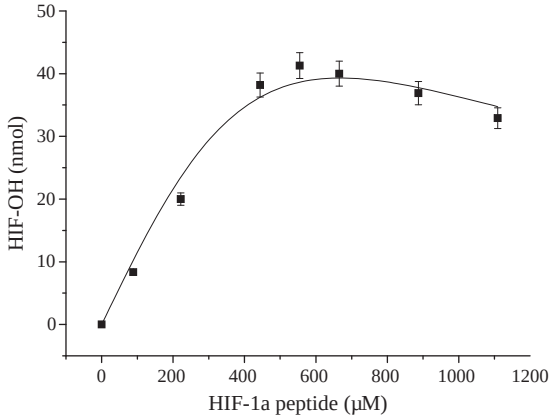


Fig. 5. Effect of concentrations of HIF-1α peptide on hydroxylation yields. The reaction mixture (100 μL), containing 14 μg PHD3, 1.0 mM DTT, 2.0 mM ascorbate, 50 μM $FeSO_4$, 0.16 mM 2-oxoglutarate, 2.0 mg/mL BSA, 0.60 mg/mL catalase, and HIF-1α peptide (0, 88.73, 221.83, 443.66, 554.57, 665.48, 887.31, and 1109.14 μM) in 20 mM phosphate buffer (pH 7.0) was incubated at 37 °C for 2 h. The data shown are means and standard deviations of three independent experiments. The lines show the least squares fit of Eq. (1) to the data.

2.6. Activity assay and selection of small molecule inhibitors

The HIF-1α peptide corresponding to residues 556–574 (DLDLEMLAPYIPMDDDFQL) was used as substrate in all activity assays [34,39].

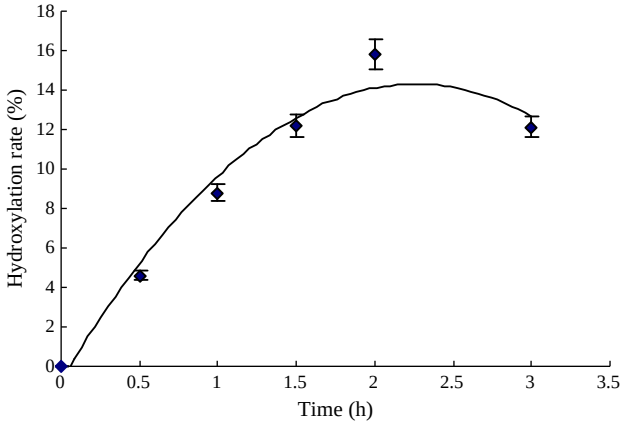


Fig. 6. Effect of incubation time on hydroxylation rate. The reaction mixture (100 μL), containing 14 μg PHD3, 1.0 mM DTT, 2.0 mM ascorbate, 50 μM $FeSO_4$, 0.16 mM 2-oxoglutarate, 2.0 mg/mL BSA, 0.60 mg/mL catalase, and 66.55 μM HIF-1α peptide in 20 mM phosphate buffer (pH 7.0) was incubated at 37 °C for 0, 30, 60, 90, 120, and 180 min. The data shown are means and standard deviations of three independent experiments.

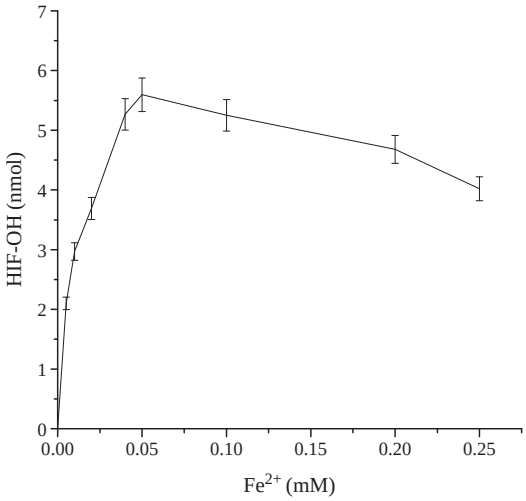


Fig. 7. Effect of concentrations of Fe^{2+} on hydroxylation yields. The reaction mixture (100 μL), containing 14 μg PHD3, 1.0 mM DTT, 2.0 mM ascorbate, $FeSO_4$ (0, 5, 10, 20, 40, 50, 100, 200, and 250 μM), 0.16 mM 2-oxoglutarate, 2.0 mg/mL BSA, 0.60 mg/mL catalase, and 66.55 μM HIF-1α peptide in 20 mM phosphate buffer (pH 7.0) was incubated at 37 °C for 2 h. The data shown are means and standard deviations of three independent experiments.

