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Investigating the Dependence of the Hypoxia-Inducible Factor Hydroxylases (Factor Inhibiting HIF and Prolyl Hydroxylase Domain 2) on Ascorbate and Other Reducing Agents

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Abbreviations: ARD, ankyrin repeat domain; CAD, *C*-terminal transactivation domain; CODD, *C*-terminal oxygen dependent degradation domain; FIH, factor inhibiting HIF; HIF, hypoxia inducible factor; IPAA, isopropylidene-L-ascorbate; LAA, L-ascorbic acid; MALDI-TOF MS, matrix-assisted laser desorption/ionisation time of flight mass spectrometry; NODD, *N*-terminal oxygen dependent degradation domain; PHD, prolyl hydroxylase domain.

SYNOPSIS

The hypoxia inducible factor (HIF) hydroxylases (prolyl hydroxylases, which in humans are PHD isoforms 1-3, and factor inhibiting HIF, FIH) regulate HIF levels and activity. These enzymes are Fe(II)/2-oxoglutarate dependent oxygenases, many of which are stimulated by ascorbate. We have investigated the ascorbate dependence of PHD2-catalysed hydroxylation of two prolyl hydroxylation sites in human HIF-1α, and of FIH-catalysed hydroxylation of asparaginyl hydroxylation sites in HIF-1α and in a consensus ankyrin repeat domain peptide. The initial rate and extent of hydroxylation was increased in the presence of ascorbate for each of these reactions. 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. We investigated whether alternative reducing agents (glutathione and dithiothreitol) could be used to promote HIF hydroxylase activity, and found partial stimulation of hydroxylation in an apparently enzyme and substrate specific manner. The results raise the possibility of developing reducing agents targeted to specific HIF hydroxylase-catalysed reactions.

INTRODUCTION

The non-heme Fe(II) and 2-oxoglutarate (2OG) dependent oxygenases are an extended enzyme family, present in most, if not all life forms. All available structures for these enzymes have a conserved double stranded beta helix core fold, which supports a highly, but not universally, conserved HXD/E…H triad of residues that is responsible for coordinating a single ferrous ion at the active site[1]. 2OG oxygenases catalyse a very wide range of oxidative reactions[1]; in humans, they are involved in a number of biological processes, ranging from DNA repair to collagen biosynthesis[2]. Their proposed consensus mechanism involves initial binding of Fe(II), followed by bidentate coordination of 2OG to the Fe(II), and proximate binding of the 'prime' substrate. This causes a structural change that stimulates binding of oxygen to the Fe(II). Oxidative decarboxylation of 2OG to give succinate then occurs, concomitant with formation of a Fe(IV)-oxo species, which effects the oxidation of the prime substrate (for review see[3, 4]). In the absence of prime substrate, or with a suboptimal substrate, many 2OG oxygenases are able to catalyse 'uncoupled' turnover of 2OG to succinate[4].

The 2OG oxygenases show varying degrees of dependence upon L-ascorbic acid (LAA), however its in vivo contribution to catalysis for most enzymes remains unclear. The role of LAA has been studied in some detail in the case of the collagen prolyl hydroxylases. Prolyl-4-hydroxylation catalysed by these enzymes stabilizes the collagen triple helix[5]; lack of ascorbate can inhibit this process and is linked to the disease scurvy. In the absence of ascorbate, the collagen prolyl hydroxylases can initially catalyse prolyl hydroxylation at a maximal rate. However, 2OG turnover that is not coupled to substrate hydroxylation is proposed to result in catalytically inert oxidised iron species and enzyme inactivation; ascorbate is proposed to reduce the oxidised iron species, thus maintaining the enzymes in their active form and promoting their full activity[6-8].

The hypoxia inducible transcription factor (HIF) plays an important role in the metazoan response to low oxygen levels (hypoxia)[9, 10]. HIF is a heterodimeric transcription factor; in human cells, levels of the HIF-α domain are regulated in an oxygen-dependent manner by four enzymes, known as the HIF hydroxylases[11-14]. These enzymes are members of the 2OG oxygenase family. Under normal oxygen conditions, the prolyl hydroxylases (PHDs 1-3) catalyse hydroxylation of two prolyl residues in HIF-α (in HIF-1α, Pro-402 and Pro-564 in the *N*- and *C*-terminal oxygen dependent degradation domains, NODD and CODD, respectively), targeting them for ubiquitination and degradation by the proteasome[15, 16]. Factor Inhibiting HIF (FIH) catalyses asparaginyl hydroxylation (Asn-803 in the *C*-terminal activation domain, CAD, of HIF-1α), a modification which prevents interaction of HIF-α with the co-transcriptional activator, p300[17]. When oxygen is limiting, HIF hydroxylase activity drops, HIF-α levels rise, and it is able to dimerise with HIF-β, enabling the upregulation of genes involved in the response to hypoxia, e.g. vascular endothelial growth factor and erythropoietin (for reviews see [18-20]). The HIF hydroxylases are therefore proposed to act as oxygen sensors.

