Many Amino Acid Substitutions in a Hypoxia-inducible Transcription Factor (HIF)-1α-like Peptide Cause Only Minor Changes in Its Hydroxylation by the HIF Prolyl 4-Hydroxylases

SUBSTITUTION OF 3,4-DEHYDROPROLINE OR AZETIDINE-2-CARBOXYLIC ACID FOR THE PROLINE LEADS TO A HIGH RATE OF UNCOUPLED 2-OXOGLUTARATE DECARBOXYLATION

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Three human prolyl 4-hydroxylases (P4Hs) regulate the hypoxia-inducible transcription factors (HIFs) by hydroxylating a Leu-Xaa-Xaa-Leu-Ala-Pro motif. We report here that the two leucines in the Leu-Glu-Met-Leu-Ala-Pro core motif of a 20-residue peptide corresponding to the sequence around Pro644 in HIF-1α can be replaced by many residues with no or only a modest decrease in its substrate properties or in some cases even a slight increase. The glutamate and methionine could be substituted by almost any residue, eight amino acids in the former position and four in the latter being even better for HIF-P4H-3 than the wild-type residues. Alanine was by far the strictest requirement, because no residue could fully substitute for it in the case of HIF-P4H-1, and only serine or isoleucine, valine, and serine did this in the cases of HIF-P4Hs 2 and 3. Peptides with more than one substitution, having the core sequences Trp-Glu-Met-Val-Ala-Pro, Tyr-Glu-Met-Ile-Ala-Pro, Ile-Glu-Met-Ile-Ala-Pro, Trp-Glu-Met-Val-Ser-Pro, and Trp-Glu-Ala-Val-Ser-Pro were in most cases equally as good or almost as good substrates as the wild-type peptide. The acidic residues present in the 20-residue peptide also played a distinct role, but alanine substitution for any of them, and in some combinations even three of them, had no negative effects. Substitution of the proline by 3,4-dehydroproline or L-azetidine-2-carboxylic acid, but not any other residue, led to a high rate of uncoupled 2-oxoglutarate decarboxylation. The data obtained for the three HIF-P4Hs in various ex-2-oxoglutarate decarboxylation with no hydroxylation.

The hypoxia-inducible transcription factors (HIFs), which are essential for the regulation of cellular and systemic oxygen homeostasis, are αβ heterodimers in which both types of subunit are basic helix-loop-helix Per-Arnt-Sim proteins. The human α subunit has three isoforms, HIF-1α to HIF-3α, of which HIF-1α and HIF-2α are expressed constitutively but are rapidly degraded under normoxic conditions (for reviews see Refs. 1–4). This degradation is mediated by the oxygen-sensitive degradation domain, which contains two critical proline residues. Hydroxylation of at least one of these to 4-hydroxyproline is essential for the binding of HIFα to the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex and for the subsequent rapid proteasomal degradation (5–8). This hydroxylation is catalyzed in humans by three recently identified cytoplasmic and nuclear HIF prolyl 4-hydroxylases (HIF-P4Hs) (9–11), which are distinct from the well characterized endoplasmic reticulum luminal collagen prolyl 4-hydroxylases (C-P4Hs) (12–16). All P4Hs belong to the family of 2-oxoglutarate dioxygenases and require Fe2+, 2-oxoglutarate, O2, and ascorbate (12–14). The Km values of the three human HIF-P4Hs for O2 are slightly above the concentration of dissolved O2 in the air (17), and thus even a small decrease in the O2 concentration will inhibit their activities, leading to stabilization of HIFα and dimer formation with HIFβ. The dimer is then translocated to the nucleus, where it becomes bound to the HIF-responsive elements in a number of hypoxia-inducible genes, such as those for erythropoietin, vascular endothelial growth factor, glycolytic enzymes, and glucose transporters (1–4).

The two critical proline residues in human HIF-1α, Pro642 and Pro664, are located in the Leu-Thr-Leu-Leu-Ala-Pro-Ala and Leu-Glu-Met-Leu-Ala-Pro-Tyr sequences, respectively. Pro642 is the principal hydroxylation site (8, 10), the Km values of HIF-P4Hs 1 and 2 for a 19-residue peptide corresponding to the N-terminal hydroxylation site being ∼20–50 times higher than those for a peptide corresponding to the C-terminal site, whereas HIF-P4H-3 did not hydroxylate the N-terminal peptide at all (17). All three HIF-P4Hs also hydroxylated 19-residue peptides corresponding to the C- and N-terminal hydroxylation sites in HIF-2α and one in HIF-3α, with the sequences Leu-Glu-Thr-Leu-Ala-Pro-Tyr, Leu-Ala-Gln-Leu-Ala-Pro-Thr, and Leu-Glu-Met-Leu-Ala-Pro-Tyr, respectively, although the second of these had distinctly higher Km values than the other two (17). These data are in agreement with the suggestion that the hydroxylation may involve a conserved core sequence Leu-Xaa-Xaa-Leu-Ala-Pro (8, 10). Initial mutagenesis experiments in fact indicated that substitution of Leu562 by Ala or Ala563 by Gly may prevent any hydroxylation (5, 9). A more recent study has demonstrated, however, that Leu562 → Val, Leu562 → Ala, and Ala563 → Ser mutants were utilized by...
the three HIF-P4Hs only marginally less effectively than a wild-type substrate, whereas Leu$^{559} \rightarrow$ Val was utilized somewhat less effectively (18). Leu$^{574}$ has recently been identified as an additional important residue for hydroxylation (19), which agrees with the previous finding that the deletion of two residues, Gln$^{572}$ and Leu$^{574}$, from the C terminus of a 19-residue HIF-1α C-terminal peptide increased the $K_m$ values for HIF-P4Hs 1 and 2 by 9- and 7-fold, respectively, although the $K_m$ for HIF-P4H-3 was unaltered, and the $V_{\text{max}}$ values for all three isoenzymes were likewise unaffected (17). Pro$^{602}$ and Pro$^{654}$ have several acidic residues in their vicinity, but mutation of Asp$^{556}$, Asp$^{558}$, Glu$^{560}$, Asp$^{669}$, or Asp$^{770}$ to asparagine appeared to have little effect on the hydroxylation (6). An Asp$^{558} \rightarrow$ Ala and Glu$^{560} \rightarrow$ Gln double mutant also served as a substrate, although less efficiently than the wild-type substrate, whereas an Asp$^{569} \rightarrow$ Asn, Asp$^{770} \rightarrow$ Asn, and Asp$^{771} \rightarrow$ Asn triple mutant failed to act as a substrate at all (18).

