The master switch of cellular hypoxia responses, hypoxia-inducible factor 1 (HIF-1), is hydroxylated by factor inhibiting HIF-1 (FIH-1) at a conserved asparagine residue under normoxia, which suppresses transcriptional activity of HIF-1 by abrogating its interaction with transcription coactivators. Here we report the crystal structure of human FIH-1 at 2.8-Å resolution. The structural core of FIH-1 consists of a jellyroll-like β-barrel containing the conserved ferrous-binding triad residues, confirming that FIH-1 is a member of the 2-oxoglutarate-dependent dioxygenase family. Except for the core structure and triad residues, FIH-1 has many structural deviations from other family members including N- and C-terminal insertions and various deletions in the middle of the structure. The ferrous-binding triad region is highly exposed to the solvent, which is connected to a prominent groove that may bind to a helix near the hydroxylation site of HIF-1. The structure, which is in a dimeric state, also reveals the putative von Hippel-Lindau-binding site that is distinctive to the putative HIF-1-binding site, supporting the formation of the ternary complex by FIH-1, HIF-1, and von Hippel-Lindau. The unique environment of the active site and cofactor-binding region revealed in the structure should allow design of selective drugs that can be used in ischemic diseases to promote hypoxia responses.

Mammalian cells adapt themselves to low oxygen conditions (hypoxia) by activating a conserved hypoxia response pathway, where the transcription factor, hypoxia-inducible factor 1 (HIF-1) plays a major role (1). The protein products of HIF-1-regulated genes are responsible for angiogenesis, vascular reactivity and remodeling, glucose and energy metabolism, cell proliferation and survival, erythropoiesis, and iron metabolism (1). HIF-1 is a basic helix-loop-helix/Per-Arnt-Sim homology domain protein composed of two (α and β) subunits. Both the half-life and transactivation function of HIF-1α are regulated by changes in the cellular oxygen level, whereas HIF-1β remains mostly unaffected (1).

Two separate domains within HIF-1α are responsible for the mechanisms by which cellular oxygen regulates HIF-1 activity. The first is the oxygen-dependent degradation (ODD) domain, which is hydroxylated by a specific proline hydroxylase (HIF-1-PH) (2–6), and then recognized by the von Hippel-Lindau (VHL) ubiquitin-protein ligase complex for targeting to the proteasome (7–11). A second hypoxic switch has recently been identified to operate in the C-terminal activation domain (CAD), where a conserved asparagine residue is hydroxylated by factor inhibiting HIF-1 (FIH-1) (12–14). Hydroxylation of the asparagine residue during normoxia suppresses interaction of CAD with transcription coactivators (15–19).

Both FIH-1 and HIF-1-PH belong to the 2-oxoglutarate (2OG)-dependent dioxygenase superfamily (2, 12–14). The family consists of a variety of enzymes catalyzing hydroxylations, desaturations, and oxidative ring closures/rearrangements (20). Crystal structures of the family members have been reported for bacterial proline 3-hydroxylase and other enzymes responsible for biosynthesis of antibiotics (21–24). Nevertheless, revelation of crystal structures of FIH-1 and HIF-1-PH is very important in understanding oxygen-dependent regulation of HIF-1, because these two enzymes serve as oxygen sensors in the hypoxia response pathway.

Here we report the crystal structure of human FIH-1, one of the two oxygen sensors. The structural core of FIH-1 consists of a β-barrel like other members of the 2OG-dependent dioxygenase family. In comparison to other family members, the structure of FIH-1 shows several distinctive features such as a unique cofactor-binding site in the wide-opened active site pocket, a dimerization domain at the C terminus, and a long and wide groove at the center of the molecule ranging from the active site of the enzyme toward the dimerization domain. The structure-based interpretation of previous biochemical analyses suggests a mechanism of hypoxia regulation by utilizing a multicomponent complex made of FIH-1, HIF-1, and VHL.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of FIH-1—The gene for FIH-1 was amplified by PCR from human colon cDNA library (Clontech) using 5′-ggg att cca tat tgt ggg gac age ggc gga g3′ as a forward primer and 5′-ctg tag atc cgg gaa gga gct ggc ggc c3′ as a reverse primer and cloned into NdeI/BamHI restriction sites of pET-28a vector (Novagen). The N-terminal His-tagged FIH-1 fusion protein containing the full-length of FIH-1 (residues 1–349) was overexpressed from Escherichia coli BL21 (DE3) and purified by nickel affinity chromatography (Qiagen). After removal of the N-terminal tag by cleavage with thrombin, FIH-1 was further purified by anion exchange chromatography using Sepharose. Protein purity was confirmed by SDS-PAGE, and concentration was determined using ε280 = 1.69 (mg/ml)/cm. FIH-1 was crystallized at 25 °C by the
Structure of Human FIH-1

Crystallographic data

|                | Peak (A1)   | Edge (A2) | Remote (A3) |
|----------------|-------------|-----------|-------------|
| A. Data collection statistics |             |           |             |
| Wavelength (Å) | 0.9792      | 0.9794    | 0.9716      |
| Space group    | P4 2 2      |           |             |
| Cell dimension (Å) | 86.89 × 86.89 × 143.42 |         |             |
| Highest resolution (Å) | 2.8         | 2.8       | 2.8         |
| Unique reflections (total) | 13,768 (91,630) | 13,563 (98,205) | 13,783 (93,608) |
| Completeness (%)* | 97.0 (91.5) | 95.9 (89.7) | 97.3 (93.7) |
| Rmerge (%) | 8.9 (20.9) | 7.2 (19.5) | 7.7 (20.8) |
| I/σI | 18.3 (4.1) | 21.0 (4.7) | 18.5 (3.9) |
| B. Refinement statistics |             |           |             |
| Resolution range (Å) | 99.2–2.8 |           |             |
| No. reflections (F > 0) | 13,563 |           |             |
| Atoms (protein/non-protein) | 2,780/5 |           |             |
| Rmerge | 22.8 |           |             |
| Rsym | 27.5 |           |             |
| Root mean square deviations |             |           |             |
| Bond lengths (Å) | 0.008 |           |             |
| Bond angles (°) | 1.5 |           |             |
| Improper (°) | 0.89 |           |             |
| Dihedrals (°) | 24.1 |           |             |

* The values in parentheses (completeness and Rmerge) are for the highest resolution bin.

