The hypoxia-inducible factors (HIFs) play a central role in oxygen homeostasis. Hydroxylation of one or two critical prolines by specific hydroxylases (P4Hs) targets their HIF-α subunits for proteasomal degradation. By studying the three human HIF-P4Hs, we found that the longest and shortest isoforms have major transcripts encoding inactive polypeptides, which suggest novel regulation by alternative splicing. Recombinant HIF-P4Hs expressed in insect cells required peptides of more than 8 residues, distinct differences being found between isoforms. All the HIF-P4Hs hydroxylated peptides corresponding to Pro \(^{464}\) in HIF-1α, whereas a Pro \(^{402}\) peptide had 20–50-fold \(K_m\) values for two isoforms but was not hydroxylated by the shortest isoform at all; this difference was not explained by the two prolines being in a \(-Pro^{402}-Ala-\) and \(-Pro^{564}-Tyr-\) sequence. All the HIF-P4Hs-hydroxylated peptides corresponding to two of three potential sites in HIF-2α and one in HIF-3α. The \(K_m\) values for \(O_2\) were slightly above its atmospheric concentration, indicating that the HIF-P4Hs are effective oxygen sensors. Small molecule inhibitors of collagen P4Hs also inhibited the HIF-P4Hs, but with distinctly different \(K_i\) values, indicating that it should be possible to develop specific inhibitors for each class of P4Hs and possibly even for the individual HIF-P4Hs.

The hypoxia-inducible transcription factors (HIFs) play a central role in the regulation of cellular and systemic \(O_2\) homeostasis. HIFs are \(αβ\) dimers in which both the \(α\) and \(β\) subunits are basic helix-loop-helix Per-Arnt-Sim proteins, and the human \(α\) subunits have three isoforms, HIF-1α to HIF-3α (for reviews see Refs. 1 and 2). HIF-1α and HIF-2α are synthesized continuously, and at least one of two critical proline residues, Pro \(^{402}\) and Pro \(^{464}\) in HIF-1α, becomes hydroxylated under normoxic conditions (3–6). The resulting 4-hydroxyproline residue is essential for the binding of HIF-α to the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex and for subsequent rapid proteasomal degradation (3–6). This hydroxylation, which requires oxygen, ceases under hypoxic conditions so that HIF-α escapes degradation and forms the \(αβ\) dimer with HIF-β. This dimer then becomes bound to the HIF-responsive elements in a number of hypoxia-inducible genes, such as those for erythropoietin, vascular endothelial growth factor, and glycolytic enzymes (for reviews, see Refs. 1 and 2).

A family of collagen prolyl 4-hydroxylases (C-P4Hs), enzymes residing within the lumen of the endoplasmic reticulum, has been extensively characterized and shown to act on Pro-\(Gly-\) sequences (7–9). The proline residues hydroxylated in HIF-1α are present in -Leu-\(X\)-Leu-Ala-Pro-Ala- and -Leu-\(X\)-Leu-Ala-Pro-Tyr- sequences, however (3–6), and the human type I and type II C-P4Hs have been shown not to hydroxylate a 19-residue synthetic peptide corresponding to the sequence around Pro \(^{464}\) in HIF-1α (4). Three human cytoplasmic P4H isoforms that hydroxylate HIF-α (HIF-P4Hs) have since been identified (10–12). These novel enzymes resemble the C-P4Hs in that they require \(Fe^{2+}\), 2-oxoglutarate, \(O_2\), and ascorbate. The three HIF-P4Hs show a 42–59% sequence identity to each other but no distinct sequence similarity to the C-P4Hs. The three critical \(Fe^{2+}\)-binding residues identified in the C-P4Hs (13, 14) are conserved, however, whereas the lysine that binds the C-5 carboxyl group of the 2-oxoglutarate (14) is replaced in the HIF-P4Hs by an arginine (10, 11). HIF-P4Hs have also been identified in Caenorhabditis elegans and Drosophila melanogaster (10, 11).

Most experiments reported so far on the HIF-P4Hs have used enzymes prepared in reticulocyte lysates and assays based on capture of the hydroxylated substrate by the VHL protein (10–12). These assays do not always allow one to distinguish between the critical requirements for hydroxylation and binding of the hydroxylated product, and they are not suitable for any detailed studies of the catalytic properties of the enzymes. Preliminary data suggest that hydroxylation of Pro \(^{402}\) may be less efficient than that of Pro \(^{564}\) and may not be performed by HIF-P4H-3 at all (11), but no kinetic data are available on possible differences in the hydroxylation of the two sites by the three isoforms, nor are any data available on possible hydroxylation of similar sequences in HIF-2α or HIF-3α. It is also unknown whether the three isoforms show differences in their kinetic constants for \(O_2\) and other co-substrates or for small molecule inhibitors.

In the present work we expressed all three human HIF-P4H isoforms as recombinant proteins in insect cells and adopted an assay based on the measurement of the amount of \(^{14}C\)CO\(_2\) released during the hydroxylation-coupled decarboxylation of 2-oxo[\(L\)-\(^{14}C\)]glutarate. This is similar to the assay used for the C-P4Hs (15) and made it possible to study differences between the isoforms in their hydroxylation of various peptide substrates and their kinetic constants for the co-substrates. We
also studied inhibition of the enzymes by several compounds known to inhibit C-P4Hs. It became evident during the early stages of this work that transcripts of two of the three isoenzymes are alternatively spliced, with major variants encoding inactive polypeptides.