The C-P4Hs catalyze an uncoupled decarboxylation of 2-oxoglutarate in the presence of all cocrystalline but in the absence of any peptide substrate at a rate that is ~0.5–1% of that of the hydroxylation reaction observed in the presence of a saturating concentration of the peptide substrate (12–14, 20). Some peptides that become bound to the C-P4Hs but do not act as substrates enhance the rate of the uncoupled decarboxylation, but it still remains below ~2% of that of the complete reaction (12–14). However, substitution of the hydroxylatable proline in a peptide substrate by 3,4-dehydroproline gave an uncoupled decarboxylation rate similar to the hydroxylation rate with the nonmodified peptide (21). No data are currently available to indicate whether substitution of the hydroxylatable proline in HIFα model peptides would lead to a similar high rate of uncoupled decarboxylation.

We studied here the sequence requirements of the three human HIF-P4Hs by systematically varying each residue preceding the proline in the Leu-Glu-Met-Leu-Ala-Pro sequence of a 20-residue peptide corresponding to the C-terminal hydroxylation site in HIF-1α. In addition, we studied in detail the effects of substitution of any of six of the seven acidic residues in this peptide by alanine, either alone or in combination with other acidic residues and substitution of the hydroxylatable proline by proline analogues. Our data indicate that the two leucines can be replaced by many residues with either no significant decrease or only a modest decrease in substrate properties and that the glutamate and methionine can well be replaced by most, although not all, amino acids, whereas the alanine is a relatively strict requirement. Any of the six acidic residues studied could be substituted by alanine with no decrease in the substrate properties, and even three of them could be substituted in some combinations with no negative effects. Peptides in which the proline was replaced by either 3,4-dehydroproline or 1-azetidine-2-carboxylic acid gave a very high rate of uncoupled 2-oxoglutarate decarboxylation. The data obtained with the three HIF-P4Hs were in most cases similar, but in some cases HIF-P4H-3 showed distinctly different properties.

MATERIALS AND METHODS

Generation of Recombinant Baculoviruses for the Expression of FLAGHis-tagged HIF-P4H Isoenzymes—The FLAGHis tag was amplified by PCR from the plasmid d2686 (Fibrogen Inc.) and cloned into the 3′ ends before the stop codons of the recombinant human HIF-P4H isoenzymes 1, 2, and 3 in the pVL baculovirus expression vector (17). The recombinant baculovirus expression vectors were cotransfected into Spodoptera frugiperda Sf9 cells with BaculoGold DNA (Pharmingen) by calcium phosphate transfection, and the recombinant baculoviruses were amplified (22).

Expression of Recombinant HIF-P4H Isoenzymes in Insect Cells and Purification of the Enzymes—Nontagged HIF-P4Hs 1 and 2 (17), glutathione S-transferase-tagged HIF-P4H-3 (17), or FLAGHis-tagged HIF-P4Hs 1–3 were expressed in Sf9 or Hs cells cultured in suspension in SF9001SF Medium-2 medium (Invitrogen). The cells, seeded at a density of 1 × 10^9/ml, were infected with the corresponding viruses at a multiplicity of 5, harvested 72 h after infection, washed with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4, and homogenized in a 0.1 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, 0.1% Triton X-100, and 0.01% Triton B, pH 7.8. The soluble fractions were used directly for enzyme activity assays or subjected to further purification.

Nontagged HIF-P4H-2 was partially purified by SE Sepharose chromatography. The cells were homogenized in 150 mM NaCl, 20 mM HEPES buffer, pH 7.5, followed by an addition of 0.25% Triton X-100 and 2.5 mM dithiothreitol. The lysate was incubated at 4 °C for 1 h, centrifuged at 20,000 × g for 20 min, and diluted 1:4 in 20 mM HEPES buffer, pH 6.5. The sample was loaded into an SP Fast Flow column (Amersham Biosciences) equilibrated with 20 mM HEPES buffer, pH 6.5. The column was washed with 50 mM NaCl, 20 mM HEPES buffer, pH 6.5, and eluted with a linear gradient of NaCl from 50 to 332 mM in HEPES buffer, pH 6.5, followed by elution with 500 mM NaCl, HEPES buffer, pH 6.5. HIF-P4H-2 was eluted at a 200 mM NaCl concentration. Recombinant HIF-P4H isoenzymes with C-terminal FLAGHis tags were purified to homogeneity with an anti-FLAG M2 affinity gel (Sigma) (23).

