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# Human oxygen sensing may have origins in prokaryotic EF-Tu prolyl-hydroxylation

**Running title (<50 characters):** Structural basis of EF-Tu prolyl-hydroxylation

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**Author Contributions:** J.S.S. designed the project, purified and crystallized proteins, determined and refined crystal structures, performed kinetic assays, and wrote the manuscript. I.K.H.L. performed NMR experiments. W.G. worked on PPHD substrate identification. M.A.B. and J.P. performed evolutionary analyses. H.P.K. performed mass spectrometry experiments. J.L. assisted in translation rate assays. W.A. assisted in kinetic experiments. H.C. computed binding energies. S.M.P. and L.A.H.B. performed cellular phenotypic assays. N.D.L. performed amino acid analysis. S.H. carried out structural analyses and made figures. C.H. and N.J.K. carried out initial studies on PPHD including protein production. C.M.T. supervised cellular assays. T.D.W.C. supervised NMR assays. G.M.P. supervised phenotypic analyses in *P. aeruginosa* and other cellular studies. M.A.M supervised crystallographic experiments and wrote the manuscript. C.J.S. designed and supervised the project and wrote the manuscript.

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**Data deposition:** Coordinates and structure factors have been deposited in the Protein Data Bank with the accession codes 4J25 for PPHD<sub>putida</sub>, 4J0Q for EF-Tu<sub>putida</sub>, and 4IW3 for the PPHD:EF-Tu complex.

## **Abstract**

The roles of 2-oxoglutarate (2OG) dependent prolyl-hydroxylases in eukaryotes include collagen stabilization, hypoxia sensing, and translational regulation. The hypoxia inducible factor (HIF) sensing system is conserved in animals but not in other organisms. However, bioinformatics imply that 2OG dependent prolyl-hydroxylases (PHDs) homologous to those acting as sensing components for the HIF system in animals occur in prokaryotes. We report cellular, biochemical, and crystallographic analyses revealing that *Pseudomonas spp.* contain a 2OG oxygenase (PPHD) related in structure and function to the animal PHDs. *P. aeruginosa* with deleted PPHD displays impaired growth in the presence of iron-chelators and increased production of the virulence factor pyocyanin. We identify elongation factor Tu (EF-Tu) as a PPHD substrate, which undergoes prolyl-4-hydroxylation on its switch I loop. A crystal structure of PPHD reveals striking similarity to human PHD2 and a *Chlamydomonas reinhardtii* prolyl-4-hydroxylase. A crystal structure of PPHD complexed with intact EF-Tu reveals major conformational changes occur in both PPHD and EF-Tu, including a >20 Å movement of the EF-Tu switch I loop. Comparison of the PPHD structures with those of HIF and collagen prolyl-hydroxylases reveals conservation in substrate recognition despite diverse biological roles and origins. The observed changes will be useful in designing new types of 2OG oxygenase inhibitors based on various conformational states, rather than active site iron chelators, which comprise most reported 2OG oxygenase inhibitors. Structurally informed phylogenetic analyses suggest that the role of prolyl-hydroxylation in human hypoxia sensing has ancient origins.

## **Significance Statement**

The Fe(II) and 2-oxoglutarate (2OG) dependent hypoxia inducible transcription factor (HIF) prolyl-hydroxylases play a central role in human oxygen sensing and are related to prolyl-hydroxylases involved in eukaryotic collagen biosynthesis and ribosomal modification. The finding that related prolyl-hydroxylases in *Pseudomonas spp.* regulate iron chelator biosynthesis supports prokaryotic origins for the eukaryotic prolyl-hydroxylases. The identification of the switch I loop of elongation factor Tu (EF-Tu) as a PPHD substrate provides new evidence of roles for 2OG oxygenases in both translational and transcriptional regulation. A structure of the PPHD:EF-Tu complex, the first of a 2OG oxygenase with its intact protein substrate, reveals major conformational changes occur in both PPHD and EF-Tu and will be useful in the design of new prolyl-hydroxylase inhibitors.

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## INTRODUCTION

The hypoxia inducible transcription factor (HIF) is a major regulator of the response to limited oxygen availability in humans and other animals (1-3). A hypoxia sensing component of the HIF system is provided by 2-oxoglutarate (2OG) and Fe(II) dependent oxygenases, which catalyze prolyl-4-hydroxylation of HIF- $\alpha$  subunits, a post-translational modification that enhances binding of HIF- $\alpha$  to pVHL, so signaling for the proteosomal degradation of HIF- $\alpha$ . The PHDs belong to a subfamily of 2OG oxygenases that catalyze prolyl-hydroxylation, which also includes the collagen prolyl-3- (CP3Hs) and prolyl-4-hydroxylases (CP4Hs) (4). Subsequently identified prolyl-hydroxylases include the ribosomal prolyl-hydroxylases (OGFOD1 and Tpa1), which catalyze ribosomal protein 23 (RPS23) prolyl-3-hydroxylation in many eukaryotes, and slime-mold enzymes, which catalyze prolyl-4-hydroxylation of Skp1, a ubiquitin ligase subunit (5-9). The HIF-PHD-VHL triad is likely present in all animals, but probably not in other organisms (3); however, structurally informed bioinformatic analyses imply the presence of PHD- homologs in bacteria (10, 11), including in *Pseudomonas spp*, suggesting prolyl-hydroxylases may have ancient origins.

## RESULTS

***Pseudomonas spp.* contain a functional prolyl-hydroxylase.** To investigate the role of a putative PHD homolog in *Pseudomonas aeruginosa* (PPHD), we initially characterized a PPHD deleted strain. Metabolic screening studies revealed that the PPHD deleted strain displays impaired growth in the presence of iron-chelators (e.g. 2,2-bipyridine) and produces increased levels of iron-chelating siderophores including pyocyanin, a bacterial virulence factor (Fig. S1)(12), implying PPHD is involved in iron regulation. Ferrous iron is essential for PHD catalysis and treatment of human cells with iron chelators, including pyocyanin (13), causes HIF- $\alpha$  upregulation via PHD inhibition, suggesting related roles for PPHD and the animal PHDs (1).