To optimize the reaction conditions, the reaction mixture (100 μL), containing 1.0 mM DTT, ascorbate (0, 0.1, 0.5, 1, 1.5, 2, 3, and 4 mM), $FeSO_4$ (0, 5, 10, 20, 40, 50, 100, 200, and 250 μM), 2OG (0, 0.04, 0.08, 0.12, 0.16, 0.20, 0.24, and 0.32 mM), 2.0 mg/mL BSA, 0.60 mg/mL catalase, HIF-1α peptide (0, 88.73, 221.83, 443.66, 554.57, 665.48, 887.31, and 1109.14 μM), and 14 μg PHD3, was incubated in a capped tube at 37 °C for 0, 30, 60, 90, 120, and 180 min. For the measurement of hydroxylation activity, the enzyme activity was assayed under standard conditions in a total volume of 100 μL containing 1.0 mM DTT, 2.0 mM ascorbate, 50 μM $FeSO_4$, 0.16 mM 2OG, 2.0 mg/mL BSA, 0.60 mg/mL catalase, 66.55 μM HIF-1α peptide, and 14 μg PHD3, pH 7.0. The samples were incubated at 37 °C for 2 h. The tag (Trx-HIS-S) and a mutant peptide (LEALAP, based on the LAMLAP motif) were used as controls in the hydroxylation assays. The reaction was quenched by boiling the samples for 5 min. Denatured proteins were removed by centrifugation and the supernatant of each sample was filtered though a 0.22-μm membrane. Peptide analyses by HPLC were carried out by using a gradient of acetonitrile from 5% to 60% in 0.05% trifluoroacetic acid on an Agilent C18 250×4.6 mm column at 1 mL/min. To determine the effects of four polynitrogen compounds (Fig. 1) on the activity of recombinant human PHD3, the hydoxylation of HIF-1α peptide was examined by recombinant human PHD3 in the absence and in the presence of the four polynitrogen compounds up to 200 μM.

The binding of polynitrogen compounds with Fe^{2+} was monitored by UV difference spectra under anaerobic conditions at room temperature. The different volumes of 500 μM Fe^{2+} were titrated into 700 μL polynitrogen compounds 1–4 (100 μM), respectively. The different titration curves from 190 nm to 400 nm were used to calculate the apparent stability constants.

3. Results and discussion

3.1. Expression and purification of recombinant human PHD3

The recombinant human PHD3 enzyme was expressed in *E. coli* with a Trx tag introduced at the N-terminus. SDS-PAGE and western-blot analyses of the recombinant human PHD3 in the cytoplasmic fraction of the IPTG induced cells revealed the presence of distinct bands with molecular masses of about 45 kDa. Factors that can influence the expression of protein PHD3 were studied, temperature, induction time, OD_{600} and IPTG concentration, for instance. The condition resulting in the best level of protein expression was 37 °C for 3 h. However, the recombinant human PHD3 protein was almost found in the inclusion bodies when the growth and the induction temperature were 37 °C, and recombinant protein purified from inclusion bodies was not isolated in catalytically active form (data not presented). The culture conditions for the highest expressed activity were 5 h of incubation at 25 °C.