**P4H Activity Assays—**HIF-P4H activity was assayed by a method based on the hydroxylation-coupled decarboxylation of 2-oxoglutarate (17, 24). The synthetic HIF-1α peptide 556-5TDLDLEMLAPMPMDDDFQ577 was used as a substrate, and its substituted variants were obtained from SynPep and Mimotopes. The nonpurified peptides used in the initial experiments were obtained from Mimotopes and were used at a concentration of 200 μM, whereas those used in the measurements of $K_m$ and $V_{\text{max}}$ values had a purity of more than 85%. $K_m$ and $V_{\text{max}}$ values for HIF-P4H isoenzymes were determined at saturating concentrations of the other components, except that the O2 concentration was that of air and thus nonsaturating (17).

Liquid Chromatography/Mass Spectrometry Analysis of Peptide Hydroxylation—The enzyme activity assays used in this set of experiments were conducted with partially purified HIF-P4H-2, following the standard protocol (17, 24), except that the concentration of 2-oxoglutarate was 100 μM, and that of the HIF-1α peptide 556-5TDLDLEMLAPMPMDDDFQ773 or peptides in which the hydroxylatable Pro$^{654}$ was substituted by 3,4-dehydroproline or L-azetidine-2-carboxylic acid was likewise 100 μM. The two methionines were substituted by alanines in all of these peptides to eliminate the possible formation of auto-oxidation products. The reaction was carried out at 37 °C for 4 h and stopped by the addition of 1 mM EDTA. Hydroxylation of the peptides was analyzed by liquid chromatography with mass spectrometric detection using a Finnigan LCQ™ DUO LC/MS instrument (Thermo Electron) with electrospray ionization in negative mode. Chromatography was carried out using a YMC Pro Pack C18 (150 × 2.0 mm, 3 μm, 120 Å) column with a gradient of 1–75% acetonitrile in 0.1% formic acid at a flow rate of 0.2 ml/min.

**RESULTS**

**Leu$^{559}$ and Leu$^{574}$ Can Be Substituted by Many Other Residues with Relatively Small Effects—**The 20-residue control peptide used as a substrate in the present study corresponds to the hydroxylation site around Pro$^{564}$ in HIF-1α and has the sequence 554-5TDLDLEMLAPMPMDDDFQ773. It differs from the 19-residue peptide used previously (17) in that it contains two additional residues in its N terminus and lacks one, Leu$^{574}$, in its C terminus. The $K_m$ values of a purified version of this peptide for HIF-P4Hs 1, 2, and 3 are 15, 25, and 5 μM, respectively (see below). Because of the high cost of synthetic peptides, all of the initial experiments were performed using crude peptide preparations of less than 50% purity. The nominal peptide concentrations used in the initial experiments were 200 μM, but because of the low degree of purity, the true concentrations were less than 100 μM. Thus the concentration of the control peptide used for HIF-P4Hs 1 and 2 may have been less than 4–6.5 times the $K_m$ and that for HIF-P4H-3 may have been less than 20 times the $K_m$. Consequently, the assays may have been insensitive for substitutions that cause
only relatively small $K_m$ effects with no changes in $V_{max}$, especially in the case of HIF-P4H-3. Enzyme activity was assayed based on measurement of the radioactivity of the $^{14}CO_2$ formed during the hydroxylation-coupled stoichiometric decarboxylation of 2-oxo-[1-$^{14}$C]glutarate using Triton X-100 buffer extracts of homogenates of insect cells expressing recombinant HIF-P4Hs as sources of the enzymes (17), or in the case of HIF-P4H-2, preparations that had been partially purified from such extracts by one chromatography step. Because the purity of the peptides was low, the reaction rates obtained in the initial experiments are given in Tables I, II, and IV only as >120, 80–120, 50–80, 30–50, 10–30, and <10% relative to that obtained with the control peptide of a similar low degree of purity.

Substitution of Leu$^{559}$ by other residues gave identical data for HIF-P4Hs 1 and 2 (Table I). Its replacement by tryptophan under the conditions used gave a reaction rate for both isoenzymes that was significantly higher than that obtained with leucine (120–130%), whereas the rates obtained with tyrosine and methionine were similar to that with leucine. Five additional amino acids, isoleucine, phenylalanine, alanine, glutamate and aspartate, gave rates that were 50–80% of that with leucine, whereas the lowest rates, −10–30%, were obtained with asparagine, threonine, histidine, arginine, and lysine, thus including all of the basic amino acids (Table I). In the case of HIF-P4H-3, there were two residues, isoleucine and phenylalanine, that gave significantly higher rates than leucine (>130%), whereas five additional residues, tryptophan, tyrosine, methionine, alanine, and valine, gave rates similar to that with leucine. Seven other residues gave rates that were 50–80% of that with leucine, and five residues gave rates that were less than 50% (Table I). It may be noted that the ranking of the peptides was not identical for HIF-P4H-3 and the other two isoenzymes (Table I).

Substitution of Leu$^{562}$ again gave identical data for HIF-P4Hs 1 and 2, and very similar, although not identical, data for HIF-P4H-3 (Table I). No residue was significantly better than leucine in this position, but four, valine, isoleucine, phenylalanine, and arginine, gave rates similar to that with leucine for isoenzymes 1 and 2, and five, valine, isoleucine, phenylalanine, tyrosine, and methionine, for isoenzyme 3 (Table I). For all three HIF-P4Hs, there were eight residues altogether that gave rates that were at least 50% of that with leucine, seven of them being the same for all three isoenzymes. The lowest rates for all three isoenzymes were obtained with glutamate, glycine, proline, and aspartate (Table I). Interestingly, these residues include the two acidic amino acids, whereas in the Leu$^{559}$ position the least effective residues were histidine, arginine, and lysine.