The PHDs have been reported to be dependent on LAA for maximal activity, in both work with isolated enzymes[16, 21, 22], and in mammalian cell cultures, where apparently physiologically relevant LAA levels (25μM) were found to suppress HIF-1α levels by promoting HIF hydroxylase activity [23], particularly in oncogenically activated normoxic cells. Cells in which LAA levels are depleted, e.g. those from L-gulono-γ-lactone oxidase knockout mice (which cannot synthesise LAA)[24], those

supplemented with angiotensin II [25], or those with elevated nickel or cobalt ions[26], have been observed to have upregulated HIF-1α protein levels. These effects are proposed to be due to inhibition of HIF hydroxylase activity, and provide indirect evidence as to the importance of LAA for proper hypoxic regulation. The depletion of LAA levels in normoxic cancer cells may be significant in the HIF upregulation observed in many of these cell types[27]. Whilst there have been associations made between high dose LAA treatment and cancer survival [28], their relevance is controversial [29], and no association has been made to date with these observations and its effect on the HIF hydroxylases.

Analysis of the effects of reducing agents on 2OG oxygenase catalysis, even in vitro, is relatively complex because of the possibility of various redox processes involving Fe(II), LAA, thiols, and oxygen [30, 31]. The role of LAA in promoting HIF hydroxylase activity has been proposed to be via either the completion of uncoupled turnover cycles (as for collagen prolyl hydroxylase), or via the maintenance of an intracellular Fe(II) pool to replenish the active site of these enzymes [23, 32]. Given the apparent physiological importance of LAA, we have carried out investigations aiming to test the selectivity of LAA in *in vitro* HIF hydroxylase catalysis, by using LAA analogues and alternative reducing agents with the objective of investigating which functional features are necessary for promotion of activity. We have focussed on PHD2, the most important of the prolyl hydroxylases under normoxic conditions[33, 34] and FIH, which acts as both a HIF hydroxylase[13] and an ankyrin repeat domain hydroxylase[35, 36], and for which LAA dependence has not previously been thoroughly investigated. We were interested to test the possibility that individual hydroxylation reactions catalysed by specific HIF hydroxylases might be selectively enhanced by particular reducing agents. Our results suggest that the requirement for LAA is not necessarily exclusive in determining HIF hydroxylase activity, and that it may be possible to selectively activate the HIF hydroxylases towards specific substrates.

EXPERIMENTAL DETAILS

Materials

Chemicals were from Sigma-Aldrich, with the exception of matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS) matrices, matrix buffers and calibrants (LaserBioLabs), and the peptide substrates HIF-1$\alpha_{556-574}$ (CODD, DLDLEMLAPYIPMDDDFQL), HIF-1$\alpha_{395-413}$ (NODD, DALTLLAPAAGDTIISLDF) and HIF-1$\alpha_{788-806}$ (CAD, DESGLPQLTSYDCEVNAPI) (Peptide Protein Research Ltd.).

Synthesis of 1CA, the synthetic ankyrin peptide

The peptide corresponding to a 'consensus' ankyrin repeat fragment substrate for FIH[37, 38] (1CA, HLEVVKLLLEAGADVNAQDK) was synthesised on a CSBio Peptide Synthesiser using aminomethylstyrene resin (0.05 mol, Polymer Labs) and rink amide linker technology (CSBio). The peptide was cleaved from the resin using trifluoroacetic acid (TFA) and purified by reverse phase-HPLC (Monolithic C18-silica column, Phenomenex) (gradient 0%-60% acetonitrile (with 0.1% TFA) in water (with 0.1% TFA)). MALDI-TOF-MS was used to identify fractions containing the desired peptide.

PHD2 and FIH purification

The catalytic domain of human PHD2$_{181-426}$ (termed PHD2 throughout) was expressed in *Escherichia coli* BL21 (DE3) cells, and purified by cation exchange and size exclusion chromatography, as reported[22]. Recombinant human FIH (with an *N*-terminal hexa-histidine tag) was produced in *E. coli* BL21 (DE3) cells, and purified by nickel affinity chromatography and size exclusion chromatography, as reported[13]. Both enzymes were shown to be >95% pure by SDS-PAGE analysis.

Hydroxylation assays

Time course assays for PHD2 were carried out in the absence/presence of LAA/analogues, by preparing a reaction mix in 50mM Tris-HCl pH 7.5 containing PHD2 (4μM), NODD/CODD substrate (200μM), 2-oxoglutarate (300μM), NH_4FeSO_4 (50μM) and LAA/analogue (4mM). Reactions were carried out at 37°C in a shaking incubator, with samples removed and quenched using cold 0.1% formic acid at regular time intervals. Stopped reaction samples were placed immediately at -20°C. Time course FIH assays were carried out in a similar fashion, except substrates were present to 1mM. All assays were performed at least in triplicate.

MALDI-TOF MS Analysis

The extent of substrate hydroxylation from the time course assays was analysed by MALDI-TOF MS. Recrystallised α-cyano-4-hydroxycinnamic acid (CHCA) MALDI matrix (1 μL) and quenched assay solution (1 μL) were 'double spotted' onto a 96 well MALDI sample plate, and analysed using a Waters Micromass™ MALDI micro MX™ mass spectrometer in negative ion reflectron mode. Instrument parameters: Laser energy 141%, Pulse 2400V, Detector 2700V, Suppression 1500. Data were analyzed using MassLynx™ v4.1.