MATERIALS AND METHODS

Cloning of the HIF-P4H Isoenzymes and Generation of Expression Vectors—The cDNAs encoding human HIF-P4Hs 1–3 were obtained by PCR amplification from colon, aorta, and lung Marathon-Ready cDNAs (Clontech). The PCR products were cloned into pUC18 using the SureClone ligation kit (Amersham Biosciences) and sequenced on an automated sequencer (ABI Prism 377, Applied Biosystems). Full-length HIF-P4H-1 and -3 cDNAs were obtained, but the HIF-P4H-2 PCR product had a 51-nucleotide insertion instead of the codons for Cys58–Pro175 (named HIF-P4H-2 PCR variant). The correct HIF-P4H-2 exon 1 sequence spanning this region was amplified from genomic DNA using Advantage-GC2 polymerase (BD Biosciences) and cloned as a blunt XhoI fragment into the HIF-P4H-2 PBC variant to generate the full-length HIF-P4H-2 cDNA. The full-length HIF-P4H-1–3 cDNAs and the HIF-P4H-2 PCR variant were cloned into the pVL and pAeGXS baculovirus expression vectors (Pharmingen), and the HIF-P4H-3 cDNA was also cloned into a pBlueBacHis baculovirus expression vector (Pharmingen), and the HIF-P4H-1 and -3 cDNA into a pMAL cDNA expression vector (New England Biolabs). Site-directed mutagenesis of the PCR product was performed using the QuickChange™ kit (Stratagene).

PCR Analysis of HIF-P4H mRNA Expression in Various Tissues—Expression of HIF-P4H-1–3 cDNAs in various tissues was studied by PCR analysis of human multiple tissue cDNA (TCTM™) panel 1 and human fetal MTC panel (Clontech) according to the manufacturer’s protocol. The primer pairs 5′-TGGCCTGGGGGTAGAAACACGTGTCCTGCCA-3′ and 5′-TCTGCGATGGCGTCGGATG-3′, amplifying the full-length HIF-P4H-1 and HIF-P4H-3, respectively, were used in the analyses. Expression of HIF-P4H-2 mRNA was studied using the primers 5′-GCCGGGCGCTGCCGGCC-3′ and 5′-ATTCTGCGGGCCATGGCTGCTG-3′, which amplified a 941-bp PCR product starting from the 3′ end of exon 1, with a 51-nucleotide insertion encoding -Leu-Leu-Gly-Gly-Arg-Pro175 (named HIF-P4H-2 PCR variant). The correct HIF-P4H-2 PCR variant lacking amino acids Gly58–Pro175 was amplified using the Advantage-2 polymerase from genomic DNA but only obtained the same PCR variant as from the cDNA pools. A correct full-length PCR product could be obtained with the Advantage-2 polymerase, however. It is very likely that this DNA region, which has a high GC content, is the likely origin of aberrant variants.

Cloning of the HIF-P4H-1–3 cDNAs—In order to express the three human HIF-P4H isoenzymes as recombinant proteins, the cDNAs encoding human HIF-P4Hs 1–3 were amplified by PCR from human colon, aorta, and lung cDNA pools using specific primers, and the PCR products were cloned into pUC18 and sequenced (the numbers used here correspond to PHD1, PHD2, and PHD3 in Ref. 11, GenBank™ accession numbers XP_040482, AAC33965, and NP_071356, respectively). The encoded amino acid sequences of the full-length HIF-P4H-1 and HIF-P4H-3 cDNAs obtained were identical to the above GenBank™ sequences, whereas the PCR product of HIF-P4H-2 corresponded to a variant (Fig. 1) in which the nucleotide encoding residues Cys58–Pro175 were missing and were replaced by a 51-nucleotide stretch encoding -Leu-Leu-Gly-Gly-Arg-Pro175. Surprisingly, this 51-nucleotide stretch was found to represent the non-coding strand of the HIF-P4H-2 cDNA between bp 450–500 in a 3′ to 5′ direction. A slightly different HIF-P4H-2 variant lacking amino acids Gly58–Gly175 has been stated previously (10) to represent a splicing variant and shown to hydroxylate Pro in vitro. Neither of these two HIF-P4H-2 PCR variants was found to follow the GT/AG consensus rule of intron splicing, however. We also attempted to amplify the HIF-P4H-2 sequence encoding amino acids Cys58–Pro175 with the Advantage2 polymerase from genomic DNA but only obtained the same PCR variant as from the cDNA pools. A correct full-length PCR product could be obtained with the Advantage-2 polymerase, however. It is very likely that this DNA region, which has a high GC content, shows a strong tendency to form secondary structures, which makes it difficult to amplify correct products and increases the likelihood of aberrant variants.

Analysis of the Expression of mRNAs for the Three Human HIF-P4H Isoenzymes Indicates Alternative Splicing in Various Tissues—In addition to the HIF-P4H-2 PCR variant and the full-length HIF-P4H-1 and -3 cDNAs obtained by PCR amplification of colon, aorta, and lung cDNA pools, smaller fragments originating from HIF-P4H-2 and -3 were also obtained in the same reactions. In order to analyze whether they represented alternatively spliced forms, the expression of the HIF-P4H mRNAs in various tissues was studied further by PCR amplification of adult and fetal human multiple tissue cDNA panels. For HIF-P4Hs 1 and 3 specific primers were used to amplify the full-length cDNAs, whereas in the case of HIF-P4H-2 the forward primer was from the 3′ end of exon 1, as this exon contains a GC-rich region that easily generates PCR errors.

