The Length of Peptide Substrates Has a Marked Effect on Hydroxylation by the Hypoxia-inducible Factor Prolyl 4-Hydroxylases*

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Three hypoxia-inducible factor prolyl 4-hydroxylases (HIF-P4Hs) regulate the HIFs by hydroxylation of prolines at two separate sites in the oxygen-dependent degradation domain (ODDD) of their α subunits. We compared in vitro hydroxylation by purified recombinant human HIF-P4Hs of 19–20- and 35-residue peptides corresponding to the two sites in HIF-α and purified recombinant HIF-1α and HIF-2α ODDDs of 248 and 215 residues. The increase in the length of peptides representing the C-terminal site from 19 to 20 to 35 residues reduced the $K_m$ values to 90–800 nM, i.e. to 0.7–11% of those for the shorter peptides, whereas those representing the N-terminal site were 10–470 μM, i.e. 10–135%. The $K_m$ values of HIF-P4H-1 for the recombinant HIF-α ODDDs were 10–20 nM, whereas those of HIF-P4H-2 and -3 were 60–140 nM, identical values being found for the wild-type HIF-1α ODDD and its N site mutant. The $K_m$ values for the C site mutant were about 5–10 times higher but only 0.2–3% of those for the 35-residue N site peptides, and this marked difference suggested that the HIF-P4Hs may become bound first to the C-terminal site of an ODDD and that this binding may enhance subsequent binding to the N-terminal site. The $K_m$ values of HIF-P4H-2 for oxygen determined with the HIF-1α ODDD and both its mutants as substrates were all about 100 μM, being 40% of those reported for the three HIF-P4Hs with a 19-residue peptide. Even this value is high compared with tissue O$_2$ levels, indicating that HIF-P4Hs are effective oxygen sensors.

The hypoxia-inducible factors (HIFs) are master regulators of the transcription of more than 100 hypoxia-regulated genes and play central roles in cellular oxygen homeostasis. HIFs are heterodimers that consist of an oxygen-regulated α subunit (HIF-α) and a stable β subunit (HIF-β), and both types of subunits are members of the basic helix-loop-helix Per-Arnt-Sim protein family. The human α subunit has three isoforms, HIF-1α to HIF-3α (for reviews, see Refs. 1–3). HIF-1α and HIF-2α are synthesized continuously, and hydroxylation of at least one of two critical proline residues in their oxygen-dependent degradation domain (ODDD), Pro$^{402}$ and Pro$^{564}$ in HIF-1α, generates a binding site for the von Hippel-Lindau (VHL) ubiquitin-protein isopeptide ligase complex that targets them for rapid proteasomal degradation under normoxic conditions (4–7). This hydroxylation is catalyzed in humans by three recently identified cytoplasmic and nuclear HIF prolyl 4-hydroxylases (HIF-P4Hs 1–3, also named PHD1–3, HPH3-1, and EGLN2, -1, and -3, respectively) (8–10). These are distinct from the well characterized collagen prolyl 4-hydroxylases (C-P4Hs) that reside within the lumen of the endoplasmic reticulum and likewise have three human isoenzymes (11–13). All P4Hs are 2-oxoglutarate dioxygenases and require Fe$^{2+}$, 2-oxoglutarate, O$_2$, and ascorbate (2, 3, 11, 14). The lack of oxygen inhibits these hydroxylations so that HIF-α is no longer recognized by the VHL protein and degraded but instead dimerizes with HIF-β. These dimers then become bound to the HIF-responsive elements in various hypoxia-regulated genes (1–3).

Hydroxylation of a specific asparagine in the C-terminal transactivation domain of an HIF-α prevents its interaction with the transcriptional coactivator p300, thus inhibiting its full transcriptional activity (15). The asparaginyl hydroxylase responsible for this modification is identical to a previously identified factor inhibiting HIF (FIH) (16, 17). It is also one of the 2-oxoglutarate dioxygenases (16, 17), but its catalytic properties are distinct from those of the HIF-P4Hs (18).

The proline residues to be hydroxylated in HIF-α are located in -Leu-X-X-Leu-Ala-Pro- sequences. It was therefore initially suggested that the HIF-P4Hs may require this conserved core motif (1–3). Early mutagenesis experiments supported this suggestion (8, 10), but subsequent studies indicated that the two leucines can be replaced by many residues (19, 20), alanine being the only relatively but not absolutely strict requirement in addition to the proline itself (20). The HIF-P4Hs require long substrates, the shortest HIF-α-like peptide hydroxylated by all three recombinant human isoenzymes having 11 residues (21). All three isoenzymes hydroxylated 19-residue peptides with sequences corresponding to those around the C-terminal hydroxylation site in HIF-α, with $K_m$ values of about 5–15 μM for HIF-1α and HIF-3α-like peptides and