**EF-Tu is a PPHD substrate.** We then investigated possible PPHD substrates. Mass spectrometric (MS) studies identified EF-Tu as a PPHD binding partner and potential substrate in *P. putida* (Table S3). This assignment is supported by a proteomic MS study on *Shewanella oneidensis*, reporting that EF-Tu is modified by a +16 Da mass shift (14); bioinformatics reveal *S. oneidensis* contains a potential hydroxylase closely related to PPHD (38% identity). We tested a set of 14 synthetic peptides covering the 19 prolines in the EF-Tu sequence as substrates for isolated recombinant PPHD<sub>putida</sub> (Table S4). We identified an EF-Tu peptide as undergoing a +16 Da mass shift (Fig. 1B and Table S4); MSMS analyses demonstrated hydroxylation at Pro54 (Hyp54)(Fig. S2A). Amino acid analyses reveal that PPHD<sub>putida</sub> catalyzes *trans* prolyl-4-hydroxylation of EF-Tu, as occurs for PHD-catalyzed HIF and CP4H-catalyzed collagen prolyl-hydroxylation (Fig. S2B)(1). We did not observe cross hydroxylation reactivity between the PPHD and PHD2 substrates; i.e. EF-Tu with PHD2, and the C-terminal oxygen degradation domain of HIF-1 $\alpha$  (Codd<sub>556-574</sub>) with PPHD.

EF-Tu undergoes hydroxylation in the wildtype but not in the PPHD deleted strain as shown by MS studies (Fig. S3 A and B). Since the human elongation factor 2 (eEF2) interacts with PHD2 (15), we tested peptides derived from the switch I loop of human eEF2 as potential PPHD or PHD2 substrates, but did not observe hydroxylation, within the limits of detection.

**Effect of prolyl-hydroxylation on EF-Tu function.** Kinetic analyses were then carried out using a 20-residue EF-Tu fragment (EF-Tu<sub>44-63</sub>). Compared to an analogous substrate fragment with PHD2, PPHD<sub>putida</sub> has a similar apparent  $K_m$  ( $47.2 \pm 8.5 \mu\text{M}$  for PPHD/EF-Tu<sub>44-63</sub>;  $36.7 \pm 9.0 \mu\text{M}$  for PHD2/HIF-1 $\alpha$  CODD), but a lower apparent  $K_m$  for O<sub>2</sub> ( $47.5 \pm 9.2 \mu\text{M}$  for PPHD/EF-Tu<sub>44-63</sub>;  $229 \pm 60 \mu\text{M}$  for PHD2/HIF-1 $\alpha$  CODD), and higher apparent  $K_m$  values for 2OG ( $182 \pm 22 \mu\text{M}$  for PPHD/EF-Tu<sub>44-63</sub>;  $55 \pm 11 \mu\text{M}$  for PHD2/HIF-1 $\alpha$  CODD), Fe(II) ( $6.3 \pm 0.7 \mu\text{M}$  for PPHD/EF-Tu<sub>44-63</sub>;  $<1 \mu\text{M}$  for PHD2/HIF-1 $\alpha$  CODD), and L-ascorbate ( $1,064 \pm 95 \mu\text{M}$  for PPHD/EF-Tu<sub>44-63</sub>;  $54 \pm 10 \mu\text{M}$  for PHD2/HIF-1 $\alpha$  CODD) (Fig. S4 A-E)(16-20). Although the results

with isolated enzyme may not reflect the cellular kinetics, given that PHD2 kinetics are proposed to reflect its role as a hypoxia sensor these preliminary differences are of interest. In particular the higher  $K_m$  for Fe(II) is notable given the phenotypic results on *PPHD* deletion, supporting a role for PPHD in iron regulation.

The switch I loop of EF-Tu plays a central role in bacterial translation by coupling GTP hydrolysis to conformational changes from the GTP-bound active state to the GDP-bound inactive state (21, 22). NMR was used to directly monitor kirromycin-induced GTP hydrolysis by EF-Tu<sub>putida</sub>; we observed no difference in the GTP hydrolysis rate for Hyp54 EF-Tu ( $0.71 \pm 0.02 \mu\text{M}/\text{min}$ ) and unmodified EF-Tu ( $0.76 \pm 0.08 \mu\text{M}/\text{min}$ )(Fig. S5A). However, addition of PPHD<sub>putida</sub> to the EF-Tu GTP hydrolysis reaction decreased the rate of GTP hydrolysis in a metal ion and 2OG dependent manner [Zn(II)  $0.60 \pm 0.05 \mu\text{M}/\text{min}$ ; Zn(II) and 2OG  $0.49 \pm 0.16 \mu\text{M}/\text{min}$ ][Zn(II) is a surrogate for paramagnetic Fe(II) and a PPHD<sub>putida</sub> inhibitor](Fig. S5 B-D). In *P. aeruginosa*, we observed no difference in the global translation rate between the PPHD deleted strain and the wildtype under standard growth conditions, as measured by <sup>35</sup>S-methionine incorporation (Fig. S5E). The effect of PPHD<sub>putida</sub> on the GTP hydrolysis rate is less with hydroxylated EF-Tu ( $0.83 \pm 0.08 \mu\text{M}/\text{min}$ ) (Fig. S5B), likely due to weaker binding of hydroxylated EF-Tu to PPHD.

Using a paramagnetic relaxation enhancement based NMR technique [with Mn(II) substituted for Fe(II)], we observed that addition of EF-Tu<sub>protein</sub> decreased the PPHD<sub>putida</sub> 2OG  $K_d$  by ~50-fold ( $8 \pm 4 \mu\text{M}$ ) compared to the absence of any substrate or in the presence of EF-Tu<sub>44-63</sub> ( $>400 \mu\text{M}$ )(Fig. S5F), implying a substantial interaction interface between PPHD<sub>putida</sub> and EF-Tu. These results implied very substantial interactions between PPHD<sub>putida</sub> and EF-Tu likely extend well beyond the immediate active site region. Notably, the observation that PPHD<sub>putida</sub> addition decreases the rate of GTP hydrolysis and the location of Pro54 on the switch I loop suggested PPHD<sub>putida</sub> sequesters the switch I loop. We therefore pursued structural characterization of PPHD<sub>putida</sub>, EF-Tu, and the PPHD:EF-Tu complex.