LAA/Analogue K_M determination

Assay mixtures were prepared as described above, with LAA/analogue concentrations ranging from 0-4mM. Reactions were allowed to proceed for 8

minutes before quenching and analysis of hydroxylation levels by MALDI-TOF MS as described above. Data were plotted, and the Michaelis-Menten equation fitted, using SigmaPlot 2000. All assays were performed at least in triplicate.

RESULTS

LAA significantly enhances the hydroxylation of both NODD and CODD HIF-1α substrates by PHD2

Although LAA is not essential for PHD2 catalysis, the activity of this enzyme has been shown to be increased in the presence of this reducing agent in isolated proteins[16, 22], insect cell extracts[21] and in cultured mammalian cells[23]. None of these studies directly measured the LAA dependence of hydroxylation. We therefore carried out PHD2 hydroxylation assays to quantify the proportion of substrate hydroxylation by MALDI-TOF MS over time, in the presence and absence of LAA. The effect of LAA on hydroxylation of both HIF-1$\alpha_{556-574}$ (CODD) and HIF-1$\alpha_{395-413}$ (NODD) peptide substrates was measured.

Typical MALDI-TOF MS spectra resulting from these assays at selected time points are shown in Supplementary Information S1. Figure 1 compares the effect of LAA on the extent of hydroxylation of both NODD and CODD over time, and clearly demonstrates that hydroxylation of NODD and CODD increases in the presence of LAA, under standard assay conditions. LAA (at 4 mM) increases both the extent of hydroxylation (64% compared to 15% for CODD, 63% compared to 2% for NODD), and the initial rate of hydroxylation (0.12 μM/s compared to 0.05 μM/s for CODD, 0.16 μM/s compared to 0.01 μM/s for NODD). These results are consistent with previous data for PHD2[16, 21-23]. Apparent $K_{m,LAA}$ values were determined for each reaction (Table 1) and were found to be similar (~50 μM) for the reaction of PHD2 with both NODD and CODD. Although the levels of hydroxylation were reduced, stimulation of hydroxylation was still observed at iron (II) concentrations below 50 μM (i.e. 10 and 25 μM), which are likely more representative of the physiological iron (II) levels (Supplementary Information S2). The addition of catalase to the standard incubation conditions (to remove potential hydrogen peroxide which could lead to inhibitory reactive oxidising species generated via the Haber-Weiss reaction), did not increase the extent of hydroxylation (Supplementary Information, S2).

Structural analogues of LAA reveal that the ene-diol portion of the molecule is important in promoting PHD2 activity

In order to investigate which features of the LAA molecule are important in stimulation of PHD2 activity, a set of analogues was substituted for LAA in PHD2 hydroxylation assays (Scheme 1, (ii)-(vii)). The effects of side chain stereochemistry and hydrophobicity were studied using D-isoascorbate and isopropylidene-L-ascorbate (IPAA), respectively. The significance of the ene-diol group with respect to reduction was examined using L-gulonic-γ-lactone, which lacks an ene-diol group (and associated reducing properties) but has a *cis* 1,2-diol group. The importance of the ene-diol with respect to enzyme binding was probed using a sulphate analogue (v); it was envisioned that replacement of one of the hydroxyl groups of the ene-diol with a sulphate group should block binding to this functionality as well as diminishing its reducing capacity by withdrawing electron density. Dehydroascorbate (vi) was studied because it is an oxidation product of LAA. 3,4-Dihydroxyphenylacetic acid (vii) contains an ene-diol group within a planar ring, similar in dimensions to the planar lactone ring containing the ene-diol moiety in LAA.

Figure 2 shows that the LAA analogues with altered side chains, D-isoascorbate (Scheme 1, (ii)) and IPAA (Scheme 1, (iii)), were able to replace LAA in promoting maximal PHD2 activity towards both CODD (Figure 2A) and NODD (Figure 2B) substrates (K_m values for these analogues were also similar to those seen with LAA,

Table 1, with K_m values for IPAA actually lower than for LAA itself). These results suggest that a highly specific binding pocket for LAA is unlikely. Of note, in a study showing that addition of LAA promotes degradation of HIF-α in human prostate cancer cells [23], addition of IPAA was also shown to result in degradation of HIF-α, albeit with higher levels of IPAA supplementation required than for LAA to observe this effect [32], indicating that this analogue can substitute for LAA in cells as well as with isolated proteins.

To confirm that the ability of IPAA to promote PHD2 activity similarly to LAA was not due to degradation of IPAA to LAA via hydrolysis of the acetal group, ^{1}H-nuclear magnetic resonance (NMR) experiments were conducted to monitor the levels of IPAA and to look for the presence of LAA throughout the reaction. The distinctive doublet (δ_H ~ 1.4ppm) of the two methyl groups in the IPAA side chain were shown not to degrade during the course of the reaction (Supplementary Information, S3) implying that IPAA was not hydrolysed to LAA during the reaction.

Use of the analogues in which the reducing ene-diol component was modified (Scheme 1, (iv)-(vii)) resulted in similar peptide hydroxylation levels to those observed in the absence of LAA. These results indicate the requirement of a reductant by PHD2 for full activity, but do not rule out an involvement of this region of LAA in interacting with the enzyme. A slightly lower level of hydroxylation was observed for dehydroascorbate and 3,4-dihydroxyphenylacetic acid (Figure 2A and 2B; see below).