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2 The abbreviations used are: HIF, hypoxia-inducible transcription factor; ODDD, oxygen-dependent degradation domain; VHL, von Hippel-Lindau; HIF-P4H, HIF prolyl 4-hydroxylase; C-P4H, collagen prolyl 4-hydroxylase; FIH, HIF asparaginyl hydroxylase.
Peptides and HIF Prolyl 4-Hydroxylases

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant HIF-P4Hs—** FLAG His-tagged HIF-P4H-1–3 (24) were expressed in H5 insect cells cultured in suspension or on plates in S9000ISFM serum-free medium (Invitrogen). The cells, seeded at a density of $1 \times 10^6$/ml, were infected with recombinant baculoviruses at a multiplicity of 5, harvested 72 h after infection, and then were washed with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4, and homogenized in a buffer containing 0.15 M NaCl, 0.1 M glycerine, 10 mM dithiobiotrel, 0.1% Triton X-100, 1 or 5 $\mu$M FeSO$_4$, and 0.01 M Tris, pH 7.8, supplemented with Complete EDTA-free protease inhibitor mixture (Roche Applied Science). The recombinant HIF-P4Hs were purified from the soluble fractions with an anti-FLAG M2 affinity gel (Sigma) (24).

**Expression and Purification of Recombinant ODDDs—** The primer pairs 5’-GCGCATATGCAAACAGAATGTGTCCTT-AAAACGG-3’ and 5’-GCGCTCGAGCTGGATACTGTA-CTGTCCTTTGAGG-3’, and 5’-GCGCATATGCAACT- GAATCCCTGTTCAGCCCACTCTTCC’ and 5’-GCGCTCGAGCT-GGAAGATGTTTGTCATGGCACTGAAGC-3’ (NdeI and Xhol sites underlined) were used to amplify cDNA fragments encoding the ODDDs of HIF-1$\alpha$ and HIF-2$\alpha$, residues Gl$\alpha$356–Gl$\alpha$358 and Gl$\alpha$358–Gl$\alpha$572, respectively, by PCR using the plasmids d386e and d286e containing full-length HIF-1$\alpha$ and HIF-2$\alpha$ cDNAs (a gift from FibroGen Inc.) as templates. The NdeI-Xhol-digested PCR fragments were cloned into a similarly cutPET-22b(+) *Escherichia coli* expression vector (Novagen) in-frame with a C-terminal histidine tag. Site-directed mutagenesis of the HIF-1$\alpha$ ODDD to P402A and P564A mutant ODDDs and to P402A/P564A double mutant ODDD was performed using the QuikChange™ kit (Stratagene). The expression plasmids were transformed into the *E.coli* BL21(DE3) strain (Novagen), and the cells were grown at 37°C to an absorbance of 0.4 at 600 nm, and expression was induced with 1 mM isopropyl-1-thio-β-d-galactopyranoside. Cells were harvested after a 3-h induction, solubilized, and boiled in the SDS-PAGE sample buffer for 5 min, and expression of the recombinant ODDDs was analyzed by 12% SDS-PAGE under reducing conditions followed by Coomassie Blue staining.

The recombinant ODDDs were purified from 150–300-ml cultures on a chelating Sepharose column charged with Ni$^{2+}$ (ProBond, Invitrogen). The cells were harvested after a 3-h induction, suspended in 1/20 volume of a 0.5 M NaCl, 0.1% Triton X-100, and 20 mM Tris-HCl buffer, pH 8, supplemented with Complete EDTA-free protease inhibitor mixture (Roche Applied Science), disrupted by sonication, and centrifuged at 17,000 × *g* for 20 min, and the soluble fractions were applied to a chelating Sepharose column stabilized with a solution of 0.5 M NaCl and 20 mM Tris-HCl, pH 8. The column was washed with the same buffer containing 0.02 M imidazole and eluted with a 150-mL linear imidazole gradient (0.02–0.3 M), and the fractions were analyzed by 12% SDS-PAGE. The fractions containing the recombinant ODDD polypeptides were pooled and concentrated using Amicon ultra centrifugal devices with *M*$_r$ 5,000 cut-off membrane (Millipore) and applied to a Superdex S-200 column (Amersham Biosciences) in a 0.3 M NaCl, 5 mM dithiothreitol, 20 mM Tris buffer, pH 8.0. Fractions of 2 ml were col-

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10–30 $\mu$m for an HIF-2$\alpha$-like peptide (21). A subsequent study indicated that a leucine located 10 residues downstream from the proline influences its hydroxylation by HIF-P4Hs (22), which agrees with the previous finding that deletion of two residues, a glutamine and a leucine, from the C terminus of a 19-residue peptide corresponding to the C-terminal hydroxylation site in HIF-1$\alpha$ increased its *K$_m$* values for HIF-P4H-1 and -2 but not for HIF-P4H-3 (21). As the 19-residue peptide used to study hydroxylation of the C-terminal site in HIF-2$\alpha$ ended just before this leucine (21), it is unknown whether its slightly higher *K$_m$* value relative to those of the HIF-1$\alpha$ and HIF-3$\alpha$ peptides (above) is simply because of the lack of this residue. A 19-residue peptide corresponding to the N-terminal hydroxylation site in HIF-1$\alpha$ had much higher *K$_m$* values for HIF-P4H-1 and -2 than the C-terminal site peptide and was not hydroxylated by HIF-P4H-3 to any significant extent at all, whereas a 19-residue peptide corresponding to the N-terminal site in HIF-2$\alpha$ was hydroxylated by all three isoenzymes, but again with distinctly higher *K$_m$* values than the corresponding C-terminal site peptide (21).