Full-length HIF-P4H-1 cDNA was amplified in all the tissues studied (Fig. 2A); no smaller PCR products were detected. The level of HIF-P4H-1 mRNA expression was highest in the adult brain, placenta, lung, and kidney (Fig. 2A). A full-length HIF-P4H-2 PCR product starting from the 3′ end of exon 1, with a...
PCR products were obtained with the HIF-P4H-2 (A) and -3 (B) primers. Amplification of the full-length coding sequences of HIF-P4Hs 1 and 3 was achieved using human multiple tissue cDNA panels. Primer pairs were designed to amplify genes in various tissues by PCR analysis of adult and fetal tissues; its expression level was the highest in the adult heart, brain, placenta, lung, and skeletal muscle and in the fetal liver (Fig. 2B). Two additional HIF-P4H-2 PCR products with smaller sizes of 500 bp were obtained in amplifications from several tissues, whereas the size of 476 bp, was likewise amplified in all the tissues (Fig. 2B); the levels of HIF-P4H-2 mRNA expression were the highest in the adult heart, brain, lung, and liver and in the fetal brain, heart, spleen, and skeletal muscle (Fig. 2B). Two additional HIF-P4H-2 PCR products with smaller sizes of ~350 and 400 bp were obtained in amplifications from several tissues, however (Fig. 2B), and sequencing showed that they represented splicing variants lacking the exon 3 or 4 sequences, respectively (Fig. 1). The levels of expression of these splicing variants were considerably lower in all the tissues than that of the HIF-P4H-2 mRNA containing all the exons from 1 to 4 (Fig. 2B). Full-length HIF-P4H-3 cDNA was amplified from all the tissues; its expression level was the highest in the adult heart, brain, placenta, lung, and skeletal muscle and in the fetal liver (Fig. 2A). Two additional HIF-P4H-2 PCR products with smaller sizes of 500 bp were obtained in amplifications from several tissues, whereas the size of 476 bp, was likewise amplified in all the tissues (Fig. 2B); the levels of HIF-P4H-2 mRNA expression were the highest in the adult heart, brain, lung, and liver and in the fetal brain, heart, spleen, and skeletal muscle (Fig. 2B). Two additional HIF-P4H-2 PCR products with smaller sizes of ~350 and 400 bp were obtained in amplifications from several tissues, however (Fig. 2B), and sequencing showed that they represented splicing variants lacking the exon 3 or 4 sequences, respectively (Fig. 1). The levels of expression of these splicing variants were considerably lower in all the tissues than that of the HIF-P4H-2 mRNA containing all the exons from 1 to 4 (Fig. 2B). Full-length HIF-P4H-3 cDNA was amplified from all the tissues; its expression level was the highest in the adult heart, brain, placenta, lung, and skeletal muscle and in the fetal heart, spleen, and skeletal muscle (Fig. 2C). In addition to the band representing the full-length HIF-P4H-3 cDNA, one representing a smaller product of ~500 bp was found in about a 1:1 ratio in all the tissues studied (Fig. 2C). Sequencing of this band showed that it represented a splicing variant lacking the HIF-P4H-3 nucleotides 76–357, and thus encoded a shortened polypeptide lacking amino acids 26–119 (Fig. 1). All the alternatively spliced HIF-P4H-2 and -3 mRNAs followed the GT/AG rule.

Recombinant Expression of the HIF-P4H Isoenzymes—The full-length HIF-P4H isoenzymes and the HIF-P4H-2 PCR variant with a -Leu-Leu-Gly-Gly-Tyr-Arg-Phe-Ala-Phe-Ser-Trp-Asn-Ser-Hsp-Glu-Arg-Ala- sequence replacing Cys58–Pro175 were expressed as recombinant polypeptides in insect cells. All three isoforms were also expressed as glutathione S-transferase (GST) fusion proteins, and HIF-P4H-3 was further expressed with an N-terminal histidine tag. The cells were harvested 72 h after infection and solubilized in a buffer containing Triton X-100, and the soluble and insoluble fractions were analyzed by SDS-PAGE followed by Coomassie Blue staining. Most of the HIF-P4H-2 polypeptide was found in the soluble fraction (Fig. 3B, lane 3), whereas the vast majority of the other polypeptides was found in the insoluble fraction (Fig. 3A, C, even-numbered lanes). Nevertheless, distinct bands corresponding to isoenzyme 1 (Fig. 3A, lane 3) and the GST-fused forms of isoenzymes 1 and 3 (Fig. 3A and C, lanes 5) could be seen in the soluble fraction. The HIF-P4H-2 PCR variant, GST-HIF-P4H-2 PCR variant, HIF-P4H-3 and His-HIF-P4H-3 polypeptides were not detectable in the soluble fraction in the Coomassie Blue-stained SDS-PAGE gels (Fig. 3B, lanes 5 and 7, and C, lanes 3 and 7), but a definitive level of HIF-P4H activity was found in the soluble fraction of the cells expressing these polypeptides (see below), and the presence of the histidine-tagged and GST-fused HIF-P4H-3 polypeptides in the soluble fraction could further be verified by Western blotting (Fig. 3D).