Alternative reducing agents can partially replace the role of LAA in promoting PHD2 activity in a substrate-selective manner

To investigate whether the promotion of PHD2 activity by LAA, D-isoascorbate and IPAA were due to their reduction potential, activity was monitored in the presence of different reducing agents. Dithiothreitol (DTT, Scheme 1 (viii)) was chosen as it is a small molecule reducing agent, therefore potentially able to access the active site of PHD2. Glutathione (Scheme 1, (ix)) was chosen at it is an abundant reducing agent in cells. Both of these reducing agents have more negative standard reduction potentials than LAA (-0.33V[39] and -0.24V[40] respectively, compared to +0.28V for LAA[41]) and should therefore be more capable of reducing Fe^{3+} to Fe^{2+} in free solution.

Figure 3 shows that under the experimental conditions used, neither DTT nor glutathione (at 4 mM) were able to fully replace LAA in promoting PHD2 activity towards either NODD or CODD substrates. PHD2 hydroxylation of CODD was partially stimulated by DTT (to 48% compared to 64% with LAA), as was PHD2 hydroxylation of NODD (to 26% compared to 63% with LAA); the apparent greater ability of DTT to stimulate PHD2 activity towards CODD rather than NODD is reflected in the K_m values for DTT for each of these reactions (42μM and 634μM, respectively, Table 1). PHD2-catalysed CODD hydroxylation was also promoted by, though to a lesser extent, glutathione (27%, as compared to 15% without LAA and 17% with oxidised glutathione). Notably (given its biological importance), glutathione appeared not to be able to stimulate NODD hydroxylation at all.

The effect of LAA on FIH hydroxylation of HIF is less significant than on PHD2 activity

The LAA-dependence of FIH catalysed activity has not been well documented. FIH is also a HIF hydroxylase, catalysing hydroxylation of Asn803, and therefore a comparison of the effect of LAA on the hydroxylation of HIF by FIH with PHD2 is of interest with respect to its overall effect on HIF and ankyrin hydroxylation.

Experiments to monitor hydroxylation of the HIF-1α CAD peptide by FIH, with analysis by MALDI MS, were carried out similarly to those performed with PHD2, and revealed that, under current assay conditions, the dependence of FIH hydroxylation of HIF on LAA was lower than the dependence of PHD2, with the maximal hydroxylation only increasing from 19% to 36% by the addition of LAA under standard assay conditions (Figure 4A). LAA also increased the initial rate of CAD hydroxylation (0.78 μM/s in the presence of ascorbate, compared to 0.32 μM/s in the absence of ascorbate). When the LAA analogues were studied for their effect on FIH hydroxylation of CAD, IPAA and D-isoascorbate were able to replicate the effect of LAA, similarly to the results with PHD2. L-gulonic-γ-lactone and L-ascorbic acid-2-sulphate resulted in similar CAD hydroxylation levels to those seen in the absence of LAA (Figure 4B). As for PHD2, 3,4-dihydroxyphenylacetic acid and dehydroascorbate inhibited FIH catalyzed hydroxylation. 3,4-Dihydroxyphenylacetic acid is a known iron chelator [42] and the addition of further ferrous iron partially reversed the level of inhibition observed for 3,4-dihydroxyphenylacetic acid, implying that, at least in part, the inhibition by 3,4-dihydroxyphenylacetic acid results from iron chelation in solution (Supplementary Information, S4).

Interestingly, and in contrast to PHD2, replacement of LAA with either DTT or glutathione did not result in an increase in FIH-catalysed HIF hydroxylation relative to levels seen without LAA (22% and 12% respectively, compared to 19% without LAA, Figure 4C). Irrespective of mechanism, these results suggest differences between the way these reducing agents act on PHD2 and FIH, and raise the intriguing possibility of the use of specific reducing agents to selectively activate certain HIF hydroxylases, or indeed 2OG oxygenases (DTT is able to replace LAA to approximately 80% activity for AlkB[32].

FIH hydroxylation of a consensus ankyrin peptide is stimulated by glutathione

As well Asn hydroxylation in the CAD of HIF-α, FIH is able to catalyse hydroxylation of conserved Asn residues in ankyrin repeat domain (ARD) proteins [35, 36]. Because ARD proteins can contain multiple hydroxylation sites on different ankyrin repeats, a consensus model ARD substrate has been developed to use as a model ankyrin substrate [37, 38]. A synthetic peptide fragment from this substrate (1CA), was used to study whether there were any substrate-dependent differences in the LAA/analogue-dependence of FIH-mediated Asn hydroxylation.

Time course experiments showed that hydroxylation of 1CA by FIH was promoted by LAA, similarly to FIH-mediated hydroxylation of HIF-α CAD (Figure 5A); the initial rate of 1CA hydroxylation in the presence of LAA was notably faster than 1CA hydroxylation in the absence of ascorbate (1.45 μM/s compared to 0.25 μM/s). 1CA hydroxylation by FIH was also faster than hydroxylation of HIF-α CAD (1.45 μM/s compared to 0.78 μM/s). Interestingly, and in contrast to the slight inhibitory effect seen for HIF-α CAD hydroxylation, reduced glutathione appeared able to stimulate hydroxylation of 1CA to a small degree (from ~12% to ~20%, Figure 5B). This effect was apparently due to its reducing capacity, as the use of oxidised glutathione resulted in no significant change in hydroxylation level (~14%). This substrate-dependent effect of glutathione on FIH activity is similar to that seen with the effect of glutathione on PHD2 activity towards NODD and CODD (described above).