Culture conditions and the vector all contribute to modulating the proportion of soluble and insoluble forms of arsenic methyltransferase, β-galactosidase, phospholipase A1, and so on [38,40–43]. Low induction temperature decreases the rate of protein synthesis and tends to raise the percentage of target protein found in soluble form [44]. Growth at 37 °C causes some proteins to accumulate as inclusion bodies, while incubation at 30 °C leads to a soluble, active protein [45]. Growth and induction at 25 °C were optimal for the expression of a soluble and active form of recombinant human PHD3. The Trx•Tag™ fusion tag is a highly soluble polypeptide that can potentially enhance solubility of

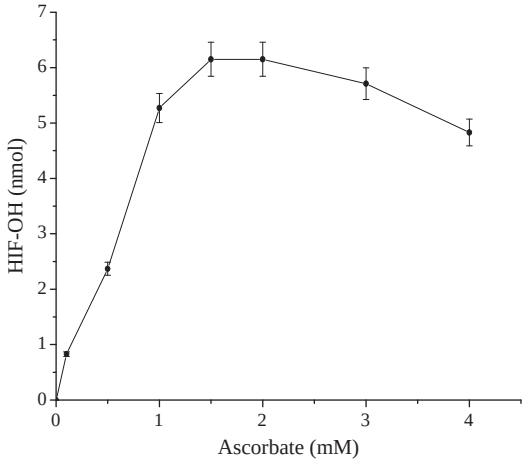


Fig. 8. Effect of concentrations of ascorbate on hydroxylation yields. The reaction mixture (100 μL), containing 14 μg PHD3, 1.0 mM DTT, ascorbate (0, 0.1, 0.5, 1, 1.5, 2, 3, and 4 mM), 50 μM $FeSO_4$, 0.16 mM 2-oxoglutarate, 2.0 mg/mL BSA, 0.60 mg/mL catalase, and 66.55 μM HIF-1α peptide in 20 mM phosphate buffer (pH 7.0) was incubated at 37 °C for 2 h. The data shown are means and standard deviations of three independent experiments.

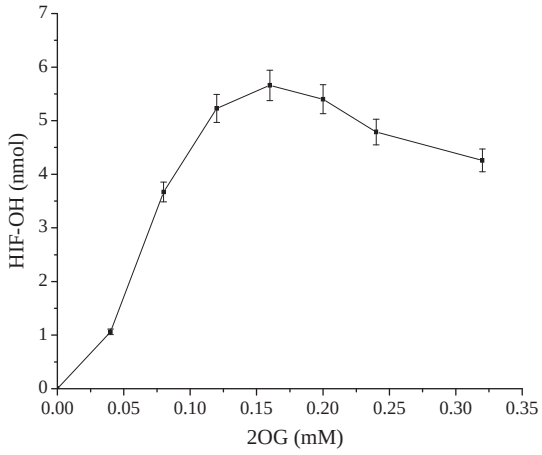


Fig. 9. Effect of concentrations of 2-oxoglutarate on hydroxylation yields. The reaction mixture (100 μL), containing 14 μg PHD3, 1.0 mM DTT, 2 mM ascorbate, 50 μM $FeSO_4$, 2-oxoglutarate (0, 0.04, 0.08, 0.12, 0.16, 0.20, 0.24, and 0.32 mM), 2.0 mg/mL BSA, 0.60 mg/mL catalase, and 66.55 μM HIF-1α peptide in 20 mM phosphate buffer (pH 7.0) was incubated at 37 °C for 2 h. The data shown are means and standard deviations of three independent experiments.