Development of a Simple Activity Assay and Measurement of the Activities of the HIF-P4Hs in Cell Extracts—The most commonly used assay for the C-P4Hs is based on the hydroxylation of a synthetic peptide substrate such as (Pro-Pro-Gly)10 and measurement of the radioactivity of the 14CO2 formed during the hydroxylation-coupled decarboxylation of 2-oxo[1-14C]glutarate (15). We adopted this assay for measurement of the activities of the HIF-P4Hs using as a standard substrate a.

---

**Fig. 1.** Schematic representation of the HIF-P4H isoenzymes 1–3. The numbers of amino acids and the GenBank accession numbers for the three polypeptides are shown on the right. The catalytically critical residues, two histidines and one aspartate that bind the Fe2+ atom and one arginine that binds the C-5 carboxyl group of 2-oxoglutarate, are marked by a dashed underline. The HIF-P4H-2 and -3 genes consist of four coding exons. The encoded amino acids are shown by an N-terminal histidine tag. The cells were harvested 72 h after infection and solubilized in a buffer containing Triton X-100, and the soluble and insoluble fractions were analyzed by SDS-PAGE followed by Coomassie Blue staining. Most of the HIF-P4H-2 polypeptide was found in the soluble fraction (Fig. 3B, lane 3), whereas the vast majority of the other polypeptides was found in the insoluble fraction (Fig. 3, A–C, even-numbered lanes). Nevertheless, distinct bands corresponding to isoenzyme 1 (Fig. 3A, lane 3) and the GST-fused forms of isoenzymes 1 and 3 (Fig. 3A and C, lanes 5) could be seen in the soluble fraction. The HIF-P4H-2 PCR variant, GST-HIF-P4H-2 PCR variant, HIF-P4H-3 and His-HIF-P4H-3 polypeptides were not detectable in the soluble fraction in the Coomassie Blue-stained SDS-PAGE gels (Fig. 3B, lanes 5 and 7, and C, lanes 3 and 7), but a definitive level of HIF-P4H activity was found in the soluble fraction of the cells expressing these polypeptides (see below), and the presence of the histidine-tagged and GST-fused HIF-P4H-3 polypeptides in the soluble fraction could further be verified by Western blotting (Fig. 3D).

---

**Fig. 2.** Analysis of expression of the HIF-P4H-1–3 genes and the alternatively spliced forms of HIF-P4H-2 and -3 transcripts in various tissues. The expression of HIF-P4H-1 (A), -2 (B), and -3 (C) genes in various tissues was studied by PCR analysis of adult and fetal human multiple tissue cDNA panels. Primer pairs were designed to amplify the full-length coding sequences of HIF-P4Hs 1 and 3, whereas the 5’ end of the HIF-P4H-2 PCR product was designed to start from nucleotide 532. In addition to the PCR products of the expected sizes, 1200, 480, and 750 bp, for HIF-P4Hs 1–3, respectively (A–C), smaller PCR products were obtained with the HIF-P4H-2 (B) and 3 (C) primers.

---

**Fig. 3.** Western blotting. Most of the HIF-P4H-2 polypeptide was found in the soluble fraction (Fig. 3B, lane 3), whereas the vast majority of the other polypeptides was found in the insoluble fraction (Fig. 3A, C, even-numbered lanes). Nevertheless, distinct bands corresponding to isoenzyme 1 (Fig. 3A, lane 3) and the GST-fused forms of isoenzymes 1 and 3 (Fig. 3A and C, lanes 5) could be seen in the soluble fraction. The HIF-P4H-2 PCR variant, GST-HIF-P4H-2 PCR variant, HIF-P4H-3 and His-HIF-P4H-3 polypeptides were not detectable in the soluble fraction in the Coomassie Blue-stained SDS-PAGE gels (Fig. 3B, lanes 5 and 7, and C, lanes 3 and 7), but a definitive level of HIF-P4H activity was found in the soluble fraction of the cells expressing these polypeptides (see below), and the presence of the histidine-tagged and GST-fused HIF-P4H-3 polypeptides in the soluble fraction could further be verified by Western blotting (Fig. 3D).
synthetic 19-residue peptide with a sequence identical to that around the C-terminal hydroxylation site of HIF-1α. Extensive optimization experiments resulted in the reactant concentrations and assay conditions given under “Materials and Methods.” The assay was found to be linear with increasing concentrations of cell extracts expressing the recombinant enzymes for up to about 6000–9000 dpm of 14CO2 generated (as shown for isoenzyme 1 in Fig. 4A), and with time for at least 25 min provided that the amount of recombinant enzyme used produced less than about 6000 dpm in this time (as shown for isoenzyme 3 in Fig. 4B). Variation of the concentration of the peptide substrate (as shown for isoenzyme 2 in Fig. 4C) or any of the co-substrates gave typical Michaelis-Menten kinetics (as shown for 2-oxoglutarate with respect to isoenzyme 3 in Fig. 4D). The assay is simple and rapid and was found to be suitable for all the experiments reported below.