DISCUSSION

Lack of LAA in the diet of some animals, including humans, causes the disease scurvy. This has been related to impaired activity of collagen prolyl hydroxylase which in vitro shows a strong dependence on LAA[5]. LAA is required for catalysis by the plant enzyme 1-aminocyclopropane-1-carboxylate oxidase (ACCO) [43], the ethylene forming enzyme, which does not use a 2OG cosubstrate but is structurally related to the 2OG oxygenases. LAA is commonly added to in vitro incubations of many 2OG oxygenases, including those originating from organisms that do not produce LAA, in order to promote catalysis. However, LAA is not present in many organisms that contain 2OG oxygenases, including microorganisms that use 2OG oxygenases to produce antibiotics very efficiently. Further, at least under some incubation conditions, LAA inhibits at least one 2OG oxygenase (proline-4-hydroxylase from *Streptomyces griseoviridus* P8648 [44]). Thus, LAA cannot be a universal 'activator' for the 2OG oxygenases and structurally related enzymes.

We aimed to investigate the extent to which LAA is selective in HIF hydroxylase catalysis in vitro. We initially studied how LAA itself affected the different reactions catalysed by the HIF hydroxylases and found that LAA enhances the hydroxylation activity of PHD2 towards both its NODD and CODD substrates to similar extents. This result is in agreement with previous in vitro data studying the effects of LAA on 2OG turnover by these enzymes [16, 21, 22], and cellular data demonstrating that the addition of LAA can reduce HIF levels [23]. LAA also promotes FIH activity towards both HIF-1α CAD and the 1CA consensus ankyrin peptide to similar final hydroxylation levels. In all cases (FIH and PHD2), the presence of LAA increased the initial rate of hydroxylation under our standard conditions.

Structural analogues of LAA were used to investigate whether a specific interaction with the HIF hydroxylases was likely, and the importance of the ene-diol reducing moiety of LAA. The ability of D-isoascorbate and IPAA to effectively replace LAA with both PHD2 and FIH suggested that the side chain moiety of LAA is not functionally significant, and that if an interaction does occur between LAA and either PHD2 or FIH, that this is not blocked by the hydrophobic nature of the IPAA side chain. In fact, the lower $K_{m,apparent}$ values for IPAA than for LAA for PHD2 catalysis of both NODD and CODD hydroxylation suggests an enhancement of the interaction between this analogue and PHD2. These results are in agreement with studies investigating LAA analogues on collagen prolyl-4-hydroxylase catalysis [45, 46], yet are interesting given the likely enclosed nature of the PHD2 active site [47, 48], at least compared to some other human 2OG oxygenases [49]. The inability of the analogues with alterations to the ene-diol moiety to replace LAA suggests that the role of LAA involves either its reducing capacity, and/or that this region of the molecule is important in interacting with the enzyme.

Compared to the effect of LAA, neither DTT nor glutathione were able to fully substitute for LAA in promoting hydroxylation by either FIH or PHD2, despite the fact that both are stronger reducing agents than LAA. However, given that glutathione is present to high levels in the cell (0.5-10 mM [50]), it is notable that glutathione was able to promote CODD hydroxylation by PHD2 and 1CA hydroxylation by FIH. The inability of DTT to fully substitute for LAA is consistent with previous work carried out on collagen prolyl hydroxylase, where DTT was only able to promote enzyme activity to a small and variable degree (<20%) [51]. Differences were noted for the effects of DTT and glutathione on PHD2 and FIH. PHD2-catalysed CODD hydroxylation was promoted by both reducing agents, though to a greater degree by

DTT (the $K_{m,apparent}$ value for DTT promoted hydroxylation of CODD was similar to that for LAA), whereas NODD hydroxylation was not promoted by glutathione (within the limits of detection under our standard assay conditions) and the $K_{m,apparent}$ value for DTT in this case was significantly higher. Although FIH-catalysed 1CA hydroxylation was promoted by glutathione, interestingly FIH-catalysed CAD hydroxylation was not promoted by either glutathione or DTT. Although the results are obtained under conditions likely far from those in cells, they suggest that selective HIF hydroxylation reactions (e.g. CODD vs. NODD hydroxylation or ODD vs. CAD hydroxylation) could be promoted by specific reducing agents. Further, although we have not found that a thiol reducing agents can replace LAA at similar levels of activity, the levels of HIF hydroxylase stimulation seen with glutathione were substantially above controls suggesting that in cells, at least when LAA is limiting, thiols may enhance activity. The enhancement of activity by different types of reducing agent is also of interest with respect to the proposal that the HIF hydroxylases may act as signal integration points for redox sensing in cells.