As the *K$_m$* values determined for the 19-residue HIF-2$\alpha$-like peptides are relatively high (21), and as residues such as a leucine located 10 residues downstream of the proline to be hydroxylated (22) and also some of the acidic residues present in various parts of the 19-residue peptides (19, 20) have been shown to have distinct effects, we studied whether the *K$_m$* values for 35-residue HIF-2$\alpha$-like peptides and recombinant HIF-1$\alpha$ and HIF-2$\alpha$ fragments of 248 and 215 residues, respectively, differ from those determined for shorter peptides. The HIF-2$\alpha$ fragments correspond to the ODDDs and contain both proline hydroxylation sites in the same polypeptide, which made it possible to study whether this situation differs from the presence of the two sites in separate peptides. Hydroxylation of only one of the two sites in the HIF-1$\alpha$ ODDD could be studied using its two mutants, P402A and P564A. A recent study has indicated that Pro$_{P402}$ in HIF-1$\alpha$ is hydroxylated before Pro$_{P564}$ in cultured cells and that hydroxylation of Pro$_{P402}$ is inhibited at higher oxygen tensions than that of Pro$_{P564}$ (23). We therefore also determined the *K$_m$* values of HIF-P4H-2 for O$_2$ in the hydroxylation of the wild-type and the two mutant HIF-1$\alpha$ ODDDs. Our data indicate that increasing the peptide length from 19 to 20 to 35 residues has a marked effect on the *K$_m$* values for peptides representing the C-terminal hydroxylation site in all three HIF-2$\alpha$ isoforms, whereas more modest or no effects are seen in the case of peptides representing the N-terminal site. The *K$_m$* values determined for the C-terminal hydroxylation site in the recombinant HIF-1$\alpha$ ODDD are in most cases still somewhat lower than those for the corresponding 35-residue peptides, whereas the *K$_m$* values for its N-terminal site are markedly lower than those for the 35-residue N site peptides, and this site had a low *K$_m$* value even for HIF-P4H-3, which had very high *K$_m$* values for the corresponding 20- and 35-residue peptides. The *K$_m$* value of HIF-P4H-2 for O$_2$ determined with the HIF-1$\alpha$ ODDD was lower than that reported with a 19-residue peptide, but was still high, about 100 $\mu$m, and no difference was found in this *K$_m$* value when determined with the wild-type or the two mutant ODDDs.
**Peptides and HIF Prolyl 4-Hydroxylases**

lected and analyzed by 12% SDS-PAGE. Those containing the pure ODDD polypeptides were pooled, and their buffer was changed to 50 mM Tris-HCl, pH 8, by concentrating as before, and the protein concentrations were measured by RotiQuant (Carl Roth GmbH).

**HIF-P4H Activity Assays**—HIF-P4H activity was assayed by a method based on measurement of the hydroxylation-coupled stoichiometric release of $^{14}$CO$_2$ from 2-oxo[1-14C]glutarate in a final reaction volume of 0.5 ml (21). The $K_m$ values of the purified enzymes for the synthetic HIF-1α, HIF-2α, and HIF-3α peptides (Table 1), the recombinant ODDD polypeptides, and of HIF-P4H-2 for O$_2$, with the wild-type HIF-1α ODDD and its P402A and P564A mutants as substrates were determined as described previously (21). The purities of the synthetic HIF-α peptides (Innovagen) were about 80%. The $K_m$ values for the 19–20-residue and 35-residue peptides and the ODDD polypeptides were determined using 2-oxoglutarate with a specific activity of 1100 or 11,100, 11,100 or 55,500, and 55,500 dpm/nmol, respectively. The relative $V_{max}$ values of each HIF-P4H for the various peptide substrates were determined with respect to that obtained with the 19-residue peptide representing the C-terminal hydroxylation site of HIF-1α in the same experiment.

In additional experiments HIF-P4H-3 activity was assayed by using [3H]proline-labeled peptides and measuring the amount of 4-hydroxy-[3H]proline formed. The cDNAs for the wild-type, P402A and P564A mutant, and the P402A/P564A double mutant HIF-1α ODDDs in pET-22b(+) were translated in the presence of [2,3,4,5-3H]proline (85 mCi/mm; PerkinElmer Life Sciences) in rabbit reticulocyte lysate using a TNT® Quick-coupled transcription/translation system (Promega). The translation products were analyzed by 12% SDS-PAGE followed by fluorography. Aliquots of 45 μl of the translation products containing $\sim 2 \times 10^6$ cpm of incorporated radioactive proline were used as substrates for the purified recombinant HIF-P4H-3 in a final reaction volume of 1 ml under the reaction conditions described previously (21), except that the 2-oxoglutarate was nonlabeled. In control experiments the recombinant HIF-P4H-3 was omitted. The enzyme reactions were dialyzed extensively to remove any remaining free [2,3,4,5-3H]proline, and the 4-hydroxy[3H]proline formed in the substrate was analyzed by a specific radiochemical procedure (25).