**Structure of PPHD.** To investigate the structural similarity of PPHD<sub>putida</sub> to PHD2, we determined a crystal structure of PPHD<sub>putida</sub> complexed with Mn(II) and *N*-oxalylglycine (NOG), which are inert substitutes for Fe(II) and 2OG, respectively (2.0 Å resolution; space group *P*1). Like other 2OG oxygenases, PPHD<sub>putida</sub> contains a double-stranded  $\beta$ -helix (DSBH) core fold comprising eight  $\beta$ -strands (I-VIII) folded into major and minor  $\beta$ -sheets that enclose the metal-/2OG-binding sites (Fig. 2A)(23, 24). Five antiparallel  $\beta$ -strands [ $\beta$ 1- $\beta$ 2(I)- $\beta$ 4(III)- $\beta$ 7(VI)- $\beta$ 9(VIII)] form the major  $\beta$ -sheet and four antiparallel  $\beta$ -strands [ $\beta$ 3(II)- $\beta$ 5(IV)- $\beta$ 6(V)- $\beta$ 8(VII)] form the minor  $\beta$ -sheet. Three  $\alpha$ -helices ( $\alpha$ 1- $\alpha$ 3) pack against the major  $\beta$ -sheet of the DSBH. A disordered region (residues 47-71) of PPHD<sub>putida</sub> corresponds to the  $\beta$ 2- $\beta$ 3 finger loop of PHD2, which is conformationally flexible and involved in HIF- $\alpha$  binding (10, 25). PPHD<sub>putida</sub> contains a HXD...H triad of metal-binding residues (His124, Asp126, His183), which with the NOG oxalyl group and a water complete octahedral metal-coordination. Although non-identical, the PPHD<sub>putida</sub> active site is very similar to that of PHD2 (10); NOG is positioned between the major and minor  $\beta$ -sheets of the DSBH such that its C-5 carboxylate forms a salt bridge with Arg192 (Arg383<sub>PHD2</sub>)(Fig. 2 B and C). The clear similarity between PPHD<sub>putida</sub> and PHD2 supports the proposal of common ancestry for the two-hydroxylases (Fig. 2B).

**Structure of a PPHD:EF-Tu complex.** We determined a *P. putida* EF-Tu structure (2.3 Å resolution; space group *C*2). EF-Tu<sub>putida</sub> and *E. coli* EF-Tu have very similar overall folds (r.m.s.d. 1.5 Å over 353 C $\alpha$  atoms) and Mg(II) binding sites in their inactive GDP-bound states (Fig. 3A and Fig. S6 A and B)(26). The switch I loop of EF-Tu<sub>putida</sub> (residues 40-62) is disordered; this is notable because the prolyl-hydroxylated regions of HIF- $\alpha$  are also predicted to be disordered in solution (18).

To obtain a structure of a PPHD:EF-Tu protein-protein complex (Fig. 3 A-C), PPHD and EF-Tu were expressed separately to prevent EF-Tu hydroxylation, then mixed, purified and crystallized using Mn(II) and NOG to stabilize and inactivate/inhibit PPHD, and Mg(II) and GDP in order to stabilize EF-Tu. In the resultant PPHD:EF-Tu complex structure (2.7 Å resolution; *P*3<sub>1</sub> 2 1), EF-Tu is in its translationally inactive

GDP-bound conformational state, consistent with the observed PPHD-mediated inhibition of GTP hydrolysis. PPHD<sub>putida</sub> binds EF-Tu between domain I and domains II/III, occupying a position similar to that observed for domain I of EF-Tu in its GTP-bound active state relative to domains II/III (Fig. 3D)(27). A Mg(II) ion is present in the EF-Tu nucleotide binding site as in the EF-Tu structure without PPHD<sub>putida</sub>. Mn(II) and NOG are present in the PPHD<sub>putida</sub> active site.

In contrast to the isolated EF-Tu<sub>putida</sub> structure, in the complex most EF-Tu switch I loop residues are ordered (16 residues disordered in isolated EF-Tu; 6 in the PPHD:EF-Tu complex). In a structure of isolated *E. coli* EF-Tu (in its inactive GDP-bound state), all the switch I loop residues are ordered and located on the surface of EF-Tu domain I, adopting a loop-helix-loop-strand conformation (Fig. 3E)(26). Comparison of the *P. putida* PPHD:EF-Tu complex crystal structure with that of *E. coli* EF-Tu (78% identity; r.m.s.d. 1.2 Å over 338 C $\alpha$  atoms) reveals major differences. Relative to the isolated *E. coli* EF-Tu structure, in the PPHD:EF-Tu complex structure, the entire switch I loop pivots around residues Gly41<sub>EF-Tu</sub> and Ile61<sub>EF-Tu</sub>, such that Pro54<sub>EF-Tu</sub> moves 22 Å and is positioned at the apex of an extended loop projecting into the PPHD<sub>putida</sub> active site (Fig. 3E).

In contrast to the isolated PPHD<sub>putida</sub> structure, in the PPHD:EF-Tu complex, the PPHD<sub>putida</sub>  $\beta$ 2- $\beta$ 3 finger loop (residues 51-74) is ordered and encloses the EF-Tu switch I loop within the active site (Fig. 3C). The residues at the apex of the flexible  $\beta$ 2- $\beta$ 3 finger loop of PPHD<sub>putida</sub> adopt a  $3_{10}$ -helix conformation (residues 58-60). An arginine (Arg57), prior to the  $3_{10}$ -helix, is positioned to form a salt bridge with Asp130 of the  $\beta$ 5(II)- $\beta$ 6(III) 'thumb' loop (7 residues, 127-133), forming a tunnel through which the EF-Tu switch I loop is threaded (Fig. 3C). The  $\beta$ 5(II)- $\beta$ 6(III) thumb loop of PPHD<sub>putida</sub> contains three arginines, three aspartates (including Asp130), and one phenylalanine, the backbone and side chains of which make interactions with domain II of EF-Tu and undergo clear conformational changes relative to their positions in the isolated PPHD<sub>putida</sub> structure (r.m.s.d. 1.4 Å)(Fig. 3F and Fig. S6C).