Proposals for the role of LAA in 2OG oxygenase catalysis include action as a specific reductant [51], to maintain free Fe(II) in solution [23] and/or to prevent/reverse enzyme inactivation brought about from oxidised iron following uncoupled 2OG turnover, as observed for collagen prolyl hydroxylase [5]. Whilst our results do not define the mode of interaction or mechanism(s) of action of LAA in catalysis by the HIF hydroxylases, they do indicate certain features of the role of this cofactor important for its stimulatory role. The observation that LAA and similar activating analogues stimulate the initial rate (at least as apparent by our current assays) of the HIF hydroxylases suggests that the prevention/reversal of enzyme inactivation is not the primary role of LAA for FIH and PHD2 (or that enzyme inactivation in the absence of LAA was undetectable under our experimental conditions). The inability of the alternative reducing agents DTT and glutathione to fully replace LAA suggests that the role of LAA is not (solely) to maintain a free pool of Fe(II) (although this may differ in cellular conditions, where it is possible that glutathione stimulates activity). The ability of D-isoascorbate and IPAA to promote activity of both FIH and PHD2 also suggests that any interaction between LAA and these enzymes is not highly specific.

The finding that certain reducing agents can promote, e.g. CODD hydroxylation but not NODD hydroxylation by PHD2 and 1CA hydroxylation but not HIF-1α CAD hydroxylation by FIH, raises the interesting possibility that modified reducing agent analogues could be designed which are targeted to specific HIF hydroxylase-catalysed reactions, in particular targeting their substrate selectivity. Promotion of FIH-catalysed ankyrin repeat domain protein hydroxylation may even target FIH away from HIF, thus decreasing its activity towards this substrate [52]. The HIF hydroxylases are current pharmaceutical targets for the treatment of ischemic diseases, typically using 2OG analogue HIF hydroxylase inhibitors to upregulate HIF [53]. By the use of 'activating' LAA analogues, it may be possible to increase the activity of the HIF hydroxylases in, for example cancer cells, and thus provide another opportunity for therapeutic intervention via these enzymes.

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FIGURE LEGENDS

Figure 1. Hydroxylation of (A) CODD, and (B) NODD HIF-1α peptide substrates by PHD2 with time. Assays were carried out in the presence (black circles) and absence (white circles) of LAA. n=3, error bars show standard deviations from the mean.

Figure 2. Hydroxylation of (A) CODD, and (B) NODD HIF-1α peptide substrates by PHD2 with time in the presence of different LAA analogues. LAA, black circles; no LAA, white circles; D-isoascorbate, white squares; IPAA, grey triangle; L-gulonic-γ-lactone, grey hexagon; L-ascorbic acid-2-sulphate, black square; dehydroascorbate, white triangle; 3,4-dihydroxyphenylacetic acid, black diamond. n=3, error bars show standard deviations from the mean.

Figure 3. Hydroxylation of (A) CODD, and (B) NODD HIF-1α peptide substrates by PHD2 with time in the presence of different reducing agents. LAA, black circles; no LAA, white circles; DTT, black squares; reduced glutathione, white triangles; oxidised glutathione, grey triangles. n=3, error bars show standard deviations from the mean.

Figure 4. Hydroxylation of HIF-1α CAD peptide substrate by FIH. (A) in the presence (black circles) and absence (white circles) of LAA; (B) in the presence of LAA and different LAA analogues: LAA, black circles; no LAA, white circles; D-isoascorbate, white squares; IPAA, grey triangle; L-gulonic-γ-lactone, grey hexagon; L-ascorbic acid-2-sulphate, black square; dehydroascorbate, white triangle; 3,4-dihydroxyphenylacetic acid, black diamond; (C) in the presence of different reducing agents: LAA, black circles; no LAA, white circles; DTT, black squares; reduced glutathione, white triangles; oxidised glutathione, grey triangles. n=3, error bars show standard deviations from the mean.

Figure 5. Hydroxylation of 1CA, a synthetic consensus ankyrin repeat domain peptide, by FIH. (A) in the presence (black circles) and absence (white circles) of LAA; (B) in the presence and absence of LAA (as in (A)) and in the presence of reduced glutathione (white triangles) and oxidised glutathione (black triangles). n=3, error bars show standard deviation.

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FIGURES

Figure 1.

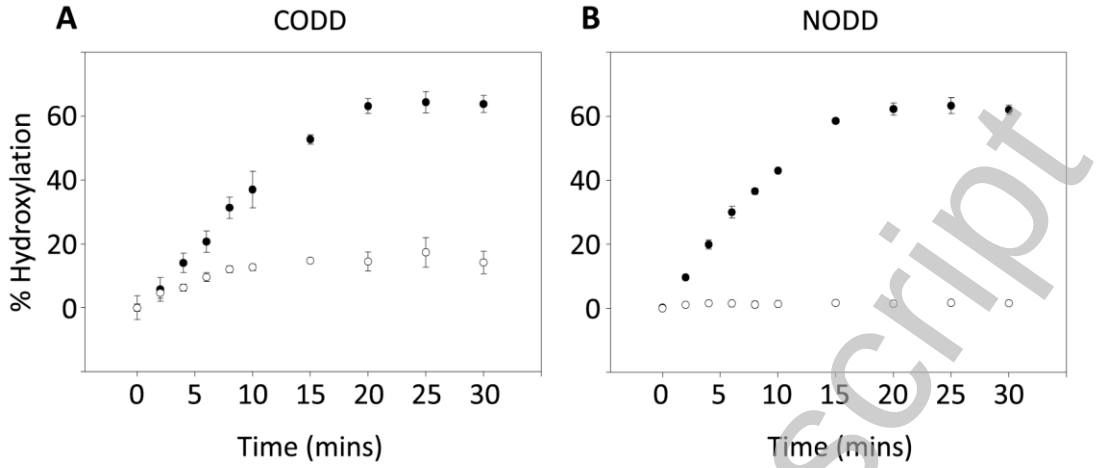


Figure 2.