**RESULTS**

**$K_m$ Values of the Three HIF-P4Hs for 35-Residue Peptides Representing the C-terminal Hydroxylation Site in HIF-1α, HIF-2α, and HIF-3α Are Markedly Lower than Those for the Corresponding 19–20 Residue Peptides**—The human HIF-P4H-1–3 were expressed as FLAG His-tagged recombinant enzymes in insect cells and purified to near homogeneity by anti-FLAG affinity chromatography (24). The 35-residue peptides were designed to contain 17 amino acids on each side of the proline to be hydroxylated (Table 1). The $K_m$ values of the HIF-P4Hs were determined by a method based on measurement of the hydroxylation-coupled stoichiometric release of $^{14}$CO$_2$ from 2-oxo[1-14C]glutarate (21, 24). These values were compared with those determined previously for the corresponding 19–20-residue peptides (21), and additional values were determined for some of the short peptides. As the 19-residue HIF-2α peptide studied previously ended just before a leucine subsequently reported to influence hydroxylation (22), we also determined the $K_m$ values for a 20-residue peptide containing this leucine (Table 1). Its $K_m$ values were about 50–70% of those for the 19-residue peptide, indicating that the leucine had a distinct although relatively modest effect (Table 2).

The $K_m$ values of the three isoenzymes for the 35-residue peptides were markedly lower than those for the corresponding 19–20-residue peptides, ranging in all but one case from 90 to 600 nm and being only about 0.7–5% of those for the shorter peptides, the only exception being the $K_m$ of HIF-P4H-2 for the 35-residue HIF-1α peptide, which was 800 nm and 11% (Table 2; Fig. 1A). The $K_m$ values of HIF-P4H-2 for the 35-residue peptides were higher than those of the other two isoenzymes (at least $p < 0.01$), the lowest $K_m$ being that of HIF-2α.

**TABLE 1**

| Peptide | Amino acid sequence |
|---------|---------------------|
| HIF-1αC19 | DDLLEMGLAPYPMDDDFQGL |
| HIF-1αC35 | KNIPSPGSTDDLLEMGLAPYPMDDDFQLSRFPQDSQLS |
| HIF-2αC19 | ELDDLETAPYPMDDDFEQ |
| HIF-2αC35 | QCSTQCTDFNELLLETLAAYPMDDDFEQPLICPE |
| HIF-3α20 | DALDLALEMLAPYMDDFQ |
| HIF-3α35 | LLDLEQCADDDLLEMGLAPYTMDDDFQLNASEQLP |
| HIF-1N20 | DALTLLRAPGDTITLSDFG |
| HIF-1N20A566P | DALTLLRAPGDTITLSDFG |
| HIF-1N20A558E | DALTLLRAPGDTITLSDFG |
| HIF-1N35 | SLFLKLRRPFDACLTLAAPGDTITLSDFGSNDTE |
| HIF-2N20 | EPEELAQLAPGDTITLSDFG |
| HIF-2N35 | FLFTKLEELPRESLAPGDTITLSDFG |

**TABLE 2**

| Peptide | $K_m$ (μM) | $V_{max}$ (pmol/min/mg) | $n$ |
|---------|-----------|-------------------------|-----|
| HIF-1αC19 | 10 ± 5 | 100 | 10 |
| HIF-1αC35 | 0.3 ± 0.06 | 100 ± 10 | 6 |
| HIF-2αC19 | 30 ± 10 | 70 ± 15 | 7 |
| HIF-2αC20 | 20 ± 5 | 105 ± 10 | 3 |
| HIF-3αC20 | 0.15 ± 0.05 | 80 ± 15 | 4 |
| HIF-3αC20 | 8 ± 5 | 115 ± 3 | 3 |
| HIF-3αC35 | 0.2 ± 0.06 | 120 ± 25 | 6 |

$^a$ The $V_{max}$ values are expressed relative to that obtained for each enzyme with the HIF-1αC19 peptide.

$^b$ The results shown for these peptides include those determined previously (21), in most cases together with some additional values determined in this study.
P4H-3 for the 35-residue HIF-1α peptide, 90 nM (Table 2). Interestingly, the increase in peptide length from 19–20 to 35 residues had no significant effect on the $V_{\text{max}}$ values, although there was a tendency for slightly lower $V_{\text{max}}$ values with the longer peptides in some cases (Table 2).