As indicated by the kinetic analyses in solution, the PPHD:EF-Tu structure reveals substantial enzyme-substrate contacts beyond the active site. PPHD and EF-Tu share a total interaction interface of 1662 Å<sup>2</sup> (24 hydrogen bonds; 10 salt bridges), resulting in a calculated  $\Delta G_{\text{binding}}$  of  $\sim -9.7$  kcal/mol, of which only 965 Å<sup>2</sup> (13 hydrogen bonds; 3 salt bridges) is accounted for by direct interactions of PPHD<sub>putida</sub> with the EF-Tu switch I loop. Other interactions include positioning the PPHD<sub>putida</sub>  $\alpha 3$ -helix into a hydrophobic pocket in the  $\beta$ -barrel of EF-Tu domain II formed by Phe222<sub>EF-Tu</sub>, Ile224<sub>EF-Tu</sub>, and Val230<sub>EF-Tu</sub>, and burying Phe265<sub>EF-Tu</sub> in a hydrophobic pocket formed by the side chains of Leu101<sub>PPHD</sub>, Leu103<sub>PPHD</sub>, and Leu177<sub>PPHD</sub> (Fig. 3A inset). These interactions collectively order the PPHD<sub>putida</sub> thumb loop, suggesting analogous interactions between PHD2 and full-length HIF $\alpha$ , not observed in a PHD2:HIF- $\alpha$  fragment structure (25), may occur.

**Conservation of substrate binding by prolyl-hydroxylases.** The positions of the  $\beta 2$ - $\beta 3$  finger loop residues in the complex are strikingly similar to those observed for PHD2 in complex with HIF- $\alpha$  and to a collagen prolyl-4-hydroxylase related protein in *Chlamydomonas reinhardtii* (CrP4H) when complexed with a proline-rich substrate (Fig. 4 A and B and Fig. S6 D and E)(25, 28). In PHD2 and CrP4H, the  $\beta 2$ - $\beta 3$  finger loop (PHD2: 23 residues, 236-258; CrP4H: 24 residues, 76-99) and  $\beta 5$ (II)- $\beta 6$ (III) thumb loop (PHD2: 6 residues; 316-321; CrP4H: 15 residues, 146-160) are of similar length and in the same locations as those of PPHD<sub>putida</sub> (Fig. 4 A and B and Fig. S7 A and B); however, an analogous Arg57:Asp130 salt bridge that connects the finger and thumb loops is not present in PHD2 or CrP4H.

The PHD2 C-terminal  $\alpha$ -helix is involved in substrate binding and catalysis (16, 25). The PPHD<sub>putida</sub> C-terminal loop (residues 201-207, corresponding to PHD2 392-408) directly interacts with EF-Tu as does the PHD2 C-terminal  $\alpha$ -helix with HIF- $\alpha$  CODD (Fig. 4A inset), suggesting evolutionary conservation of a role for the C-terminus in substrate recognition.

Comparison of the substrate complexes reveals that conservation is not limited to the enzymes, but extends to the bound substrates. Strikingly, the unrelated substrates all adopt closely related conformations in the active site regions when bound to their respective prolyl-hydroxylase partners; EF-Tu (residues 50-57)



complexed with PPHD shares a similar orientation and conformation to that of HIF- $\alpha$  (residues 560-567) complexed with PHD2 (backbone atoms r.m.s.d. 0.1 Å) and to that of a proline-rich peptide complexed with CrP4H (backbone atoms r.m.s.d. 0.1 Å)(Fig. 4 A and B and Fig. S6 D and E). However, there are differences in observed substrate binding for the prolyl-4- (PPHD, PHDs, CP4Hs) and that predicted for prolyl-3-hydroxylases (OGFOD1/Tpa1)(Fig. S7 A and B). The prolyl-4-hydroxylases have a conserved arginine at their active site opening positioned to hydrogen bond to the carbonyl of the substrate proline (Arg134<sub>PPHD</sub>, Arg322<sub>PHD2</sub>, Arg161<sub>CrP4H</sub>). Prolyl-4-hydroxylases also have a conserved tyrosine (Tyr121<sub>PPHD</sub>, Tyr310<sub>PHD2</sub>; Tyr140<sub>CrP4H</sub>), the hydroxyl of which points towards the nitrogen of the substrate proline (Fig. 4 A and B and Fig. S6 D and E and Fig. S7B). In contrast, the prolyl-3-hydroxylases have a smaller hydrophobic leucine at the equivalent position to Tyr121<sub>PPHD</sub> (Leu156<sub>Tpa1</sub>; Leu152<sub>OGFOD1</sub>)(29), which may promote prolyl-3- over prolyl-4-hydroxylation by adjusting the position of the proline within the active site.

In the PPHD<sub>putida</sub> active site, the pyrrolidine ring of Pro54<sub>EF-Tu</sub> is observed in a C-4 *endo* conformation, poised for hydroxylation as observed in the PHD2:HIF- $\alpha$  and CrP4H:proline-rich peptide complexes (Fig. 4 A and B and Fig. S6 D and E)(25, 28, 30), and in contrast to the C-4 *exo* conformation as observed for prolyl-hydroxylated HIF-1 $\alpha$  when complexed with pVHL (1).

## DISCUSSION

The combined results suggest that roles for prolyl-hydroxylation are ancient, extending from EF-Tu in bacteria to, at least, collagen, HIF- $\alpha$ , and RPS23 in eukaryotes. The results support growing evidence for the importance of 2OG oxygenases, including prolyl-hydroxylases, in the regulation of translation as well as of transcription (5-8, 29, 31-35). It is notable that JmjC subfamily 2OG oxygenases also play roles both in histone/chromatin and ribosomal protein modification (5, 36).