Significant levels of HIF-P4H activity were found in the soluble fractions of insect cells expressing any of the three recombinant isoenzymes (Table I). The differences in the activity values shown do not represent differences in the specific activities of the three isoenzymes, as their expression levels and solubilities in the extraction buffer were not equal (see Fig. 3). The presence of a GST fusion partner slightly increased the activity levels obtained with HIF-P4H-1 and HIF-P4H-2 PCR variant, whereas a marked increase was obtained with HIF-P4H-3 (Table I). These increases were most probably because of improved solubility of the GST fusion proteins in the extraction buffer (see Fig. 3).

The highest observed activity values for the three isoenzymes ranged to about 0.16–0.22 nmol of 2-oxoglutarate decarboxylated per μg of protein/20 min. If the purities of the isoenzymes in the cell extracts were 100%, these values would correspond to about 0.4–0.55 mol of 2-oxoglutarate decarboxylated per mol of enzyme/min, depending on the isoenzyme. The purities of the enzymes in the soluble fractions are likely to be far less than 1%, however, and thus the true specific activities are likely to be much more than 100-fold, i.e. much more than 40–55 mol/mol/min. Although these estimates are highly inaccurate, it should be noted that a highly purified HIF-P4H-1 produced in E. coli had a specific activity of only 12 × 10−6 mol/mol/min (18), which agrees with our data (below), indicating that HIF-P4Hs expressed in E. coli are completely or essentially completely inactive.

Purification of the recombinant HIF-P4H isoenzymes 1 and 3 was attempted by means of GST or histidine tags, but in both cases the vast majority of the recombinant enzyme did not become bound to the affinity column or the enzyme became very loosely bound and was already eluted during the washing steps (data not shown). The HIF-P4H isoenzymes 1 and 3 were also expressed in E. coli as maltose-binding protein fusion proteins, but no enzyme activity could be detected with the purified polypeptides (data not shown).
of the hydroxylation-coupled decarboxylation of 2-oxo-[1-14C]glutarate under the conditions described under "Materials and Methods." A, effect of increasing amounts of the Triton X-100-soluble fraction of cells expressing HIF-P4H-1 on the hydroxylation of 0.05 μmol of the peptide DLDLEMLAPYIPMDDDFQL. B, time course for the hydroxylation of 0.05 μmol of DLDLEMLAPYIPMDDDFQL by HIF-P4H-3. C, effect of the concentration of the DLDLEMLAPYIPMDDDFQL peptide substrate on the HIF-P4H-2 reaction velocity. The $K_m$ of HIF-P4H-2 for the peptide, 10 μM, was calculated from the double-reciprocal plot (inset). D, effect of the concentration of 2-oxoglutarate on the HIF-P4H-3 reaction velocity. The $K_m$ of HIF-P4H-3 for 2-oxoglutarate, 55 μM, was calculated from the double-reciprocal plot (inset).

**TABLE I**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>HIF-P4H activity (dpm/100 μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-P4H-1</td>
<td>4390</td>
</tr>
<tr>
<td>GST-HIF-P4H-1</td>
<td>5590</td>
</tr>
<tr>
<td>HIF-P4H-1Arg$^{367}$ → Lys</td>
<td>0</td>
</tr>
<tr>
<td>HIF-P4H-2</td>
<td>16,360</td>
</tr>
<tr>
<td>HIF-P4H-2 PCR variant</td>
<td>7070</td>
</tr>
<tr>
<td>GST-HIF-P4H-2 PCR variant</td>
<td>8410</td>
</tr>
<tr>
<td>HIF-P4H-2-Δexon3</td>
<td>0$^a$</td>
</tr>
<tr>
<td>HIF-P4H-2-Δexon4</td>
<td>0</td>
</tr>
<tr>
<td>HIF-P4H-3</td>
<td>2780</td>
</tr>
<tr>
<td>His-HIF-P4H-3</td>
<td>1560</td>
</tr>
<tr>
<td>GST-HIF-P4H-3</td>
<td>6940</td>
</tr>
<tr>
<td>HIF-P4H-3-Δ26–119</td>
<td>0</td>
</tr>
<tr>
<td>His-HIF-P4H-3-Δ26–119</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ Values are given in dpm/100 μg of cell protein. Similar results were obtained in more than 10 experiments. Differences in the activity values do not represent differences in the specific activities of the recombinant polypeptides, as the polypeptides differ in their solubility in the extraction buffer (see Fig. 3).

$^b$ P4H activity of this variant was not determined experimentally, as lack of one of the iron-binding histidines and the arginine that binds the C-5 carboxyl group of 2-oxoglutarate inevitably inactivates the polypeptide.

**The Short Splicing Variants of Isoenzymes 2 and 3 Encode Inactive Polypeptides**—The HIF-P4H-2 splicing variant lacking the amino acids encoded by exon 3 is inevitably an inactive form, as it does not contain the second iron-binding histidine and the arginine that binds the C-5 carboxyl group of 2-oxoglutarate, and it was therefore not studied any further. In order to study the activity of the splicing variants of HIF-P4H-2 lacking the amino acids encoded by exon 4 and of HIF-P4H-3 lacking residues 26–119, the corresponding cDNAs were expressed in insect cells. The cells were infected and harvested as described above, and expression of the polypeptides was analyzed by SDS-PAGE (data not shown). To verify the presence of the alternatively spliced HIF-P4H-3 polypeptide in the soluble extract, it was also expressed with an N-terminal histidine tag, and the cell extracts were analyzed by Western blotting (data not shown). No enzyme activity was detected in cell extracts expressing any of the alternatively spliced HIF-P4H-2 or -3 forms (Table I).