Glu$^{560}$ and Met$^{561}$ Can Be Substituted by Most Amino Acids, whereas Ala$^{563}$ Is a Relatively Strict Requirement—The requirements for Glu$^{560}$, Met$^{561}$, and Ala$^{563}$ were studied by means of experiments similar to those above. Glu$^{560}$ could be substituted by most amino acids in the case of HIF-P4H-2 with no significant change in the reaction rate (Table II), with seven additional residues giving rates of 50–80%, whereas lysine and proline were the only ones that gave a rate less than 50% (~40%). The data obtained with HIF-P4H-1 were very similar, although not identical, to those with HIF-P4H-2 (details not shown). In the case of HIF-P4H-3 any amino acid in this position gave a rate that was similar to or even slightly higher (up to 140%) than that obtained with glutamate (Table II). Interestingly, arginine and lysine were among the best substitutes for HIF-P4H-3, whereas they were among the least effective ones for HIF-P4Hs 1 and 2 (Table II).

Met$^{561}$ could be replaced by almost any amino acid with no significant change in the reaction rate for HIF-P4H-2, proline being the only residue that gave a rate less than 80% (~50%) (Table II). The data for HIF-P4H-1 were again similar, although not identical, to those for HIF-P4H-2 (details not shown). Most amino acids also gave rates similar to or slightly higher (up to ~130%) than that with methionine for HIF-P4H-3, whereas three residues, aspartate, glutamate, and proline, gave rates below 80%, with proline giving the lowest rate (~60%) (Table II).

Ala$^{563}$ could be substituted only by serine with no significant change in the reaction rate for HIF-P4H-2, whereas threonine and lysine gave rates of 50–80% (Table II). Valine and glutamine gave rates of 30–50%, and four other amino acids gave rates of 10–30%, whereas all of the others gave rates less than 10% (Table II). The ranking of residues was similar for HIF-P4H-1, but the percentages obtained with some amino acids were even lower than for HIF-P4H-2, the rate obtained with serine being only ~60% and that with valine 20%, whereas the rate obtained with lysine was the same as for HIF-P4H-2 (details not shown). In the case of HIF-P4H-3, isoleucine, valine, and serine gave rates similar to that with alanine, six others gave rates of 50–80%, five 30–50%, and four gave rates of less than 30% (Table II).

Analysis of Purified Peptides, Including Those with More than One Substituted Residue, Confirms Data Obtained in the Initial Experiments—To study whether the data obtained with peptides of low purity can be confirmed with those of a high degree of purity, many purified peptides were studied as substrates of the three HIF-P4Hs using either enzymes similar to those in the initial experiments or enzymes purified to homogeneity by an affinity chromatography based on their FLAG tags. Identical $K_m$ and relative $V_{max}$ values were obtained with both enzyme sources.

Substitution of Leu$^{559}$ by tryptophan gave for all three HIF-P4Hs a $V_{max}$ of 120% with essentially unchanged $K_m$ values relative to the control peptide (Table III). These data are in agreement with the increased reaction rates found for iso-
The relative reaction rates with peptides representing the C-terminal hydroxylation site of HIF-1α with 19 substitutions for the Glu560, Met561, and Ala563 residues

The reaction rates were determined using partially purified recombinant HIF-P4H-2 and soluble extracts of insect cells expressing HIF-P4Hs 1 and 3 as sources of the enzyme. The 19 amino acid residues are: Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val.

| Activity | HIF-P4H-2<sup>a</sup> | HIF-P4H-3 | HIF-P4H-2<sup>b</sup> | HIF-P4H-3 | HIF-P4H-2<sup>c</sup> | HIF-P4H-3 |
|----------|----------------------|------------|----------------------|------------|----------------------|------------|
| >120%    | Glu<sup>560</sup> (LEMLAP) replaced by | Met<sup>561</sup> (LEMLAP) replaced by | Ala<sup>563</sup> (LEMLAP) replaced by | | | |
| 80–120%  | Most amino acids | Other amino acids | Pro | Most amino acids | Asp, Glu, Pro | Ala, Ser, Thr, Lys, Arg, Ile, Lys, His, Cys, Thr, Val, Glu, Met, Phe, Gly, Leu |
| 50–80%   | Trp, Val, Phe, Tyr, Cys, Arg, His | 60–50% | Lys, Pro | | 300–600 | |
| 30–50%   | 60–50% | ND | 500 | ND | 100–500 | |
| 10–30%   | | | | | | |
| <10%     | | | | | | |

<sup>a</sup> The reaction rates are relative values with respect to that determined for the nonmodified control peptide 554DTDLLEMLAPYIPMDDFQ<sup>573</sup>.<br><sup>b</sup> The values were determined using highly purified recombinant HIF-P4Hs 1–3 or soluble extracts of insect cells expressing HIF-P4H-1 as sources of the enzyme. The 19 amino acid residues are: Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val.<br><sup>c</sup> Similar, although not identical, data were obtained for HIF-P4H-1. In the case of Ala<sup>563</sup>, no residue gave a rate of 80–100%, with serine giving a rate of only about 60%. The rate obtained with valine was also distinctly lower than with HIF-P4H-2, only 20%.