**An Increase in the Peptide Length from 19–20 to 35 Residues Has Relatively Small Effects on the $K_m$ Values for Peptides Representing the N-terminal Hydroxylation Site in HIF-1α and HIF-2α.** The $K_m$ values for 19–20-residue peptides representing the N-terminal hydroxylation site in HIF-1α and HIF-2α are much higher than those for the 19-residue peptides representing the C-terminal site (21). As the 19-residue peptide representing the N-terminal site in HIF-1α was subsequently found to erroneously lack an aspartate located in position +10 with respect to the proline to be hydroxylated, a new 20-residue peptide containing this aspartate (Table 1) was used here as the reference peptide (Table 3). The $K_m$ values of HIF-P4H-1 and -2 for this 20-residue peptide were about 50 μM, i.e. 5–7 times those for the corresponding C-terminal site peptide, whereas the $K_m$ of HIF-P4H-3 was 620 μM, i.e. more than 100 times that for the C site peptide (Tables 2 and 3). The $K_m$ values of the three HIF-P4Hs for the 20-residue HIF-2α N site peptide were about 60–110 μM (21). The $V_{\text{max}}$ values for the 20-residue HIF-1α N site peptide were similar to those for the HIF-1α C site peptide, whereas those for the 20-residue HIF-2α N site peptide were 30–70% of those for its C site peptide (Table 3).

As the $K_m$ of HIF-P4H-3 for the 20-residue N site HIF-1α peptide was much higher than that for the HIF-2α peptide, an attempt was made to identify the reasons for this difference by studying two mutants of the HIF-1α peptide, one containing a proline instead of an alanine in position +2 with respect to the proline to be hydroxylated, and the other a glutamate instead of an alanine in position +6; the two mutant residues correspond to those present in HIF-2α (Table 1). The alanine to proline mutation reduced the $K_m$ from 620 to 330 μM, whereas the alanine to glutamate mutation gave no decrease in the $K_m$, but rather a slight tendency to increase to 760 μM (Table 3).
Peptides and HIF Prolyl 4-Hydroxylases

TABLE 3

$K_m$ and $V_{max}$ values of the HIF-P4H isoenzymes for peptides representing the N-terminal hydroxylation site in HIF-1α and HIF-2α

The values are means ± S.D.

| Peptide               | HIF-P4H-1 |               | HIF-P4H-2 |               | HIF-P4H-3 |               |
|-----------------------|-----------|---------------|-----------|---------------|-----------|---------------|
|                       | $K_m$     | $V_{max}$     | $n$       | $K_m$         | $V_{max}$ | $n$           | $K_m$         | $V_{max}$     | $n$           |
|                       | $\mu M$   | %             |           | $\mu M$       | %         |           | $\mu M$       | %             |           |
| HIF-1αN20             | 50 ± 9    | 110 ± 5       | 3         | 50 ± 10       | 120 ± 20  | 3           | 620 ± 160     | 85 ± 20       | 4           |
| HIF-1αN20A566P        |           |               |           | 233 ± 110     | 90 ± 20   | 4           | 760 ± 130     | 85 ± 20       | 4           |
| HIF-1αN20A558E        |           |               |           | 470 ± 130     | 70 ± 10   | 10          | 290 ± 130     | 70 ± 10       | 10          |
| HIF-1αN35             | 10 ± 1    | 125 ± 10      | 3         | 25 ± 6        | 120 ± 15  | 4           | 110 ± 40      | 25 ± 2        | 3           |
| HIF-2αN20             | 100 ± 40  | 70 ± 15       | 9         | 60 ± 15       | 65 ± 6    | 4           | 150 ± 50      | 50 ± 15       | 5           |

* The $V_{max}$ values are expressed relative to that obtained for each enzyme with the HIF-1αC19 peptide.

\footnote{The values shown for this peptide include those determined previously (21).}

The $K_m$ values of HIF-P4H-2 for the 35-residue HIF-1α and HIF-2α N site peptides were about 25 and 50 $\mu M$, respectively, and those of HIF-P4H-3 about 470 and 150 $\mu M$, respectively.

Nevertheless, these HIF-P4H-1 $K_m$ values were 50–135% of those for the corresponding 20-residue peptides (Table 3; Fig. 1B). The $K_m$ values of HIF-P4H-1 for the two 35-residue N site peptides were distinctly lower, about 10 $\mu M$, being 20 and 10% of those for the 20-residue peptides. Nevertheless, these HIF-P4H-1 $K_m$ values were still 30- and 50-fold lower than those for the corresponding 35-residue C site peptides, whereas the corresponding $K_m$ value differences of HIF-P4H-2 were about 30- and 125-fold and those of HIF-P4H-3 5200- and 750-fold (Tables 2 and 3).