Structurally guided bioinformatic analyses are informative with regard to the distribution of prolyl-hydroxylases. Comparison of the topologies of the prolyl-3- and -4-hydroxylases for which crystal

structures are available reveals conservation not only of the DSBH, Fe(II), and 2OG binding sites, but also of other functionally important elements including the  $\beta$ 2- $\beta$ 3 finger loop and  $\alpha$ -helices that pack against the DSBH (Fig. S7 A and B), enabling assignment of putative prolyl-hydroxylases with a high confidence level. All identified subtypes of prolyl-4-hydroxylases (CP4Hs and PHDs) and prolyl-3-hydroxylases [Leprecans (mammalian collagen prolyl-3-hydroxylases) and OGFOD1s] are ubiquitous in animals, and all but the Leprecans are present in choanoflagellates. However, their presence in prokaryotes is sporadic and they are apparently absent in archaea (Fig. 5 and Fig. S8).

Given that HIF and collagen exist in animals but not bacteria, protein prolyl-hydroxylases (and other enzymes catalyzing post-translational modifications) may have first evolved to catalyze oxidation of highly abundant proteins, such as the switch I loop of EF-Tu, which are part of the translational machinery. Such protein hydroxylases possibly derived from 2OG oxygenases acting on small-molecules, e.g. proline-hydroxylases (37), some of which have simpler structures than the protein-hydroxylases (23). In eukaryotes, the prolyl-hydroxylases evolved divergently to catalyze hydroxylation of collagen and other proteins involved in translational and transcriptional regulation, e.g. Skp1 and RPS23 (6-9). Their role in HIF- $\alpha$  hydroxylation evolved in animals, possibly via fusion of a basic helix-loop-helix domain transcription factor with an EF-Tu, collagen, or Skp1 related, or derived substrate. The observation that PHD2 interacts with, but apparently does not hydroxylate, eEF2, the eukaryotic counterpart to prokaryotic EF-G, an EF-Tu homolog (15), is interesting because it may reflect an ancestral relationship between PPHD and EF-Tu. Competition between HIF- $\alpha$  and eEF2 [and potentially other PHD substrates, e.g. pyruvate kinase M2 (PKM2)(38) or centrosomal protein 192 (Cep192)(39)], may help to regulate the hypoxic response in higher animals. Analogous competition is proposed for factor inhibiting HIF (FIH) catalyzed HIF-1 $\alpha$  asparagine hydroxylation, which reduces HIF activity by blocking its interaction with coactivator proteins (1). FIH also takes ankyrin repeat domains as substrates, in a manner proposed to enable a robust and tuneable hypoxic response (40).

The conformational changes observed on substrate binding in the PPHD:EF-Tu complex reveal that major induced fit during 2OG oxygenase catalyzed protein-modification can occur. The observed changes will be useful in designing new types of 2OG oxygenase inhibitors based on various conformational states, rather than active site iron chelators, which comprise most reported 2OG oxygenase inhibitors including PHD inhibitors in clinical trials (41).

The finding that PPHD regulates the production of siderophore pyocyanin implies that iron regulation in *P. aeruginosa* may be important in terms of clinically relevant infections. The role of the PHDs in the hypoxic response has been highlighted, in part due to medicinal opportunities relating to the treatment of ischaemic and related diseases such as anaemia (41). While regulation of PHD activity by iron availability is well-established [cobaltous ion induced erythropoiesis is proposed to be via PHD inhibition (41)], it has had less prominence. *P. aeruginosa* infections, which often occur in hypoxic niches, such as in the cystic fibrosis lung (42), can induce a HIF-mediated hypoxic response in eukaryotic cells by production of (a) small molecule factor(s), which has been proposed to be cyanide (43). Pyocyanin induces HIF- $\alpha$  upregulation in human cells (13), likely via PHD inhibition, at least in part, by iron sequestration. Thus, it seems that during some infections prolyl-hydroxylases in both human and bacterial cells may be involved in 'cross talk' involving regulation limited not only by oxygen availability, but also by that of iron and, possibly, 2-oxoglutarate (or other TCA cycle intermediate or related compound)(44). Therefore, the biological roles of the PHDs and PPHDs likely extend beyond hypoxic sensing in an isolated organism.

From a clinical perspective the work is relevant to ongoing trials involving PHD inhibitors for anaemia treatment (41). At least some PHD inhibitors inhibit other 2OG oxygenases (41, 45). There is also increasing use of metal chelators, including orally active compounds (which inhibit the PHDs by iron chelation)(46), for iron overload diseases. While our work does not predict what effect, if any, application of clinically applied PHD inhibitors/iron chelators will have on the course of infections by bacteria containing PHD homologs, the potential for effects of PHD inhibitors on infection should be considered.

## Materials and Methods

### Protein purification and crystallization.

Recombinant PPHD<sub>putida</sub> and EF-Tu<sub>putida</sub> were produced in *E. coli* and purified using Ni-affinity and size exclusion chromatography. For purification of the PPHD:EF-Tu complex, cell pellets containing either PPHD<sub>putida</sub> or EF-Tu<sub>putida</sub> were co-lysed with Mn(II) and NOG to prevent EF-Tu hydroxylation and purified in the same way. For details of crystallization conditions, data collection and processing, and refinement statistics, see SI Methods, Table S1, and Table S2.

### *P. aeruginosa* phenotypic analyses.

*P. aeruginosa* PAO1 wildtype and PPHD deletion strain were grown (28 °C) in various concentrations of 2,2-bipyridine (0, 1, 5, and 25 mM); the optical density (600 nm, OD<sub>600</sub>) was assayed every 60 mins. For pyocyanin quantification, 5 mL cultures of *P. aeruginosa* wildtype and PA0310 deleted strains grown to stationary phase were extracted with 3 mL of CHCl<sub>3</sub>, then back extracted with 1 mL of 0.2 HCl, and the aqueous phase decanted and its absorbance measured at 520 nm. For global translation rate assays, 1 mL cultures of *P. aeruginosa* wildtype and PA0310 deleted strains were grown to log phase (OD<sub>600</sub> = 0.5) and the rate of incorporation of <sup>35</sup>S-methionine (10 µCi/mL) was measured by scintillation counting.