The effects of substitution of more than one residue were studied using five purified peptides, three double mutant peptides, Leu<sup>559</sup>→Trp, Leu<sup>662</sup>→Val (WEMVAP), Leu<sup>559</sup>→Ty, Leu<sup>662</sup>→Ile (YEMIAIP), and Leu<sup>559</sup>→Ile, Leu<sup>662</sup>→Ile (YEMIAP), one triple mutant peptide, Leu<sup>559</sup>→Trp, Leu<sup>662</sup>→Val, and Ala<sup>563</sup>→Ser (WEMVSP), and one four-substitution mutant, Leu<sup>559</sup>→Trp, Met<sup>656</sup>→Ala, Leu<sup>652</sup>→Val, and Ala<sup>563</sup>→Ser (WEAVSP). All five peptides gave <i>K<sub>m</sub></i> values for the three HIF-P4Hs that were similar to or less than twice that of the control peptide, and in the case of HIF-P4H-3 all five peptides gave <i>V<sub>max</sub></i> values that were 90–120% of that of the control (Table III). In the case of HIF-P4H-2 four of the peptides gave
The reaction rates were determined using partially purified recombinant HIF-P4H-2 as a source of the enzyme.

| Activity | One substitution | Two substitutions | Three substitutions | Four substitutions |
|----------|-----------------|------------------|---------------------|-------------------|
| >80%     | Asp^556         | Asp^556 + Asp^558| Asp^556 + Asp^558 + Asp^570 | Asp^556 + Asp^558 + Asp^570 + Asp^571 |
|          | Asp^558         |                  |                     |                   |
|          | Asp^558 + Glu^560 | Asp^558 + Asp^558 + Asp^570 |                   |                   |
|          | Glu^560         |                  |                     |                   |
|          | Asp^569         | Asp^569 + Asp^570 |                   |                   |
|          | Asp^569 + Asp^570 | Asp^569 + Asp^570 + Asp^571 |                   |                   |
|          | Asp^570         |                   |                     |                   |
|          | Asp^570 + Asp^571 |                   |                     |                   |
|          | Asp^571         |                   |                     |                   |
|          | Asp^571 + Glu^560 |                   |                     |                   |
|          | Glu^560 + Asp^570 |                   |                     |                   |
| 50–80%   | Asp^556 + Asp^569 | Asp^556 + Asp^556 + Asp^570 |                   |                   |
|          | Asp^558         | Asp^558 + Asp^569 |                   |                   |
|          | Asp^558 + Glu^560 | Asp^558 + Asp^570 + Asp^571 |                   |                   |
|          | Glu^560 + Asp^569 |                   |                     |                   |
|          | Asp^569         | Asp^569 + Asp^570 |                   |                   |
|          | Asp^569 + Asp^570 | Asp^569 + Asp^571 |                   |                   |
| 30–50%   | Asp^556 + Asp^556 + Glu^560 |                   |                   |
|          | Asp^556 + Glu^560 + Asp^569 |                   |                   |
|          | Asp^556 + Glu^560 + Asp^570 |                   |                   |
|          | Asp^556 + Glu^560 + Asp^571 |                   |                   |
| 10–30%   | Asp^556 + Asp^556 + Asp^569 + Asp^570 |                   |                   |
|          | Asp^556 + Asp^556 + Asp^569 + Asp^570 |                   |                   |
|          | Asp^556 + Asp^556 + Asp^569 + Asp^571 |                   |                   |
| <10%     | Asp^556 + Asp^556 + Asp^556 + Asp^570 |                   |                   |
|          | Asp^556 + Asp^556 + Asp^556 + Asp^571 |                   |                   |
|          | Asp^556 + Asp^556 + Asp^556 + Asp^572 |                   |                   |
|          | Asp^556 + Asp^556 + Asp^556 + Asp^573 |                   |                   |

* The reaction rates are expressed relative to that determined for the nonmodified control peptide DTDLDEMLAPYIPDFFQ.

$V_{\text{max}}$ values identical to that of the control, one giving a $V_{\text{max}}$ of 70%, whereas in the case of HIF-P4H-1 two peptides gave $V_{\text{max}}$ values of 80–90% and three of 60–70% (Table III). Our data thus indicate that the three HIF-P4Hs, and especially HIF-P4H-3, act well on sequences with cores distinctly different from the conserved Leu-Xaa-Xaa-Leu-Ala-Pro core motif (8, 10).

Substitutions of Alanine for Acidic Residues Cause Either No Effect Or Only Minor Effects in Many Cases—The 20-residue peptide contains seven acidic residues, six of which, excluding the N-terminal aspartate, were subjected to alanine substitution studies with peptides of a similar degree of purity as in the other initial experiments. As indicated in Table IV, three of the acidic residues, Asp^556, Asp^558, and Glu^560, were on the N-terminal side of the Pro^564 to be hydroxylated, and three, Asp^569, Asp^570, and Asp^571, were on the C-terminal side. The data are shown only for HIF-P4H-2, the results obtained with the other two isoenzymes being very similar.

Any single acidic residue could be substituted by alanine with no decrease in the substrate properties of the peptide under the conditions used (Table IV). Also, any two acidic residues could be substituted with no decrease in the reaction rate unless one of them was Asp^569, i.e. the first of the three consecutive aspartates in the PYIPD sequence, in which case the rate was 50–80% (Table IV). Interestingly, even three acidic residues could be substituted with no significant decrease in the reaction rate when two of them were on the N-terminal side of Pro^564 and one on the C-terminal side, but not Asp^569 (Table IV), whereas substitution of three residues, of which two were on the C-terminal side and one on the N-terminal side, gave rates of 50–80%, unless the pair on the C-terminal side was Asp^556 and Asp^570, in which case the rate was lower (Table IV). Substitution of all three acidic residues on the N-terminal side, and some other combinations of three and four residues, gave rates that were 30–50% of the control, whereas some combinations of four residues gave rates that were only 10–30% (Table IV). Substitution of all three residues on the C-terminal side or substitution of four residues, of which three were on the C-terminal side, gave the lowest rates, less than 10% (Table IV).