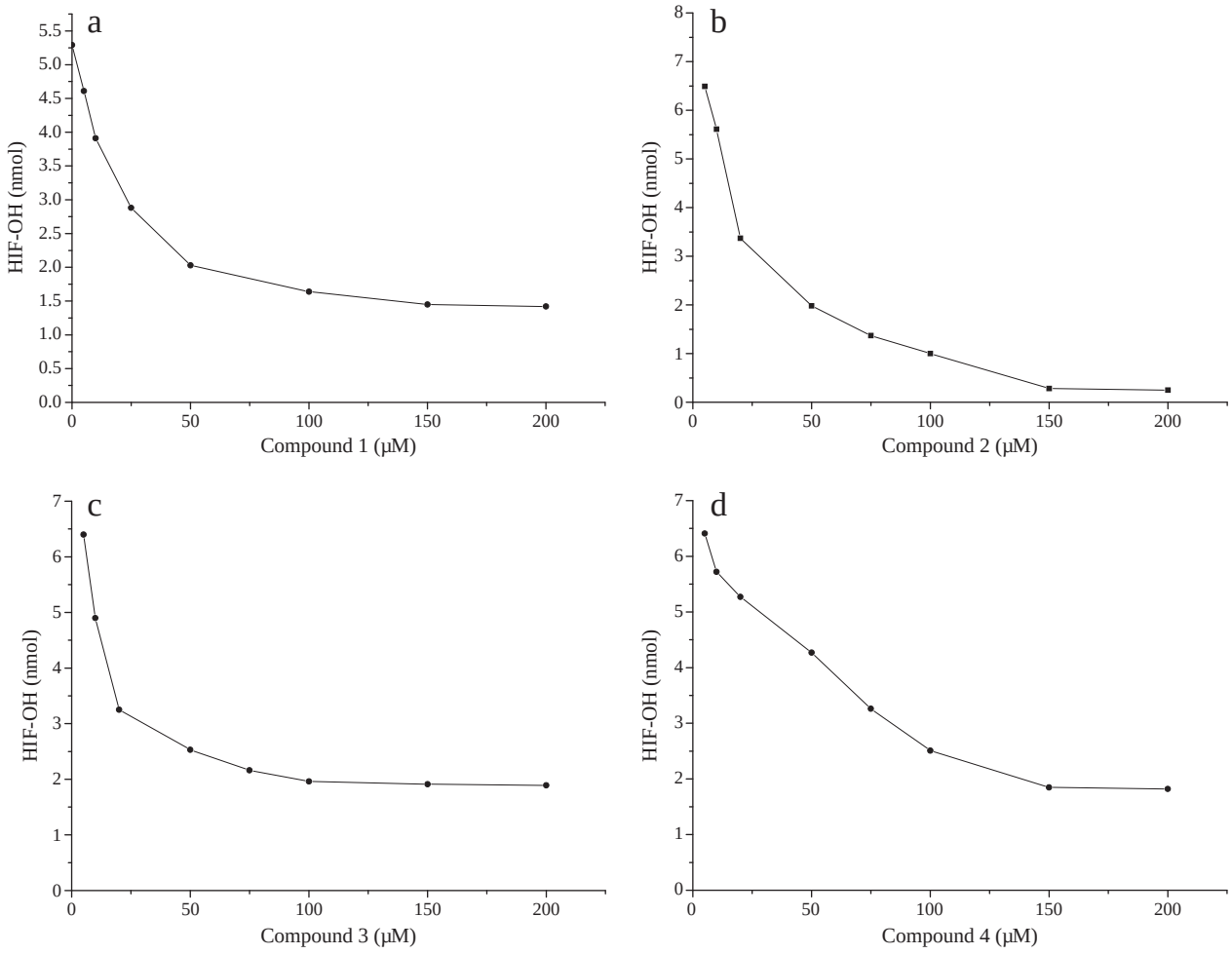


Fig. 10. Modulation of hydroxylation activity of recombinant human PHD3 by polynitrogen compound 1–4 (a–d). The reaction mixture (100 μL), containing 14 μg PHD3, 1.0 mM DTT, 2.0 mM ascorbate, 50 μM $FeSO_4$, 0.16 mM 2-oxoglutarate, 2.0 mg/mL BSA, 0.60 mg/mL catalase, and 66.55 μM HIF-1α peptide in 20 mM phosphate buffer (pH 7.0) was incubated at 37 °C for 2 h.