### Determination of EF-Tu as a PPHD substrate.

Hydroxylation assays for PPHD<sub>putida</sub> contained 10 µM PPHD, 100 µM EF-Tu<sub>44-63</sub> (H<sub>2</sub>N-IVEFDKIDSAPEEKARGITI-CONH<sub>2</sub>), 50 µM Fe(II)(prepared from (NH<sub>4</sub>)<sub>2</sub>Fe(SO<sub>4</sub>)<sub>2</sub>), 2 mM 2OG, 4 mM sodium L-ascorbate in 50 mM HEPES pH 7.5, 500 mM NaCl, 5% (v/v) glycerol and quantified by MALDI MS. MALDI MS/MS was performed to determine the site of hydroxylation. To determine the stereochemistry of hydroxylation, amino acid hydrolysate of Hyp54 modified EF-Tu<sub>44-63</sub> was derivatized and compared with the elution times of hydroxyproline standards. To analyze EF-Tu Pro54 hydroxylation, cells were lysed, analyzed by SDS-PAGE, and the EF-Tu containing band was excised and subjected to proteosomal digestion (ArgC) and LC-MS/MS. For purification of recombinant Hyp54 EF-Tu, PPHD<sub>putida</sub>, Fe(II), 2OG, and sodium L-ascorbic acid were added to the cell lysate at a final concentration of 1 mg/mL, 50 µM, 2 mM, and 4 mM, respectively, and incubated for 1 hour at room temperature. The hydroxylation status of Pro54 was then analyzed by trypsinolysis and MSMS analysis.

### NMR experiments

Measurements of Mn(II) and 2OG *K<sub>d</sub>* to PPHD<sub>putida</sub> employed a Bruker AVII 500 instrument (total sample volume 160 µL). Solutions were buffered using ~30 mM Tris-d11 and ~20 mM HEPES pH 7.5 in 36.25% H<sub>2</sub>O and 63.75% D<sub>2</sub>O (Fig. S5 F and G). Solutions contained 10 µM unmodified or Hyp54 EF-Tu, 50 µM kirromycin, 100 µM GTP, 50 µM PPHD<sub>putida</sub> (where applicable), 100 µM ZnCl<sub>2</sub> (where applicable), 100 µM 2OG (where applicable), 10 mM MgCl<sub>2</sub>, and 200 mM NaCl. Solutions were buffered in 50 mM HEPES pH 7.5 dissolved in 90% H<sub>2</sub>O and 10% D<sub>2</sub>O. <sup>1</sup>H NMR spectroscopy was used to monitor the conversion of GTP to GDP in real-time. For ZnCl<sub>2</sub> inhibition experiments, mixture contained 10 µM *apo* PPHD<sub>putida</sub>, 50 µM FeCl<sub>2</sub>, 500 µM 2OG, 600 µM EF-Tu<sub>44-63</sub>, 400 µM ZnCl<sub>2</sub> (where applicable), 1.25 mM ascorbate and 100 mM NaCl buffered using 50 mM Tris-D11 (pH 7.5)(Fig. S5D).

See SI for detailed Materials and Methods.

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## Figure Legends

**Fig. 1.** Identification of EF-Tu as a PPHD<sub>putida</sub> substrate. (A) PPHD<sub>putida</sub>, human HIF (PHDs) and collagen (CP4Hs) prolyl-4-hydroxylases catalyze 4-*trans* prolyl-hydroxylation. (B) EF-Tu is a PPHD<sub>putida</sub> substrate; MS spectrum showing a +16 Da peak revealing PPHD<sub>putida</sub>-dependent prolyl-4-hydroxylation of EF-Tu<sub>44-63</sub>.

**Fig. 2.** PPHD<sub>putida</sub> is structurally similar to human PHD2. (A) Ribbons representation of the overall structure of PPHD<sub>putida</sub>; DSBH-associated  $\beta$ -strands (pale cyan), roman numerals indicate strands in the DSBH, DSBH-extension  $\beta$ -strands (dark blue),  $\alpha$ -helices (red), loops (white), NOG (yellow), Mn(II) (pink sphere). (B) Superposition of the PPHD<sub>putida</sub>(red):NOG(yellow) and human PHD2(blue):1(gray) (PDB ID: 2G1M) (r.m.s.d. 1.7 Å over 181 C $\alpha$  atoms) structures. (C) Superimposition of active site residues of PPHD<sub>putida</sub>(green):NOG(yellow) and human PHD2(brown):1(gray) (PDB ID: 2G1M) [1: *N*-(4-hydroxy-8-iodoisoquinolin-3-yl)carbonylglycine].

**Fig. 3.** A PPHD:EF-Tu complex structure reveals major conformational changes in both proteins. (A) View from a PPHD<sub>putida</sub>(magenta):EF-Tu(cyan) complex crystal structure reveals EF-Tu in its GDP-bound state; the EF-Tu switch I loop is located in the PPHD active site positioning Pro54 in its *endo*-conformation for *trans*-4-hydroxylation. 6 residues (43-48) in the switch I loop are disordered (dotted line). Inset: details of how PPHD interacts with EF-Tu domain II. (B) The EF-Tu switch I loop is enclosed in the PPHD catalytic site; the electron density  $|F_o - F_c|$  map is in green mesh contoured to 3.0  $\sigma$ . (C) Surface representation of the PPHD<sub>putida</sub>(magenta):EF-Tu(cyan) complex revealing enclosure of the PPHD<sub>putida</sub>  $\beta$ 2- $\beta$ 3 finger loop and the  $\beta$ 5(II)- $\beta$ 6(III) thumb loop around the EF-Tu switch I loop. (D) Superimposition of domains II and III of *P. putida* EF-Tu (cyan) in complex with PPHD (r.m.s.d. 0.6 Å)(PPHD not shown) and *E. coli* EF-Tu (yellow) (PDB ID: 2BVN)(27) bound to enacyloxin IIA (blue sticks) and a non-hydrolyzable GTP-analogue GDPNP (red). (E) Superimposition of *E. coli* EF-Tu (PDB ID: 1DG1)(26) and *P. putida* EF-Tu from the PPHD<sub>putida</sub>:EF-Tu complex; the switch I loop is highlighted (*P. putida* EF-Tu, blue; *E. coli* EF-Tu, red). (F) Superimposition of the  $\beta$ 5(II)- $\beta$ 6(III) thumb loop as in isolated PPHD (green) and in the PPHD<sub>putida</sub>(magenta):EF-Tu(cyan) complex reveals reorganization of thumb residues on substrate binding: Phe128<sub>PPHD</sub> rotates 90° to position its phenyl ring directly behind Pro54<sub>EF-Tu</sub>; Asp131<sub>PPHD</sub> forms a salt bridge with Arg133<sub>PPHD</sub>; Asp132<sub>PPHD</sub> rotates 180° to salt bridge to Arg234<sub>EF-Tu</sub> of domain II allowing Arg134<sub>PPHD</sub> to hydrogen bond to Pro54<sub>EF-Tu</sub>.