Substitution of Pro^564 by 3,4-Dehydroproline or L-Azetidine-2-Carboxylic Acid, but Not by Other Proline Analogues or Other Amino Acids, Leads to a High Rate of Uncoupled 2-Oxoglutarate Decarboxylation—Peptides in which Pro^564 was substituted by 3,4-dehydroproline or l-azetidine-2-carboxylic acid (Fig. 1), gave rates for the decarboxylation of 2-oxoglutarate in the initial experiments with all three HIF-P4Hs that were similar to or even higher than the hydroxylation-coupled decarboxylation rate obtained with the control peptide (details not shown). These effects were highly specific, because the decarboxylation rates found with peptides in which the proline had been replaced by $\beta$-thioproline, l-homoproline (l-pipecolic acid), l-trans-4-hydroxyproline, 3-azetidine-carboxylic acid, nipecolic acid, or l-tetrahydroisoquinoline carboxylic acid were less than 5% (details not shown). Also, replacement of the proline by serine, cysteine, methionine, asparagine, glutamine, or histidine caused no significant decarboxylation (details not shown).

Further experiments were carried out with highly purified peptides to determine the $K_m$ and $V_{\text{max}}$ values and to assess whether the high 2-oxoglutarate decarboxylation rates ob-
served with the 3,4-dehydroproline and azetidine-2-carboxylic acid peptides indeed represented uncoupled decarboxylation. To avoid the formation of auto-oxidation products of methionine, the two methionines present in the control peptide were substituted by alamines in all of these peptides, including the control peptide itself.

The \( V_{\text{max}} \) values of the 3,4-dehydroproline peptide for all three HIF-P4Hs were 120–140%, whereas the \( K_m \) values were similar to that of the control peptide or slightly lower (Table V). The \( K_m \) values of the azetidine-2-carboxylic acid peptide for the HIF-P4Hs were 3–6-fold relative to that of the control peptide, the \( V_{\text{max}} \) values for HIF-P4Hs 1 and 2 being 70%, whereas that for HIF-P4H-3 was 110% (Table V).

Possible hydroxylation of the peptides was studied by performing enzyme reactions in which the concentrations of the peptide and 2-oxoglutarate were each 100 \( \mu M \), and the reaction was run for 4 h, in which time most of the 2-oxoglutarate had been consumed. Liquid chromatography with mass spectrometric detection was then used to separate the peptide from the other reaction components and analyze its mass spectral features. In agreement with our previous data (details not shown), the elution position of the hydroxylated control peptide (retention time, 40.92 min; Fig. 2A) differed distinctly from that of the nonhydroxylated peptide (retention time, 41.40 min; Fig. 2B), whereas the mobilities of the 3,4-dehydroproline (Fig. 2, C and D) and azetidine-2-carboxylic acid (details not shown) peptides were identical with and without enzyme incubation. The corresponding mass spectral data, which represent the −2 charge state of the peptides, are shown as insets in the four panels in Fig. 2. In such analyses the mass to charge ratio \( m/z \) of the nonhydroxylated control peptide should be 1137.2, and that of the hydroxylated control peptide should be 1145.2. The values 1137.92 and 1145.95 obtained here (Fig. 2, A and B) are identical with the theoretical values within the bounds of experimental error and indicate that the control peptide had become hydroxylated. The corresponding theoretical values for the nonhydroxylated and hydroxylated 3,4-dehydroproline peptide are 1136.2 and 1144.2, respectively. The values 1136.84 and 1136.67 obtained here (Fig. 2, C and D) are identical with each other and with the theoretical value for the nonhydroxylated peptide within the bounds of experimental error. Mass spectral analysis of the azetidine-2-carboxylic acid peptide likewise showed that it was not chemically altered by the enzyme incubation (details not shown). It is thus evident that the high 2-oxoglutarate decarboxylation rates observed with these two peptides were not due to any hydroxylation-coupled decarboxylation.

Experiments similar to the above were also carried out to study whether all of the 2-oxoglutarate decarboxylation found with peptides in which residues other than the proline had been modified indeed represented hydroxylation-coupled decarboxylation. Two peptides with sequences DTDLDEALAPYPADDFFQ and DTDLDELALKPYIPADDFFQ were selected for these experiments. The mobilities of both peptides were altered by the enzyme incubation, as in the case of the control peptide (above), and mass spectrometry indicated that they had become hydroxylated (details not shown).

| Pro\(^{564}\) replaced by | HIF-P4H-1 | HIF-P4H-2 | HIF-P4H-3 |
|-------------------------|-----------|-----------|-----------|
| \( K_m \) \( \mu M \) | \( V_{\text{max}}^\alpha \) | \( K_m \) \( \mu M \) | \( V_{\text{max}}^\alpha \) | \( K_m \) \( \mu M \) | \( V_{\text{max}}^\alpha \) |
| None | 45 | 100 | 40 | 100 | 4 | 100 |
| 3,4-Dehydroproline | 30 | 120 | 30 | 120 | 6 | 140 |
| Azetidine-2-carboxylic acid | 150 | 70 | 240 | 70 | 40 | 110 |

\(^\alpha\) The \( V_{\text{max}} \) values are expressed relative to that obtained for each enzyme with the nonmodified control peptide \(^{564}\)DTDLDEALAPYPADDFFQ (taken as 100%).