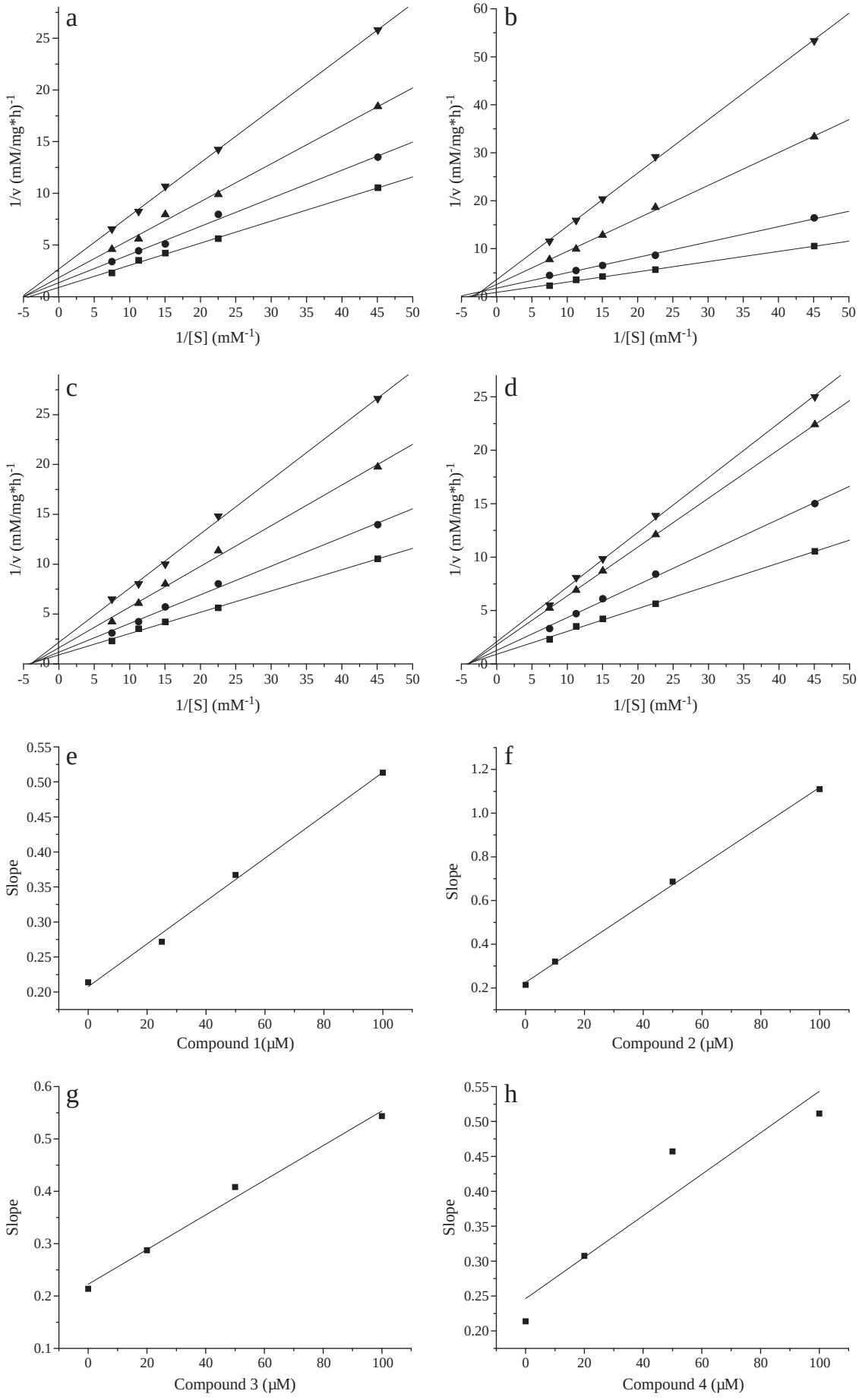
target proteins. Many proteins that are normally produced in an insoluble form in *E. coli* tend to become more soluble when fused with the N-terminal thioredoxin (Trx•Tag) sequence [46]. When pET-32a(+) vector designed for cytoplasmic expression is used, the Trx•Tag not only enhances the solubility of many target proteins, but also catalyzes the formation of disulfide bonds in the cytoplasm [47]. The combination of low expression temperature and coexpression of thioredoxin produced maximum levels of soluble, active, properly folded recombinant human PHD3.

The catalytically active recombinant human PHD3 was purified by Ni-NTA agarose affinity chromatography. The purity of the purified recombinant human PHD3 was above 94%. Fig. 2 shows a representative SDS-PAGE gel and western blot (using a monoclonal antibody against the 6×His-tag) in which the supernatant and purified recombinant human PHD3 appear as single bands with an apparent molecular mass of 45 kDa. The mass of recombinant human PHD3 was determined to be 45,651 ± 31 Da. The human PHD3 cDNA had a 717-bp open reading frame that encoded a 239 amino acid protein [36]. The recombinant human PHD3 in this study was a fusion protein which had a Tag (Trx-HIS-S) in the N-terminal and a His·Tag in the C-terminal. Thus, the calculated molecular mass for the recombinant human PHD3 is 45,642 Da. The results of molecular mass measurements performed by MALDITOF-MS confirmed the identity of our purified protein as recombinant human PHD3 (Fig. 3).