**The 2-Oxoglutarate-binding Arginine in HIF-P4H-1 Cannot Be Replaced by a Lysine**—One distinct difference between the HIF-P4Hs and C-P4Hs is that the residue that binds the C-5 carboxyl group of the 2-oxoglutarate is an arginine in the HIF-P4Hs but a lysine in the C-P4Hs (14). To study whether the arginine present in the HIF-P4Hs can be replaced by a lysine, we mutated Arg$^{367}$ in HIF-P4H-1 to lysine and expressed the mutant enzyme in insect cells as above. No P4H activity was found in the soluble fraction of cells expressing this mutant, indicating that arginine is an absolute requirement (Table I). This result differs from that obtained when the 2-oxoglutarate-binding lysine in the type I C-P4H was altered to arginine, as this mutation inactivated the enzyme by 85% under standard assay conditions and increased the $K_m$ for 2-oxoglutarate about 15-fold (14).

**The HIF-P4H Isoenzymes Require Long Peptide Substrates**—Our initial experiments demonstrated that none of the three HIF-P4H isoenzymes hydroxylated the peptides Leu-Ala-Pro, Leu-Ala-Pro-Tyr, Leu-Glu-Met-Leu-Ala-Pro, and Leu-Glu-Met-Leu-Ala-Pro-Tyr (details not shown). We therefore decided to focus on a 19-residue peptide corresponding to the C-terminal hydroxylation site in HIF-1α. All three isoenzymes were found to hydroxylate this peptide, with $K_m$ values of about 5–10 μM (Table II). Omission of two residues from the N terminus, resulting in a 17-residue peptide, had little effect on hydroxylation by any of the isoenzymes, whereas omission of four residues markedly increased the $K_m$ values in the cases of HIF-P4Hs 1 and 2 (Table II). Shortening of the peptide by two additional residues, to yield a 13-residue peptide, led to increased $K_m$ values with all three isoenzymes, although the
The Tyr565 not due to differences in the residue after the proline (Table III). The N-terminal hydroxylation of the N-terminal and C-terminal sites are not due to differences in the residue after the proline (Table III). These results differ distinctly from those obtained in two studies...
ertheless found no significant difference between a wild-type and Tyr<sup>256</sup> → Ala mutant peptide (3).

The Human HIF-P4H Isoenzymes Also Hydroxylate Peptides Related to Human HIF-2α and HIF-3α and C. elegans HIF-α—

The $K_m$ values of the three human HIF-P4H isoenzymes for a 19-residue peptide corresponding to the C-terminal hydroxylation site in HIF-2α were slightly to distinctly higher than those for the corresponding HIF-1α peptide, whereas the $V_{max}$ values were in two of three cases slightly lower (Table IV). A 20-residue peptide corresponding to a potential N-terminal hydroxylation site also became hydroxylated, although it was a less good substrate than the peptide corresponding to the C-terminal site (Table IV). Nevertheless, the difference between the two HIF-2α peptides was much smaller than in the case of the HIF-1α peptides, and all three isoenzymes acted on the N-terminal HIF-2α peptide (Table IV). HIF-2α has an additional sequence TNFQPAPVPHSCFLLLD with an LPV motif, but a corresponding peptide could not be hydroxylated by any of the HIF-P4Hs (Table IV).

HIF-3α also has a sequence highly similar to that around the C-terminal hydroxylation site in HIF-1α, and a 20-residue peptide corresponding to this sequence was found to act as a good substrate for all three HIF-P4Hs (Table IV).

The HIF system has also been identified in C. elegans, but the human HIF-P4Hs have been reported not to act on the only proline residues in the oxygen-dependent degradation domain of this nematode (11). However, we obtained hydroxylation of a 20-residue peptide corresponding to the C. elegans hydroxylation site by all three human HIF-P4Hs, although with high $K_m$ values (Table IV).

The HIF-P4H Isoenzymes Have Much Higher $K_m$ Values for O<sub>2</sub> than the C-P4Hs—the three HIF-P4H isoenzymes retained partial activity even in the absence of added Fe<sup>2+</sup>, suggesting that the iron atom may be more tightly bound to the HIF-P4Hs than the C-P4Hs (details not shown). It was therefore not possible to determine the $K_m$ values for Fe<sup>3+</sup>. The $K_m$ values for 2-oxoglutarate of the three HIF-P4Hs were about 3-fold and those for ascorbate about half relative to those of the type I C-P4H, and there were no significant differences in these values between the three isoenzymes (Table V).

A striking difference between the two classes of P4H was found in the $K_m$ values for O<sub>2</sub>, which were determined here for both the HIF-P4Hs and the type I C-P4H in the same experiments. The $K_m$ values of the HIF-P4Hs were slightly above the concentration of dissolved O<sub>2</sub> in air (about 200 μM), and there were no significant differences between the three isoenzymes (Table V). The $K_m$ of the type I C-P4H was much lower, only about one-sixth of these values (Table V).