**DISCUSSION**

Our data clearly indicate that the two leucines in the Leu-Xaa-Xaa-Leu-Ala-Pro core motif can be substituted by many amino acids with essentially no decrease or only a modest decrease in the properties of the 20-residue HIF-1α-like peptide as a substrate for the three HIF-P4Hs. Furthermore, substitution of Leu\(^{559}\) in the cases of isoenzymes 1 and 2 by one residue, tryptophan, and in the case of isoenzyme 3 by three residues, isoleucine, phenylalanine, or tryptophan, even slightly improved the substrate properties of the peptide. As expected, the two Xaa positions could be occupied by almost any amino acid, but a surprising finding was that in the case of Glu\(^{566}\) eight amino acids were even better for HIF-P4H-3 than the authentic residue. These amino acids included arginine and lysine, whereas in the cases of HIF-P4Hs 1 and 2 arginine, histidine and lysine were among the four least effective substitutes. The position of Met\(^{561}\) could likewise be occupied by residues that improved the substrate properties for isoenzyme 3, namely tyrosine, tryptophan, leucine, and valine, but surprisingly, three residues, aspartate, glutamate, and proline, were less effective than methionine for isoenzyme 3, whereas only proline was less effective than methionine for isoenzymes 1 and 2. Ala\(^{563}\) was found to be by far the strictest requirement, as it could be fully substituted by no residue in the case of HIF-P4H-1, by only one residue, serine, in the case of HIF-P4H-2, and by three residues, isoleucine, valine, and serine, in the case of isoenzyme 3, whereas only four additional residues gave a rate of more than 30% with isoenzyme 2 under the conditions tested.

Hydroxylation by a C-P4H requires an Xaa-Pro-Gly triplet, in which Xaa can be any amino acid and the glycine can in some cases be replaced by alanine or glutamine (13). A tripeptide with this sequence fulfills the minimum requirement, but longer peptides are much better substrates, because amino acids in other parts of the peptide also affect enzyme-substrate interaction. Furthermore, the conformation of the peptide has a major effect in that the triple-helical conformation of collagen-like peptides completely prevents hydroxylation (19). Our present data indicate that the HIF-P4Hs require an Ala-Pro sequence, in which no other residue can fully replace the alanine in the case of isoenzyme 1, and only one or three residues may do so in the cases of isoenzymes 2 and 3. Previous work has demonstrated that the HIF-P4Hs require even longer peptide substrates than the C-P4Hs, the minimum requirement for any hydroxylation being a peptide of more than eight residues (17). The present and previous (18) data suggest that the requirement for a long peptide may be due to the marked redundancy in the substrate sequence, which has preferences for certain positions such as those occupied by Leu\(^{559}\) and Leu\(^{562}\), those occupied by acidic residues, and that occupied by Leu\(^{574}\) (19),
and consequently the HIF-P4Hs may have a long peptide-substrate-binding site with multiple interactions (17). A specific peptide conformation may not be a requirement for binding, because NMR studies have shown that a 19-amino acid peptide corresponding to the C-terminal hydroxylation site of HIF-1α in which Pro564 is replaced by 3,4-dehydroproline, was substituted by the theoretical mass to charge ratios of the 2-oxoglutarate had been consumed, and hydroxylation of the peptides was analyzed by liquid chromatography (A and C) and mass spectroscopy (insets in A and C). The inset in A shows the mass spectroscopy data of the peak with the retention time of 40.92. In control experiments, the peptides were subjected to hydroxylation (B and D). The mass spectroscopy data shown in the insets represent the −2 charge state of the peptides. The theoretical mass to charge ratio m/z of the DTDLDEALAPYIPADDDFQ peptide and the dehydroproline peptide are 1137.2 (B) and 1136.2 (D), respectively, and those of the corresponding hydroxylated peptides are 1145.2 (A) and 1144.2 (C). The observed m/z values are given in the insets in A–D.

The three-dimensional structures of the HIF-P4Hs and the full-length HIF-1α are currently unknown, but the structure of a HIF-1α-peptide-pVHL complex has been solved (25, 26). Residues 560–577 of the hydroxylated peptide were found to become bound to one of the β sheets of the pVHL in an extended β strand-like conformation as if the peptide was a complementary β strand, although there is no extensive main chain hydrogen bonding to define it as such (25, 26). The β sheet-like interactions contribute to the stability of the complex, but the optimized hydrogen bonding to the hydroxyproline discriminator between binding of hydroxylated and unmodified HIF-1α to pVHL (25, 26). Crystal structures of various 2-oxoglutarate dioxygenases, including the HIF asparaginyl hydroxylase have shown that their catalytic sites are all located in a jelly roll β barrel structure formed by eight β strands, the cosubstrates, and the peptide substrate becoming bound in a cavity that is located between the two sheets of the jelly roll and exposed at one side of the β barrel (14, 27–29). It therefore seems very likely that the catalytic site of the HIF-P4Hs has a similar structure (10) and that HIF-1α may become bound to these enzymes by forming an intermolecular β sheet-like structure and inserting the proline to be hydroxylated into the β barrel cavity, the binding being thus analogous to the interaction of HIF-1α with pVHL (26).

Previous studies have demonstrated clear differences between the three HIF-P4Hs in their substrate requirements, especially between isoenzyme 3 and the two others. One such difference is that shortening of a 19-residue HIF-1α-like peptide at its N or C terminus influences the $K_m$ and $V_{max}$ values for the three isoenzymes differently, isoenzyme 3 being the least sensitive to this (17). On the other hand, isoenzyme 3 failed to hydroxylate a 19-residue peptide corresponding to the N-terminal hydroxylation site around Pro562 in HIF-1α, whereas this peptide was hydroxylated by HIF-P4Hs 1 and 2, although with $K_m$ values −20–50 times higher than those for a peptide corresponding to the C-terminal site (17). This difference could not be explained by the residue following the proline being alanine in the N-terminal peptide but tyrosine in the C-terminal one, because a Tyr565 → Ala substitution in the C-terminal peptide had only very minor effects on the $K_m$ values and no effect on the $V_{max}$ with any of the three isoenzymes (17). A Tyr565 → Gly substitution had a slightly larger effect on $K_m$, but again none on $V_{max}$ with any of the isoenzymes (17). The present data further indicate that isoenzyme 3...
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differed very distinctly in its sequence requirements from isoenzymes 1 and 2 in some cases, the latter two having identical or very similar requirements in most cases but not all.