The Presence of Both Hydroxylation Sites in a Single Polypeptide Has a Marked Effect on $K_m$ Values for the N-terminal Site—Hydroxylation of the two sites in a single polypeptide was studied by using recombinant HIF-1α and HIF-2α fragments of 248 and 215 residues, respectively, which correspond to their ODDDs (Fig. 2). These recombinant polypeptides were expressed in *E. coli* in forms containing a C-terminal His tag and purified to homogeneity from the soluble fraction of the cell homogenates on a Ni$^{2+}$-charged chelating Sepharose column followed by gel filtration on Superdex S-200 (Fig. 3).

Hydroxylation of only one of the two prolines in the HIF-1α ODDD was studied using its two mutants, P402A and P564A (Fig. 2), which were expressed and purified to homogeneity in the same manner as the wild-type fragment (Fig. 3).

The $K_m$ of HIF-P4H-1 for the wild-type recombinant HIF-1α ODDD was 10–20 $\mu M$, being too low to be determined accurately with the assay used, whereas the $K_m$ values of HIF-P4H-2 and -3 were 140 and 70 $\mu M$, respectively (Table 4; Fig. 1C); the values for these three enzymes were 3–7, about 20 and 80% of those for the 35-residue HIF-1α C site peptide (Table 2). The $K_m$ values of HIF-P4H-1 and -3 for the HIF-2α ODDD were very similar to those for the HIF-1α ODDD, whereas the $K_m$ value of HIF-P4H-2 for the HIF-2α ODDD was slightly lower ($p < 0.001$) than that for the HIF-1α ODDD (Table 4); this isoenzyme also had a slightly lower $K_m$ value for the 35-residue HIF-2α than for the HIF-1α peptide (Table 2). The $K_m$ values determined for the P402A mutant HIF-1α ODDD were identical to those for the wild-type ODDD within the limits of experimental error (Table 4), indicating that the values determined for the wild-type ODDD probably represented those for its C-terminal site. Surprisingly, the $V_{max}$ values of the HIF-P4Hs for the wild-type HIF-1α and HIF-2α ODDDSs were only 50–65% of those for the 19-residue HIF-1α and 20-residue HIF-2α C site peptides (Table 4).

The $K_m$ values of the three HIF-P4Hs for the P564A mutant HIF-1α ODDD were about 5–13 times those for the P402A mutant and the wild-type ODDD (Table 4; Fig. 1D), indicating...
a major difference in the hydroxylation of the two sites in a single ODDD. Nevertheless, the $K_m$ values of the three HIF-P4Hs for the P564A mutant HIF-1α ODDD were only about 1, 3, and 0.2% of those for the 35-residue HIF-1α N site peptide (Table 3). The $V_{\text{max}}$ values for HIF-P4H-1 and -2 for the P564A mutant were similar to those for the wild-type ODDD, whereas the $V_{\text{max}}$ values for HIF-P4H-3 was only about 50%, indicating that this site is hydroxylated especially poorly by HIF-P4H-3.

As the difference in the $K_m$ values of the HIF-P4Hs for the P564A mutant and wild-type ODDDs (Table 4) and 2-fold difference in the $K_m$ values of the HIF-P4Hs, which decrease with increasing chain length, whereas the $V_{\text{max}}$ values show only minor changes and, surprisingly, decrease rather than increase when the values for the 19–20-residue peptides are compared with those for the ODDDs. A similar situation has been reported previously for the C-P4Hs in that their $K_m$ values for (X-Pro-Gly)$_n$ peptide substrates and nonhydroxylated collagen fragments decrease markedly with inreas-

### TABLE 5

| Peptide                  | $K_m$ (μM) | $V_{\text{max}}$ (dpm) | $n$ |
|--------------------------|------------|------------------------|-----|
| HIF-1αODDD               | 0.01       | 55 ± 10                | 5   |
| HIF-1αODDD/P402A         | 0.01       | 45 ± 10                | 4   |
| HIF-1αODDD/P564A         | 0.1        | 55 ± 15                | 3   |
| HIF-2αODDD               | 0.01       | 50 ± 10                | 3   |

$^a$ The $V_{\text{max}}$ values are expressed relative to that obtained for each enzyme with the HIF-1αC19 peptide.

### TABLE 6

$K_m$ values of HIF-P4H-2 for O$_2$ with different substrates

| Substrate                 | $K_m$ (μM) |
|---------------------------|------------|
| HIF-1αC19                 | 250$^a$    |
| HIF-1αODDD                | 100 ± 20   |
| HIF-1αODDD/P402A          | 100 ± 20   |
| HIF-1αODDD/P564A          | 100 ± 40   |

$^a$ See Ref. 21.

*Peptides and HIF Prolyl 4-Hydroxylases*

$V_{\text{max}}$ values between the P564A mutant ODDD and the P402A mutant and wild-type ODDDs (Table 4).