**Fig. 4.** *Pseudomonas* PPHD, human PHD2, and *C. reinhardtii* P4H (CrP4H) share a conserved mechanism for substrate-binding. Overall and active site superimpositions of the PPHD<sub>putida</sub>(magenta):EF-Tu(cyan) complex and both (A) human PHD2 (sand) in complex with HIF- $\alpha$  CODD (residues 556-574; PDB ID: 3HQR) (green) and (B) CrP4H (green) in complex with proline-rich peptide substrate (PDB ID: 3GZE) (yellow) reveal conservation of the roles of the  $\beta$ 2- $\beta$ 3 finger loop and the  $\beta$ 5(II)- $\beta$ 6(III) thumb loop, the orientation of the substrate backbone in the active site, and other key substrate binding interactions. Note that while CrP4H does not share a structurally conserved Arg133<sub>PPHD</sub> with PPHD as does PHD2 (see inset), CrP4H does share a conserved Glu109<sub>PPHD</sub> (Glu127<sub>CrP4H</sub>; Thr296<sub>PHD2</sub>). Inset: the PPHD<sub>putida</sub> and PHD2 C-termini interact differently with their substrates. In contrast, the PHD2 C-terminus interacts directly with HIF- $\alpha$  CODD (25).

**Fig. 5.** Distribution of prolyl-hydroxylases in Kingdoms of life. Tracking putative homologs of the CP4Hs, PHDs, Leprecans (CP3Hs), and OGFOD1s in representative organisms throughout the Kingdoms of life (for phylogenetic analyses encompassing a wider distribution, see Fig. S8). CP4Hs and PHDs catalyze prolyl-4-hydroxylation. Human OGFOD1 catalyzes a single prolyl-3-hydroxylation, whereas its yeast homolog Tpa1 catalyzes a double prolyl-3- and prolyl-4-hydroxylation (7).