Because many of the observations reported here are based on experiments with peptides of low purity, the possibility cannot be excluded that some of the findings with respect to a specific amino acid substitution may be slightly erroneous and that the true values obtained with a purified peptide would be slightly higher or lower than those shown in Tables I, II, and IV. It should be noted, however, that the data obtained with various peptides for a single position showed a remarkable consistency with respect to the nature of the amino acid, in that the three basic amino acids histidine, arginine, and lysine were the least effective substitutes for Leu\textsuperscript{560}, for instance, aspartate and glutamate were among the least effective substitutes for Leu\textsuperscript{562}, and arginine, histidine, and lysine were among the four least effective substitutes for Glu\textsuperscript{560} in the cases of HIF-P4Hs 1 and 2. Also, the seven Leu\textsuperscript{559} substitutes that gave a rate for isoenzyme 3 that was similar to or higher than that with the Glu-Met-Leu-Ala-Pro motif, the peptide for HIF-P4Hs 2 and 3 than the control peptide with the Leu-Met-Ile-Ala-Pro, Trp-Glu-Met-Val-Ser-Pro, and Trp-Glu-Ala-Val-Ser-Pro were essentially as good or even better substrates for HIF-P4Hs 2 and 3 than the control peptide with the Leu-Glu-Met-Leu-Ala-Pro motif, the linearity between rates of hydroxylation with the nonmodified peptides at 90 and 80% of that of the control, whereas those of the latter two were ~60 and 70%, evidently because of the unfavorable effect of the alanine to serine substitution in the case of this isoenzyme. The peptide Ile-Glu-Met-Ile-Ala-Pro was a slightly worse substrate than the control peptide for all three isoenzymes, its \(V_{\text{max}}\) values for HIF-P4Hs 3, 2 and 1 being 90, 70, and 60%, respectively, with only a minor effect on the \(K_m\) for any of the isoenzymes. It is possible that the HIF-P4Hs may have additional \textit{in vivo} substrates that are regulated by oxygen-dependent proline hydroxylation and subsequent binding to pVHL, ubiquitination, and proteasomal degradation, with one suggested substrate being the large subunit of RNA polymerase II (30). Previous data reporting that a conserved Leu-Xaa-Xaa-Leu-Ala-Pro core motif may be an important requirement for hydroxylation (8, 10) suggest that it might be possible to find additional HIF-P4H substrates by means of computerized protein sequence searches and verification of the results by hydroxylation experiments with synthetic peptides. Our present data indicate, however, that such an approach is not feasible because of the lack of specificity of the sequence of the core motif.

None of the six acidic residues studied was found to be critical for the substrate properties of the 20-residue peptide, thus confirming data obtained previously for five of these residues (6). Acidic residues were nevertheless found to play a distinct role in the substrate properties of the peptide, although as many as three of them could be substituted simultaneously by alanines in some combinations with no adverse effects. The data further indicate that Asp\textsuperscript{560} may be more critical than the other acidic residues studied and that acidic residues on the C-terminal side of the hydroxylatable proline may be more critical than those on the N-terminal side. Although confirmatory experiments using purified peptides having alanine substitutions for the acidic residues were not undertaken, remarkable consistency was found in the data with respect to the effects of mutations of more than one residue, suggesting that purified peptides would have given at least very similar, if not identical results.

Substitution of the hydroxylatable proline in a peptide substrate for the C-P4Hs by 3,4-dehydroproline has been found to lead to uncoupled decarboxylation of 2-oxoglutarate at a rate similar to the rate of hydroxylation with the nonmodified peptide (21). Our present data indicate that the HIF-P4Hs are similar to the C-P4Hs with respect to the effect of such a substitution and extend those reported for the C-P4Hs by demonstrating that replacement of the proline by azetidine-2-carboxylic acid also leads to a high uncoupled decarboxylation rate, the effects of the two proline analogues being highly specific in that no other replacement of the proline residue studied caused any significant uncoupled decarboxylation.

Ascorbate is not needed in the hydroxylation reaction catalyzed by the C-P4Hs, but it is an absolute requirement for the C-P4Hs both \textit{in vitro} and \textit{in vivo} (12–14). The reaction requiring ascorbate has been shown to be the uncoupled 2-oxoglutarate decarboxylation, in which ascorbate is consumed stoichiometrically with the decarboxylation (31, 32). The C-P4Hs catalyze this uncoupled decarboxylation in the absence of any peptide substrate at a rate that is ~0.5–1% of the rate of the hydroxylation reaction (13, 20). In the uncoupled decarboxylation the highly reactive ferryl ion formed at the catalytic site during the first half-reaction of the catalytic cycle is probably converted to Fe\textsuperscript{3+}\textsuperscript{−}O\textsuperscript{−}, making the enzyme unavailable for new catalytic cycles until reduced by ascorbate (13, 31, 32). The C-P4Hs catalyze occasional uncoupled decarboxylation cycles even in the presence of a saturating concentration of their peptide substrates, and the main biological function of ascorbate in their reactions both \textit{in vitro} and \textit{in vivo} is believed to be that of serving as an alternative oxidizable substrate in the uncoupled decarboxylation cycles (12–14, 31, 32). The HIF-P4Hs also catalyze an uncoupled 2-oxoglutarate decarboxylation in the absence of their peptide substrates, at ~3% of the rate of the hydroxylation-coupled decarboxylation, and the presence of a 3,4-dehydroproline or azetidine-2-carboxylic acid residue at the catalytic site markedly increases this rate, as found here. It therefore seems highly likely that the main function of ascorbate in the HIF-P4H reaction is similar to that in the C-P4H reaction, \textit{i.e.} to serve as an alternative oxygen acceptor in the uncoupled decarboxylation cycles.

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