3.2. Optimization of the hydroxylation condition of HIF-1α peptide by recombinant human PHD3

The recombinant human PHD3 could hydroxylate the proline of the HIF-1α peptide to form hydroxyproline in vitro. Mass spectrometry analysis of the resultant peptide product indicated a mass increase of 16 daltons, consistent with hydroxylation of the proline residue [10] (Fig. 4a). However, the tag (Trx-HIS-S) could not hydroxylate the proline of the HIF-1α peptide, and the result was shown in Fig. 4b. The recombinant human PHD3 could catalyze the proline of LEALAP (635 Da, LEALAP + Na^+) to form the hydroxylated peptide (651 Da, LEALAP-OH + Na^+) (Fig. 4c). The native and hydroxylated HIF-1α peptide can be separated by HPLC using a gradient of acetonitrile from 5% to 60% in 0.05% trifluoroacetic acid. The retention time of native and hydroxylated HIF-1α peptide was 28.67 min and 29.18 min,

Fig. 11. Double reciprocal plots for the inhibitor of the recombinant human PHD3 by polynitrogen compounds 1–4. Double-reciprocal plots for Compound 1 (a), Compound 2 (b), Compound 3 (c), and Compound 4 (d), respectively. Compound 1: Plots for (■) 0 μM, (●) 25 μM, (▲) 50 μM, and (▼) 100 μM. Compound 2: Plots for (■)0 μM, (●)10 μM, (▲)50 μM, and (▼)100 μM. Compound 3: Plots for (■) 0 μM, (●) 20 μM, (▲) 50 μM, and (▼) 100 μM. Compound 4: Plots for (■) 0 μM, (●) 20 μM, (▲) 50 μM, and (▼) 75 μM. Replot of slopes of the corresponding double reciprocal plots versus concentrations of inhibitors for Compound 1 (e), Compound 2 (f), Compound 3 (g), and Compound 4 (h), respectively. The reaction mixture (100 μL), containing 14 μg PHD3, 1.0 mM DTT, 2.0 mM ascorbate, 50 μM $FeSO_4$, 0.16 mM 2-oxoglutarate, 2.0 mg/mL BSA, 0.60 mg/mL catalase, and 22.18, 44.36, 66.55, 88.72, and 133.10 μM HIF-1α peptide in 20 mM phosphate buffer (pH 7.0) was incubated at 37 °C for 2 h.


a
1/v (mM/mg*h)-1
1/[S] (mM-1)
b
1/v (mM/mg*h)-1
1/[S] (mM-1)
c
1/v (mM/mg*h)-1
1/[S] (mM-1)
d
1/v (mM/mg*h)-1
1/[S] (mM-1)
e
Slope
Compound 1(μM)
f
Slope
Compound 2 (μM)
g
Slope
Compound 3 (μM)
h
Slope
Compound 4 (μM)

respectively (Fig. S2). The generation of hydroxylated products increased with the concentration of HIF-1α peptide from 0 to 665.48 μM. The HIF-1α peptide concentration range investigated here clearly showed substrate inhibition of rate by HIF-1α peptide (Fig. 5). The rate of hydroxylation in the presence of noncompetitive substrate inhibition is defined in Eq. (1):

$$V = [S]\mathrm{Vmax} / \left[K_M + S + \left(S^2 / K_I\right)\right] \quad (1)$$

where V is the initial velocity of the reaction (mmol/(h mg protein)), [S] the substrate (HIF-1α peptide) concentration (μM), Vmax the maximal velocity of the reaction (mmol/(h mg protein)), K_M the Michaelis constant for HIF-1α peptide (μM), and K_I is the inhibition constant for HIF-1α peptide (μM) [48]. The kinetic parameters estimated by fitting the Eq. (1) are followed with apparent K_M and Vmax of 0.169 mM and 1.418 mmol/(mg h), respectively. Moreover the hydroxylation rate increased with the incubation time, reaching a maximum value at 2 h, and then decreased (Fig. 6).