Several Inhibitors of C-P4Hs Also Inhibit HIF-P4Hs—Pyridine 2,4-dicarboxylate and pyridine 2,5-dicarboxylate are well established competitive inhibitors of the C-P4Hs with respect to 2-oxoglutarate, with $K_i$ values of 2 and 0.8 μM, respectively (7, 8). Pyridine 2,4-dicarboxylate was also found to be a competitive inhibitor of the HIF-P4Hs, although its $K_i$ values ranged from 7 to 40 μM, depending on the isoenzyme (Table VI). In contrast, pyridine 2,5-dicarboxylate was only a very weak inhibitor of all three isoenzymes, with $K_i$ values exceeding 300 μM, which we regarded as a limit for any significant inhibition (Table VI). Other small molecule inhibitors of C-P4Hs were also found to inhibit HIF-P4Hs, isoenzymes 2 and 3 having the lowest and isoenzyme 1 the highest $K_i$ values, but all the compounds tested were much less effective inhibitors of the HIF-P4Hs than of the C-P4Hs (Table VI).

### DISCUSSION

The activity of the human HIF system is regulated by a series of novel oxygen-dependent post-translation modifications that are mediated by specific 2-oxoglutarate dioxygenases. In normoxia, hydroxylation of at least one of two critical proline residues in the oxygen-dependent degradation domain by HIF-P4Hs (10–12) targets HIF-α to capture by the VHL protein and subsequent ubiquitination and proteasomal degradation (3–6). In a complementary mechanism, hydroxylation of a conserved asparagine in the C-terminal transactivation domain by an asparaginyl hydroxylase (19–22) prevents binding of the transcriptional co-activator p300 (21, 23). The present data suggest still further complexity in this system, as transcripts of two of the three HIF-P4Hs, isoenzymes 2 and 3, were found to be subject to alternative splicing, with major transcripts encoding catalytically inactive polypeptides. Changes in the splicing pattern can therefore be expected to influence the amounts of the active enzymes produced.

It is well established that the C-P4Hs act more effectively on long than on short peptides, the chain length influencing the
$K_m$ values, whereas the $V_{max}$ values for short and long peptides are essentially identical (see Ref. 8). The minimum requirement for hydroxylation by the C-P4Hs is nevertheless fulfilled by tripeptides with the sequence X-Pro-Gly. The present data indicate that the HIF-P4Hs also require long substrates, but their minimum requirements are much longer peptides, as even one of two 11-residue peptides with a sequence corresponding to that around Pro$^{564}$ was hydroxylated by only HIF-P4Hs 1 and 3, whereas the other was hydroxylated by all three isoenzymes, although very ineffectively. The data thus also indicate that the three isoenzymes are not identical with respect to their requirements for the length of their substrates.

When the length requirement around Pro$^{564}$ was studied by systematically shortening a 19-residue peptide at its N-terminal or C-terminal end, all three isoenzymes hydroxylated a peptide with two residues (LA) preceding the proline, but the $K_m$ values of HIF-P4Hs 1 and 2 were very high. In the case of residues after the proline, none of the enzymes acted on a peptide with four such residues (YIPM); all three required at least five (YIPMD).

The hydroxylated peptides become bound to the VHL protein in an extended conformation, with residues 560–567 (EMLAOYIP, in which O is 4-Hyp) and 567–577 (DFQLRSF) forming two distinct binding sites (24, 25). It therefore seems likely that the peptides may also become bound to the HIF-P4Hs in a similar extended conformation. The present data indicate that all three HIF-P4H isoenzymes may require at least residues 562–569 (LAPYIPMD). However, HIF-P4H-2 did not hydroxylate the 11-residue peptide LEM LAPYIPMD and none of the isoenzymes hydroxylated the 8-residue LAPYIPMD, indicating that the hypothetical minimum lengths are not sufficient when the peptides are shortened at both ends. It should further be noted that the enzymes do not strictly require these actual residues, as a recent study (26) indicates that several single residue point mutations can be tolerated with relatively minor effects in the sequence of a 16-residue peptide corresponding to amin acids 557–572 in HIF-1α. Our data and those of Huang and co-workers (26) suggest that HIF-P4Hs may have a long peptide-binding site with multiple interactions.

The C-P4Hs have a peptide-substrate-binding domain that is located between residues 140 and 250 in their catalytic subunit (27) and is distinct from the catalytic domain, which is located in the C-terminal regions of these polypeptides of more than 500 residues. Binding studies performed with varying techniques have shown that all the main properties of peptide binding to the enzyme tetramer are similar to those of binding to a recombinant domain.2 No data are currently available to indicate whether the presumably long peptide-binding site of the HIF-P4Hs is also located in a separate domain or whether the peptides become directly bound to the catalytic domain, which probably has a jellyroll-like structure consisting of eight β-strands (11).