The $K_m$ value of HIF-P4H-2 for Oxygen Is Lower When Determined with a Recombinant ODDD than with a 19-Residue C-terminal Site Peptide — The $K_m$ values of the three HIF-P4Hs have been determined with a 19-residue peptide representing the C-terminal hydroxylation site in HIF-1α and found to be very high, 230–250 μM (21); data obtained in another study using a similar peptide but a quantitative VHL capture assay agreed with these values in that no saturation was found with any of the three isoforms at a 200 μM O$_2$ concentration (26). We determined here the $K_m$ value of HIF-P4H-2 for O$_2$ with the recombinant HIF-1α ODDD as a substrate and obtained a value of 100 μM (Table 6; Fig. 4A), which is about 40% of those determined for the three HIF-P4Hs with the 19-residue peptide. In additional experiments this $K_m$ value for O$_2$ was also determined with the C-terminal and N-terminal site in HIF-1α using the P402A and P564A mutant ODDDs as substrates. The value obtained in both cases was 100 μM (Table 6; Fig. 4B), thus identical to that determined with the wild-type ODDD.

No differences were found in the $K_m$ values of HIF-P4H-2 for Fe$^{2+}$ and 2-oxoglutarate or IC$_{50}$ for pyridine 2,4-dicarboxylate, a competitive inhibitor with respect to 2-oxoglutarate (21), when determined with either the 19-residue peptide representing the C-terminal site or the full-length HIF-1α ODDD as a substrate (details not shown).

**DISCUSSION**

The present data indicate that the length of the peptide substrates has a marked effect on the $K_m$ values of the HIF-P4Hs, which decrease with increasing chain length, whereas the $V_{\text{max}}$ values show only minor changes and, surprisingly, decrease rather than increase when the values for the 19–20-residue peptides are compared with those for the ODDDs. A similar situation has been reported previously for the C-P4Hs in that their $K_m$ values for (X-Pro-Gly)$_n$ peptide substrates and nonhydroxylated collagen fragments decrease markedly with increases...
Peptides and HIF Prolyl 4-Hydroxylases

FIGURE 4. Determination of the $K_m$ value for $O_2$ with a recombinant HIF-1α ODDD as a substrate. A, effect of $O_2$ concentration on the reaction velocity of HIF-P4H-2 with the recombinant HIF-1α ODDD as a substrate. B, effect of $O_2$ concentration on the reaction velocity of HIF-P4H-2 with the recombinant P564A mutant HIF-1αODDD as a substrate. The $K_m$ values for $O_2$ were calculated from the double-reciprocal plots (insets).

The effects of increasing the peptide length from 19–20 to 35 residues were distinctly different in the peptides representing the C-terminal and N-terminal hydroxylation sites in HIF-α. This increase reduced the $K_m$ values for peptides representing the C-terminal hydroxylation site in almost all cases to only 0.7–5% of those with the shorter peptides, whereas the corresponding changes for peptides representing the N-terminal hydroxylation site were 50–135% with HIF-P4H-2 and -3 and 10–20% with HIF-P4H-1. The $K_m$ values for the 35-residue N site peptides were markedly higher than those for the C site peptides, being 30–125-fold in the cases of HIF-P4H-1 and -2 and 750–5200-fold in the case of HIF-P4H-3, whereas the $K_m$ values for the 20-residue N site peptides were 4–7-fold relative to those for the corresponding C site peptides in the cases of HIF-P4H-1 and -2 and 20–120-fold in the case of HIF-P4H-3. Surprisingly, a further increase in peptide length from 35 to 248 residues caused no further reduction in the $K_m$ value of HIF-P4H-3 for the C-terminal hydroxylation site when studied with the P402A mutant ODDD, whereas decreases to less than 7% and about 20% were seen in the cases of HIF-P4H-1 and -2. In contrast, this increase in peptide length reduced the $K_m$ values of the three HIF-P4Hs for the N-terminal site to 0.2–3%, as studied with the P564A mutant ODDD. Nevertheless, it is not known whether this marked decrease was due entirely to the increase in peptide length or whether it was at least in part due to the presence of both hydroxylation sites in a single polypeptide, even though the C-terminal site was present in the P564A mutant form.

The $K_m$ values of the wild-type and P402A mutant HIF-1α ODDDs were essentially identical, whereas the $K_m$ values for the P564A mutant ODDD were about 5–13 times higher, indicating that the values determined for the wild-type ODDD probably represent those for the C-terminal hydroxylation site and that the HIF-P4Hs interact more effectively with the C-terminal site than with the N-terminal site. Our data thus agree...
with a recent report indicating that the C-terminal site in HIF-1α is hydroxylated before the N-terminal site in cultured cells (23). Furthermore, the V_{max} of HIF-P4H-3 for the P564A mutant ODDD was about half that for the wild-type and the P402A mutant ODDD, indicating particularly ineffective hydroxylation of the N-terminal site by HIF-P4H-3. Our hydroxylation experiments with the proline-labeled HIF-1α ODDD and its mutants support these suggestions. It seems highly likely that the HIF-P4Hs may become bound first to the C-terminal site of a full-length ODDD and that this binding may then enhance binding to the N-terminal site. This proposal would be consistent with the marked decrease in K_m values when the length of the N-terminal site peptide was increased from 35 residues to that of the P564A mutant ODDD.