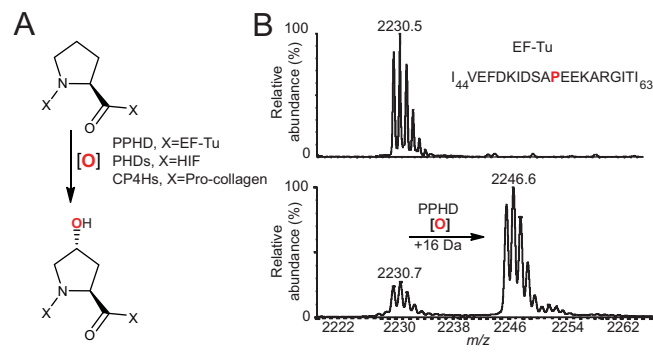


Fig. 1

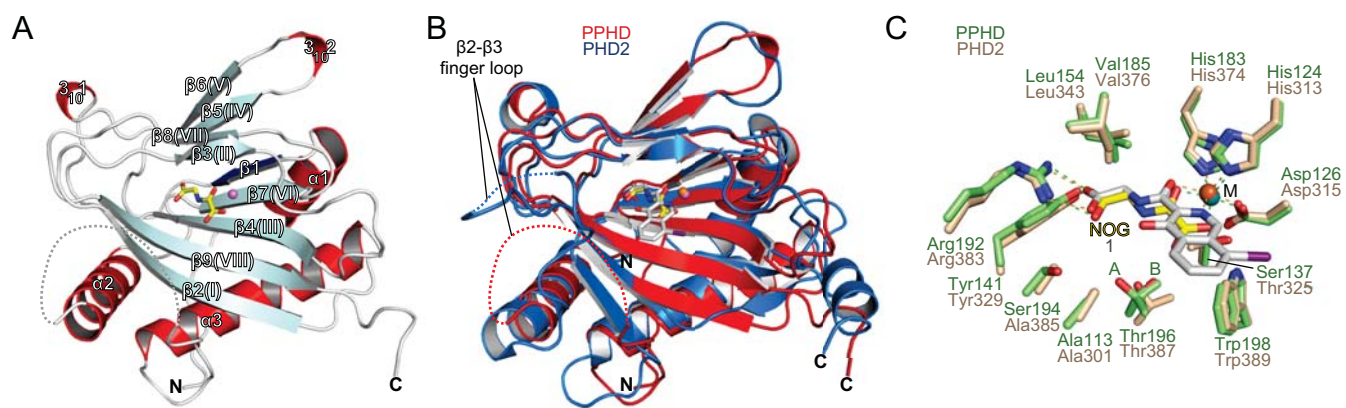


Fig. 2

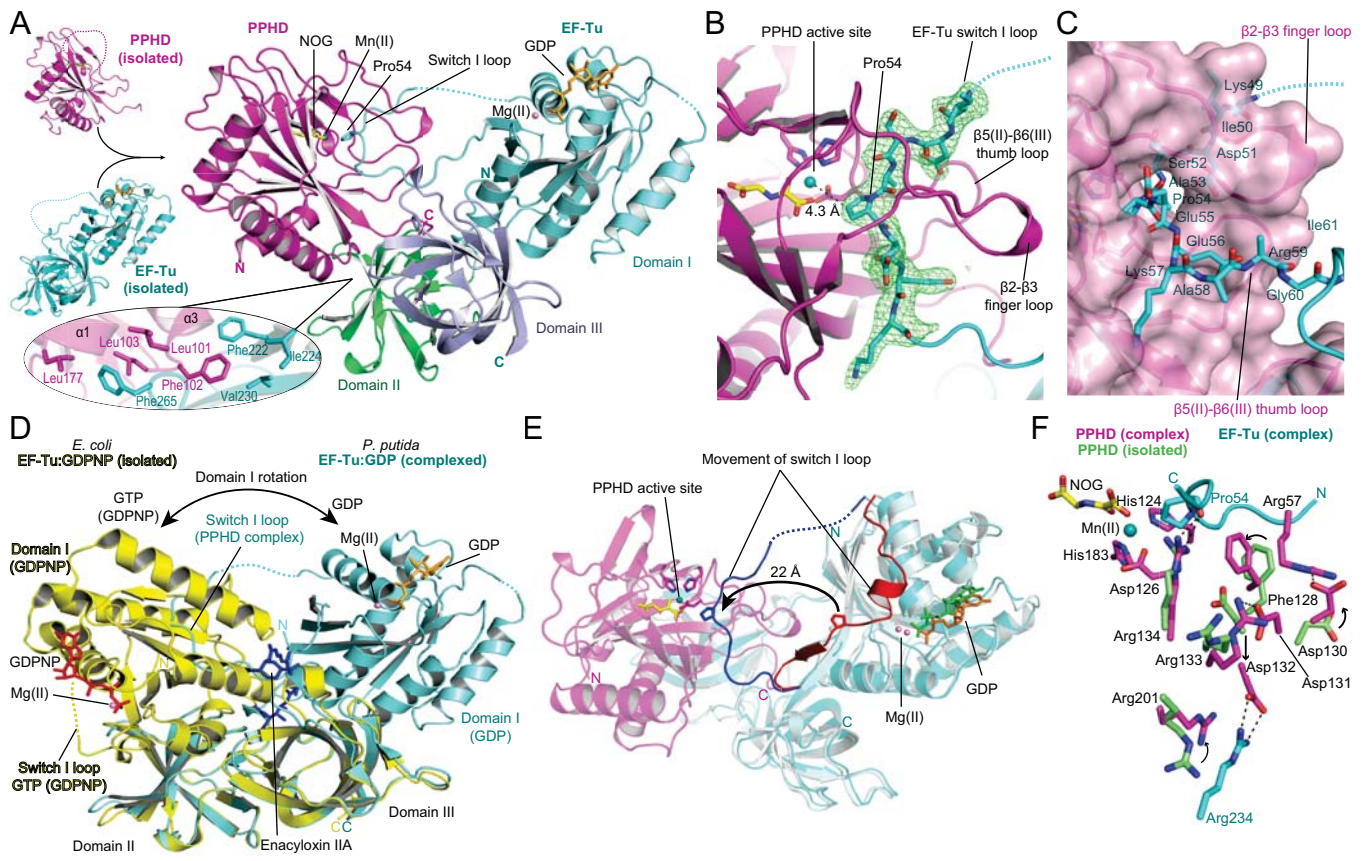


Fig. 3

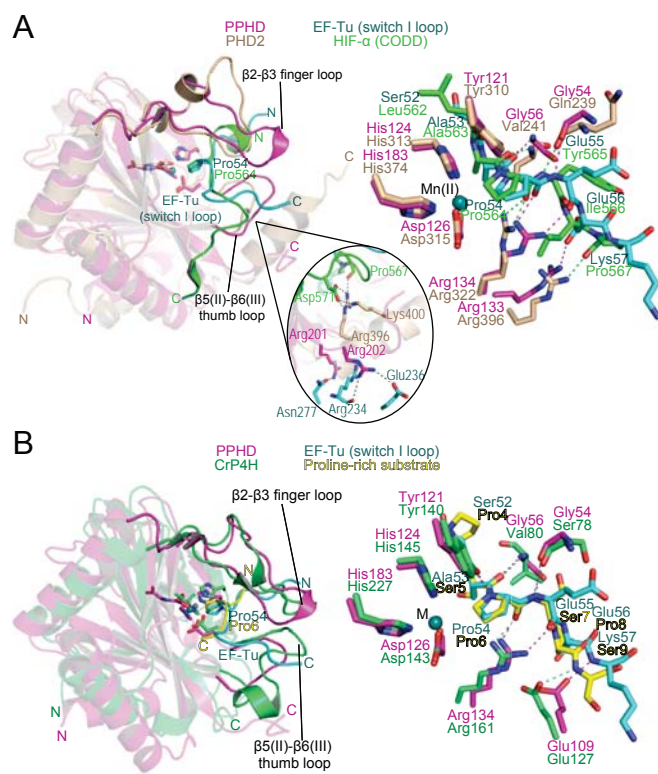


Fig. 4

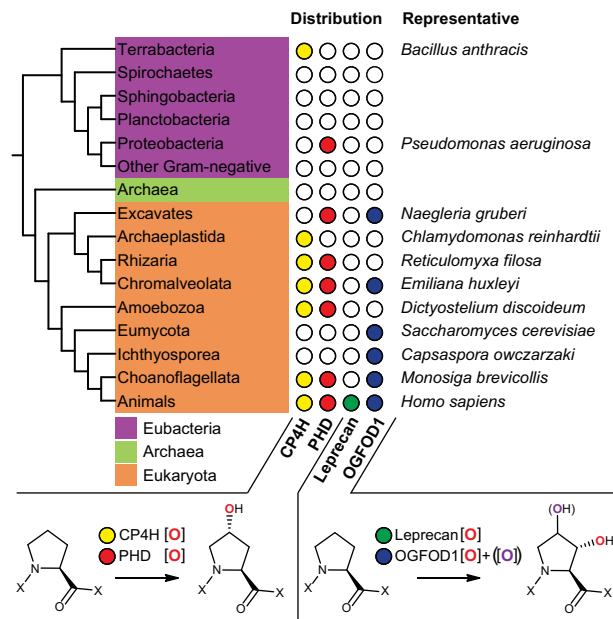


Fig. 5