Fig. 7 showed that the maximal rate of hydroxylation occurred with about 50 μM Fe^{2+}. When the concentration of Fe^{2+} was above 50 μM, the hydroxylation decreased gradually with the increasing concentrations of Fe^{2+}. The generation of hydroxylated HIF-1α initially increased with the concentration of ascorbate, reaching a maximum value at 2 mM, and then decreased (Fig. 8). PHD3 reached the maximal rate when the concentration of 2OG was about 160 μM, however, the higher concentrations of 2OG inhibited the hydroxylation reaction (Fig. 9).

PHD3 belongs to Fe^{2+}, 2OG dependent oxygenase family [16,30–32]. Fe^{2+} plays an important role in the whole hydroxylation system, and there will be no decarboxylation of 2OG in the absence of Fe^{2+}. Nicholas reported that downregulation of HIF-1α prolyl hydroxylation correlated with a decrease in intracellular iron(II) [49]. Ascorbate may be another co-factor requirement of some 2OG-dependent oxygenases that appears to be shared by the PHDs to incite the hydroxylation to HIF-1α peptide. In the hydroxylation, the ascorbate can mediate iron redox status from Fe^{III} to Fe^{II}. In the case of prolyl hydroxylase, ascorbate oxidation (either directly to dehydroascorbate or via the semi dehydroascorbate radical) is required to complete cycles in which oxidation of 2OG to succinate is uncoupled from that of substrate oxidation, leaving an oxidized ($Fe^{IV}=O$) intermediate unable to return to the reduced form and complete the catalytic cycle [12,13,50–55]. Flashman reported that the initial rate and extent of hydroxylation were increased in the presence of ascorbate in PHD2-catalyzed hydroxylation of two prolyl hydroxylation sites in human HIF-1α, and FIH-catalyzed hydroxylation of asparaginyl hydroxylation sites in HIF-1α and in a consensus ankyrin repeat domain peptide [56]. When ascorbate was replaced with structural analogues, the results revealed that the ascorbate side chain was not important in its contribution to HIF hydroxylase catalysis, whereas modifications to the ene-diol portion of the molecule negated the ability to promote hydroxylation. On the other hand, ascorbate may prevent oxidation of ferrous iron by dissolved oxygen [57]. To date, the actual role of ascorbate is not very clear, therefore, the role of ascorbate will be studied further.

3.3. Effects of polynitrogen compounds 1–4 on the activity of recombinant human PHD3

Polynitrogen compounds 1–4 inhibited the enzymatic hydroxylation of HIF-1α peptide in a concentration-dependent manner and the IC_{50} values were 29.5, 16.0, 12.8, and 60.4 μM, respectively (Fig. 10). Kinetic analysis characterized the inhibition of recombinant human PHD3 by these compounds over a range of HIF-1α peptide concentrations from 22.18 to 133.10 μM. Double-reciprocal (1/V versus 1/[HIF-1α peptide]) plots showed these compounds to be noncompetitive inhibitors of the hydroxylation of HIF-1α peptide by recombinant human PHD3 (Fig. 11a–d). The Ki values of 67.0, 25.3, 67.3, and 82.1 μM were obtained from the replot of the slopes of the corresponding double-reciprocal plots versus concentration of polynitrogen compounds 1–4 (Fig. 11e–h).

The apparent stability constants for the complexes were measured by UV difference spectra. Fig. S3a shows that compound 1 has an absorption peak at 266 nm. With the increasing concentrations of Fe^{2+}, the intensities of the absorption peak of compound 1 decreased gradually. In the complexes of the polynitrogen compounds 1–4, the ratio of ligand and Fe is 1:1 according to the crystal structures (unpublished data). The apparent stability constant of compound 1 for Fe^{2+} was deduced to be $3.4\times10^3\ M^{-1}$ according to the formula. In the same way, the apparent stability constants of other three complexes are $4.0\times10^3\ M^{-1}$, $4.2\times10^3\ M^{-1}$ and $2.5\times10^3\ M^{-1}$, respectively. Compared to the apparent stability constants of the complexes, the value of IC_{50} is lower when the apparent stability constant of a complex is higher. These results show that the polynitrogen compounds 1–4 can inhibit the activity of recombinant human PHD3 through their binding with Fe^{2+}.