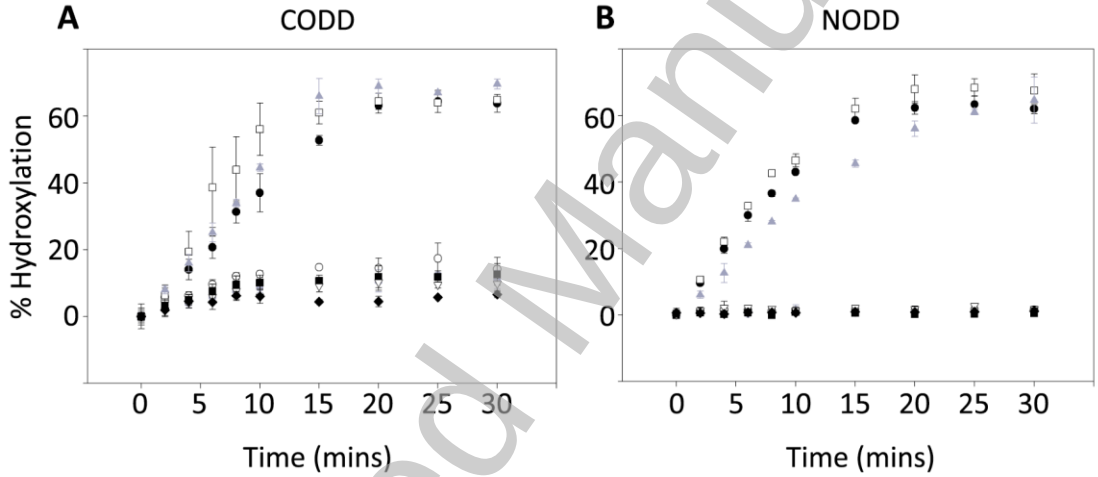


Figure 3.

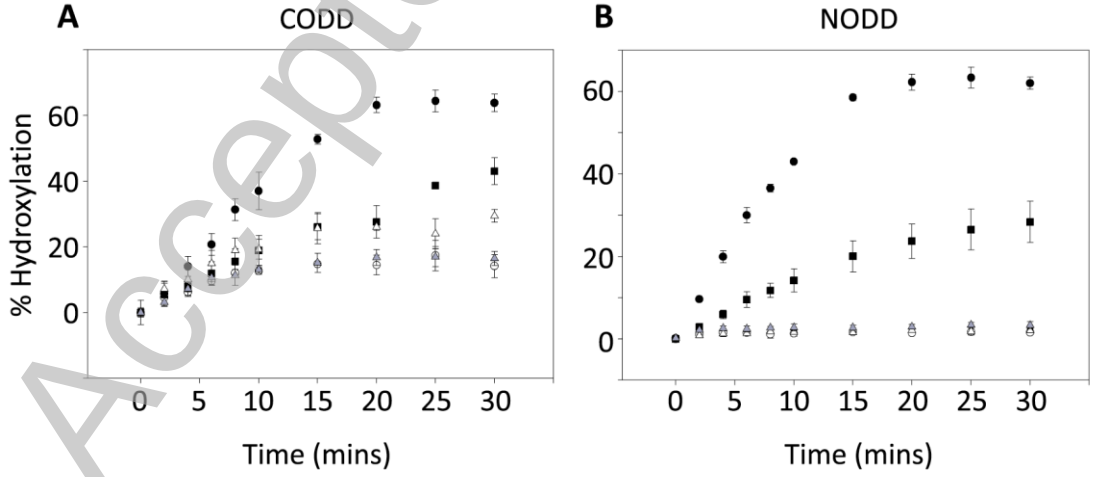
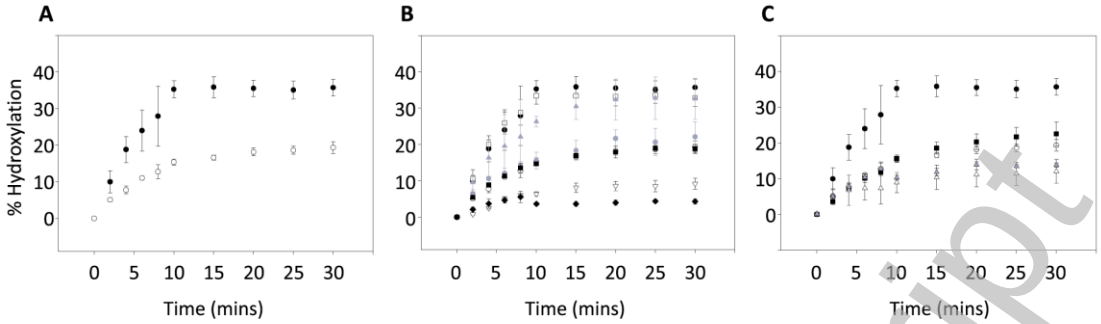


Figure 4.