Studies in cultured cells have suggested that the actions of the HIF-P4Hs on different HIF-α isoforms are not equivalent, with HIF-P4H-2 having relatively more effect on HIF-1α than on HIF-2α and HIF-P4H-3 having relatively more effect on HIF-2α than on HIF-1α (31). Our data suggest that these differences are not because of differences in the K_m values of the HIF-P4Hs for the corresponding HIF-α ODDDs, as HIF-P4H-2 had a slightly higher K_m value for the HIF-1α than for the HIF-2α ODDD, whereas HIF-P4H-3 had identical values for both ODDDs, and these values were between those of HIF-P4H-2 for the two ODDDs. According to our data, HIF-P4H-1 has the lowest K_m values for both the HIF-1α and HIF-2α ODDDs, suggesting that it might interact with them more effectively than the other two isoenzymes. It should be noted, however, that interaction of the HIF-P4Hs with HIF-α isoforms in cells is also influenced by the cellular location of the enzymes and their substrates and by the concentrations of the various enzyme proteins.

The K_m values of the HIF-P4Hs for oxygen, when determined with a 19-residue peptide representing the C-terminal hydroxylation site in HIF-1α, were very high, 230–250 μM, being slightly above the concentration of dissolved O_2 in the air (21). Although early kinetic studies suggested that the cosubstrates and the peptide substrate may become bound to the C-P4Hs in the order Fe^{2+}, 2-oxoglutarate, O_2, and the peptide substrate, the order of binding of the last two reactants was somewhat uncertain (14). A subsequent study indicated that the K_m value of a C-P4H for O_2 is distinctly higher in the uncoupled decarboxylation of 2-oxoglutarate than in the hydroxylation reaction (32), i.e. in C-P4H-catalyzed 2-oxoglutarate decarboxylation without subsequent hydroxylation of the peptide substrate, in which ascorbate acts as an alternative oxygen acceptor (14). This finding suggested that the peptide substrate may in fact become bound to the C-P4Hs in the hydroxylation reaction before O_2. Subsequent mechanistic studies based on the crystal structures of several 2-oxoglutarate dioxygenases have demonstrated that this assumption was correct (2). As HIF-P4H-2 is the most abundant, and therefore most important, HIF-P4H isoenzyme in most cell types (31, 33), we determined here its K_m value for O_2 with the recombinant HIF-1α ODDD as a substrate and obtained a value of about 100 μM, i.e. about 40% of those determined for the three HIF-P4Hs with the 19-residue peptide. In agreement with the order of addition of the cosubstrates to the enzyme, the use of ODDD instead of the 19-residue peptide as a substrate had no effect on the K_m values for Fe^{2+} and 2-oxoglutarate or the IC_{50} for the 2-oxoglutarate analogue inhibitor pyridine 2,4-dicarboxylate (21, 24), i.e. reactants that become bound to the enzyme before the peptide substrate.

The K_m value of FIH for O_2, when determined with a 35-residue peptide as a substrate, was about 90 μM (18), being about one-third of those determined for the HIF-P4Hs with the 19-residue peptide as a substrate (21). This difference suggested that a minor decrease in the O_2 concentration is likely to influence primarily the activities of the HIF-P4Hs, whereas a larger decrease in the O_2 concentration is needed for a significant decrease in the activity of FIH (18). As FIH appears to require even longer peptide substrates than the HIF-P4Hs (16, 18) and is likely to have a reaction mechanism identical to that of the HIF-P4Hs, it seems likely that the K_m of FIH for O_2 determined with a long HIF-α fragment would also be lower than that determined with the 35-residue peptide. It thus seems possible that the difference in the K_m values of the HIF-P4Hs and FIH for O_2 is a biologically relevant difference.

The K_m value of HIF-P4H-2 for O_2 determined here is still very high and is above the oxygen concentrations found in almost all tissues under normoxic conditions in vivo (34). Accordingly, the in vivo activities of the HIF-P4Hs are likely to be limited by O_2 and are likely to be altered by changes in its concentrations in a very sensitive way, making these enzymes effective oxygen sensors. It should be noted, however, that HIF-P4H-2 has considerable activity levels even under O_2 concentrations that are much lower than the K_m value, e.g. at 20 and 10 μM (Fig. 4), which correspond to about 2 and 1% O_2 in the air. This situation explains why the HIF-P4Hs have considerable activity in cultured cells even in the presence of low O_2 concentrations (23). Although hydroxylation of the N-terminal site of HIF-1α has been shown to decrease at limiting O_2 tensions in cultured cells at higher O_2 tensions than that of the C-terminal site (23), we found no difference in the K_m values of HIF-P4H-2 for O_2 when determined with the wild-type, P564A, or P402A mutant HIF-1α ODDD, suggesting that the difference seen in cultured cells may be due to the lower affinity of the N-terminal site for the enzyme, and possibly local changes in O_2 concentrations, rather than differences in the K_m values for the two hydroxylation sites.

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