It has been suggested that hydroxylation of Pro$^{102}$ in HIF-1α may be less effective than that of Pro$^{564}$ and may be promoted only by isoenzymes 1 and 2 (11). This suggestion was based on experiments involving hydroxylation of HIF-1α mutants by HIF-P4Hs produced in a reticulocyte lysate and subsequent capture of the hydroxylated polypeptides by the VHL protein. A Pro$^{102}$ → Ala mutation was found to have only a minor effect on capture when the C-terminal site (Pro$^{564}$) was intact, whereas a Pro$^{564}$ → Gly mutation with an intact N-terminal site (Pro$^{102}$) distinctly reduced the capture and appeared to block it in the case of isoenzyme 3, and mutation of both prolines abolished capture with all three isoenzymes (11). Our present data explain these findings by demonstrating that the $K_m$ values of HIF-P4Hs 1 and 2 for the peptide corresponding to the N-terminal hydroxylation site were more than about 20–50 times higher than those for the C-terminal peptide and that isoenzyme 3 did not hydroxylate the N-terminal peptide at all. It is thus likely that hydroxylation of the N-terminal site will also occur much less efficiently than that of the C-terminal site in vivo, and it even seems possible that the N-terminal site may not become hydroxylated at all in many situations. It is also evident that the patterns of hydroxylation of the two sites by the three HIF-P4Hs are not identical.

Although the HIF-2α sequence has up to three potential hydroxylation sites, a 19-residue peptide corresponding to one of them (TNIFQPLAPVPHSPFLLL) could not be hydroxylated by any of the HIF-P4Hs. A HIF-2α peptide corresponding to the C-terminal hydroxylation site in HIF-1α was a good substrate for HIF-P4H-3 and a relatively good substrate for the other two isoenzymes, whereas a peptide corresponding to the N-terminal site had 2–3-fold $K_m$ values for HIF-P4Hs 1 and 2 and a much higher $K_m$ for HIF-P4H-3. The difference in the $K_m$ values of isoenzymes 1 and 2 between the two HIF-2α sites was thus much smaller than for HIF-1α, and even isoenzyme 3 hydroxylated the N-terminal HIF-2α site. HIF-3α also had one site that became effectively hydroxylated by all three isoenzymes. The functions of HIF-3α are currently poorly understood, and an IPAS protein that functions as a dominant negative regulator of the HIF system has been shown to be one of the many splicing variants of the HIF-3α locus (28, 29). Our data and those recently reported by Maynard and co-workers (29) suggest that HIF-3α is subject to similar regulation by proline hydroxylation as HIF-1α and HIF-2α.

The $K_m$ values of the three HIF-P4Hs for O$_2$ were found to be essentially identical, indicating that changes in the O$_2$ concentration of a given cell are likely to have similar effects on the catalytic activities of all three isoenzymes. These $K_m$ values were found to be slightly above the concentration of dissolved O$_2$ in air and much higher than the $K_m$ for O$_2$ of the type I C-P4H. These data agree with the functions of the two classes of P4H, i.e. the HIF-P4Hs are effective oxygen sensors, as their $K_m$ values for O$_2$ are close to atmospheric oxygen concentrations and thus even small decreases in O$_2$ are likely to influence their activities, whereas the type I C-P4H must be able to act even in situations with low O$_2$ concentrations, as in wounds and tissues of low vascularity.

Stabilization of HIF-α polypeptides by small molecule inhibitors of HIF-P4Hs is believed to be therapeutically beneficial in diseases characterized by acute or chronic ischemia, such as myocardial infarction, stroke, peripheral vascular disease, and diabetes (10–12). Preliminary data indicate that several competitive inhibitors of C-P4Hs with respect to 2-oxoglutаратate also inhibit HIF-P4Hs, although no $K_i$ values have been available so far (11, 12). Our data on seven such compounds indicate that five of them inhibited all HIF-P4Hs with $K_i$ values of 50 μM or lower, HIF-P4H-1 having the highest values and HIF-P4Hs 2 and 3 the lowest. All the compounds were much less effective inhibitors of the HIF-P4Hs than the C-P4Hs, which is not surprising, as they were selected from those known to be effective C-P4H inhibitors (8, 30). It should be noted, however, that the $K_i$ values of the compounds for the C-P4Hs could not be used to predict their $K_i$ values for the HIF-P4Hs. For example, pyridine 2,5-dicarboxylate, one of the most effective inhibitors of the C-P4Hs, with a $K_i$ of 0.8 μM, was a particularly weak inhibitor of all three HIF-P4Hs, with $K_i$ values exceeding 300 μM. Our data thus indicate that there must be distinct differences in the structures of the 2-oxoglutarate...
utarate-binding sites between the C-P4Hs and HIF-P4Hs, and even between the members of the HIF-P4H family. It thus should be possible to develop potent small molecule inhibitors for the HIF-P4Hs that show a high degree of specificity with respect to the C-P4Hs, and it might even be possible to develop inhibitors that show some specificity with respect to the individual HIF-P4Hs.

Acknowledgments—We thank Tanja Aatsinki, Aila Holappa, Riitta Jokela, Raija Junntunen, Anne Kokko, Eeva Lehtimäki, Outi Manty, and Jaana Träskelin for excellent technical assistance and Prof. Imo Hassinen (Dept. of Medical Biochemistry and Molecular Biology, University of Oulu) for valuable suggestions.

REFERENCES
Characterization of the Human Prolyl 4-Hydroxylases That Modify the Hypoxia-inducible Factor
Maija Hirsilä, Peppi Koivunen, Volkmar Günzler, Kari I. Kivirikko and Johanna Myllyharju

doi: 10.1074/jbc.M304982200 originally published online June 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M304982200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 29 references, 18 of which can be accessed free at http://www.jbc.org/content/278/33/30772.full.html#ref-list-1