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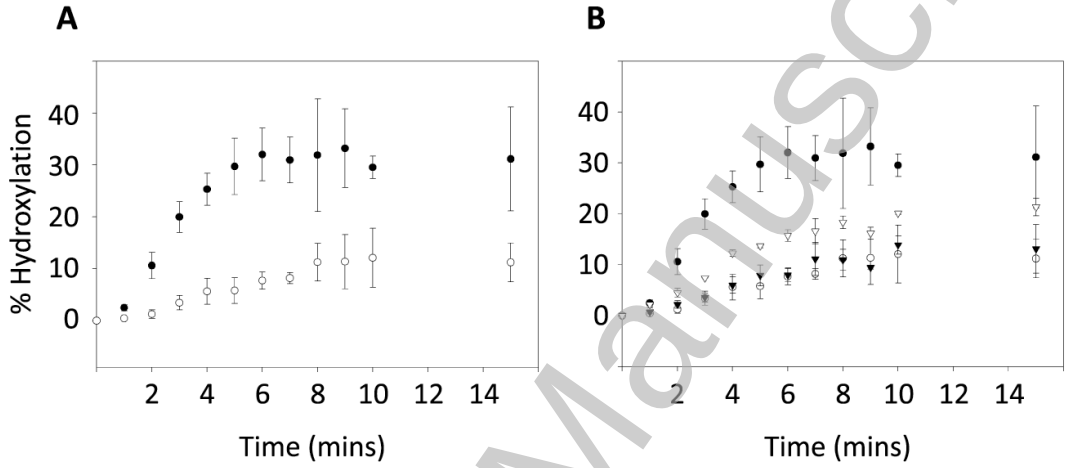
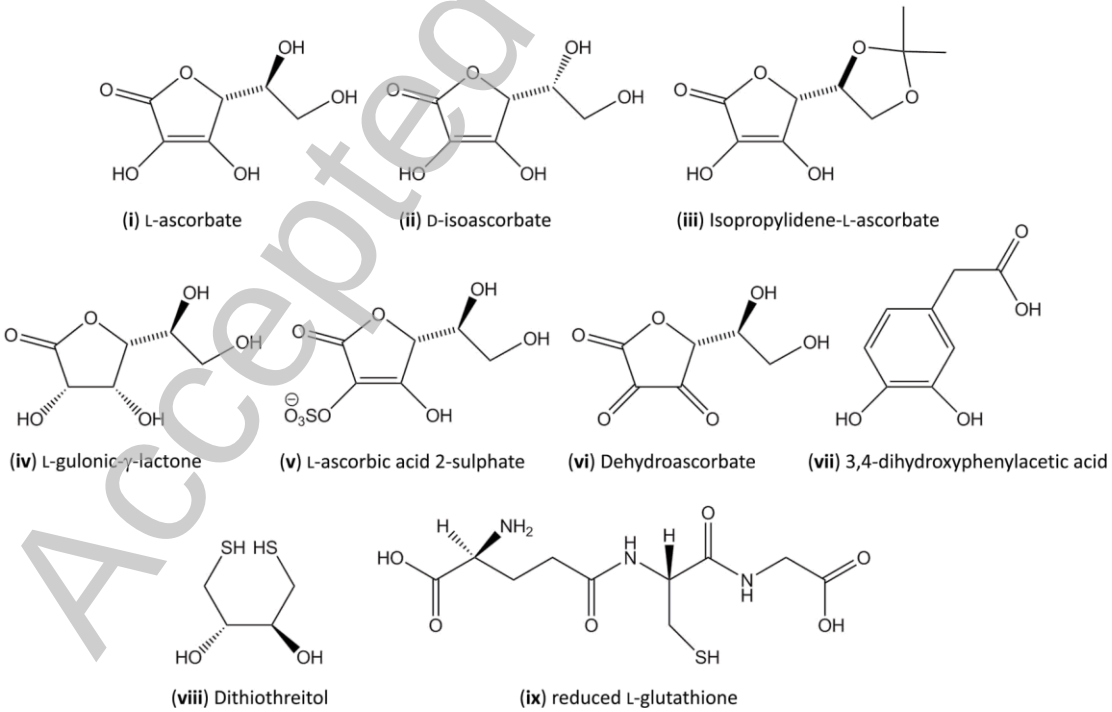


Schemes



Scheme 1. Chemical structures of (i) LAA, and (ii)-(ix) the analogues used in this study.

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Tables

Table 1. K_m (apparent) values determined for LAA and 'active' LAA analogues for PHD2 catalysed hydroxylation of CODD and NODD under standard assay conditions.

	PHD2 CODD	**PHD2 NODD**
LAA	54 ±10	53.2 ±17
D-isoascorbate	40 ±14	77.3 ±27
IPAA	18 ±4	27.3 ±7
DTT	42 ±14	635 ±319
Glutathione (reduced)	>5000	ND