Since the PHD isozymes play an integral role in oxygen homeostasis, their inhibition is attractive from the perspective of developing pharmaceuticals that induce a pro-angiogenic response for the treatment of, for example, heart disease [19]. There are mainly triformed inhibitors including 2OG analogues, small polypeptide mimic molecules and iron chelators. Macrocyclic saturated tetraamines such as a 12-membered cyclen (1, 4, 7, and 10-tetraazacyclododecane) and a 14-membered cyclam (1, 4, 8, and 11-tetraazacyclotetradecane) have long been studied from a chemical point of view [58], but biological interest has also been growing [59–62]. Furthermore, some polynitrogen complexes were measured by the same method with the polynitrogen compounds 1 and 4. The results illustrated that these complexes are not effective inhibitors of recombinant human PHD3. On the other hand, polynitrogen compounds 1–4 were the potential inhibitors of recombinant human PHD3. The 2OG-dependent-oxygenase family is the largest of several families of non-heme, Fe^{2+}-dependent enzymes that use a conserved two-histidine, one carboxylate motif to bind Fe^{2+} at the catalytic site. Mutational studies of both PHDs and FIH have confirmed the assignment and crucial importance of the predicted Fe^{2+}-coordinating residues [10,63]. In the hydroxylation mechanism, iron binding by the motif is relatively labile [16]. When iron is dissociated from the enzyme active site because of the chelating effect of the polynitrogen compounds, PHD3 may be inactivated by conversion of PHD3–Fe^{2+} complex into PHD3, which is similar to the conversion of holoenzyme into apoenzyme. If the conversion equilibrium is reversible, the inhibition of PHD3 by the polynitrogen compounds is the same as the reversible coordination equilibrium between the polynitrogen compounds and Fe^{2+}, otherwise the inhibition is irreversible. Our results show that these compounds are noncompetitive inhibitors of the recombinant human PHD3, so we draw the conclusion that the inhibition of PHD3 by the polynitrogen compounds is reversible. Indeed, the inhibition may result from binding of the inhibitor to the enzyme or from complexation of iron in the solution. Studies with FIH and other 2OG-dependent oxygenases indicate that, in most cases, the binding of 2OG to the active site occurs to form the 2OG–Fe^{2+}–enzyme complex [16]. If the polynitrogen compounds are bound to Fe^{2+} in the active site, forming enzyme–Fe^{2+}–inhibitor complex, the compounds could be classified as 2OG analogues. As is known polynitrogen compounds are perfect ligands which can form various complexes with kinds of metal ions, such as Fe, Cu, and Co. Our results also show that the value of IC_{50} is lower when the apparent stability constant of the complex is higher. Considering the relationship between the apparent stability constant and IC_{50}, the four polynitrogen compounds were classified as iron chelators. Fe^{2+} plays an important role in the hydroxylation of PHD3, forming stable complexes with polynitrogen compounds which leads to

a decrease in the concentration of Fe^{2+} gradually. Thus, PHD3 is readily inhibited by these polynitrogen ligands.

4. Conclusion

Under optimal conditions, soluble and active recombinant human PHD3 was expressed in *E. coli* with a Trx tag introduced at the N-terminus. Four novel inhibitors of human PHD3 were selected. The results showed that these polynitrogen compounds were noncompetitive inhibitors of the hydroxylation of HIF-1α peptide by the recombinant human PHD3. The inhibition mechanism of these polynitrogen compounds was attributed to their binding to iron to form stable coordination complexes.

Abbreviations

HIF-1α hypoxia inducible factor 1α
PHD3 prolyl hydroxylase 3
2OG 2-oxoglutarate
IPTG isopropyl β-D-thiogalactopyranoside
BSA bovine serum albumin
MALDI-TOF-MS matrix-assisted laser desorption ionization, time-of-flight mass spectrometry
VEGF vascular endothelial growth factor
EPO erythropoietin
DTT dithiothreitol

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jinorgbio.2010.12.001.

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