Hang-drop vapor diffusion method. Tetragonal crystals were obtained in drops containing 1.8 μl of protein solution (20 mg/ml) and 1.8 μl of reservoir solution (0.4 M lithium sulfate, 20% PEG 4000, 0.1 M Tris, pH 8.5). The crystals belonged to the P422 space group with unit cell dimensions of a = b = 86.89 Å and c = 143.42 Å.

Data Collection, Structure Determination, and Refinement—Crystals grown from selenomethionyl-derivatized protein was used for the MAD data collection at the Pohang Accelerator Laboratory beamline 6B. Data collected at three wavelengths (peak, edge, and remote) were processed and scaled with the program DENOZO and SCALEPACK (25). Seven selenium sites out of eight expected sites were located by the program SOLVE (26), and heavy atom parameters were refined by the program SHARP (27). The phases were subsequently improved by solvent-flattening using the program DM (28). The resulting experimental map was of high quality and allowed us to build the majority of the residues.

Overall Structure—The overall fold of FIH-1 is composed of a central β-barrel surrounded by eight α-helices with an approximate dimension of 60 × 45 × 35 Å (Fig. 1a). The structure of FIH-1 reveals two discrete domains. Domain I runs from N terminus to residue 300 and is mainly composed of the central FIH-1 reveals two discrete domains. Domain I runs from N terminus to residue 300 and is mainly composed of the central β-barrel (sheet 1, β2–β7, β9, β12, and β14; sheet 2, β1, β8, β10, β11, and β13) and six α-helices (α1–α6) surrounding it. Domain II (residues 301–349) comprising two consecutive α-helices (α7 and α8) stretches away from domain I. Domain II, which is completely missing in all known structures of the dioxygenase family, forms strong interactions with the same region of the 2-fold symmetry related molecule (Fig. 1b). It is further stabilized by interactions with helix α6. Dimeric association of FIH-1 revealed by the crystal structure was confirmed by gel filtration chromatography and dynamic light scattering experiments (data not shown). Dimeric interface, which is predominantly composed of hydrophobic interactions, buries 1,516 Å² that corresponds to 8.7% of the total surface of the FIH-1 monomer.
Fig. 2. **Structure-based sequence alignment of FIH-1 with CAS.** Sequences of FIH-1 and CAS were aligned by Cα-carbon superposition of the two structures. In the structural superposition, the initial transformation operator between the two structures was obtained from the result of the DALI search. The initial alignment was further refined with the program O by using the default constraints (less than 3.80 Å deviation for more than three consecutive Cα-carbon atoms). As the result, 103 residues were superposed with an overall root mean square deviation of 2.50 Å. The superposed residues are shaded yellow in the figure. The secondary structural elements of FIH-1 are shown in blue above the alignment and those of CAS are in red below the alignment. The facial triad residues (His-199, Asp-201, and His-279 of FIH-1) are indicated by inverted black triangles. A black circle below the alignment indicates the arginine residue (Arg-293 of CAS) involved in coordinating the 5-carboxylate of 2OG in CAS. A lysine residue (Lys-214 of FIH-1) implicated in the 2OG binding of FIH-1 (see text) is indicated as a black circle above the alignment.

Fig. 3. **The active site conformation.** a, the active site residues in FIH-1 are presented as superimposed with the corresponding residues of CAS in complex with 2OG and ferrous ion. The two structures were superposed as in Fig. 2. In the figure, facial triad residues (His-199, Asp-201, and His-279 of FIH-1; His-144, Glu-146, and His-279 of CAS) and the residue implicated in the 2OG binding (Lys-214 of FIH-1; Arg-293 of CAS) are presented. Side chains of FIH-1 and CAS are drawn in blue and yellow sticks, and labeled black and red, respectively. One of the facial triad residues (His-279) is common in both proteins (labeled magenta). Main chains near the presented residues are drawn as tubes of Cα-carbon trace (FIH-1, purple; CAS, gray). b, the 2Fo-Fc electron density map around the facial triad residues (His-199, Asp-201, and His-279) is presented in stereo. The map is contoured at 1.0 σ level.
Despite low sequence homology, the overall structure of FIH-1 is similar to those of other 2OG-dependent oxygenases as proposed based on the conservation of the signature motif residues (13, 14). In a search for homologous structures by using the Dali server, seven members of the 2OG-dependent oxygenases including clavaminic acid synthase (CAS), isopenicillin synthase, and anthocyanidin synthase were identified with similar z-values ranging from 9.0 to 7.0. Structural similarities are mainly found in the central $\beta$-barrel region. When we aligned the structure of FIH-1 with that of CAS, 103 C atoms were superimposed with a root mean square deviation of 2.50 Å (Fig. 2). Although there are good alignments in the region of the central $\beta$-barrel region, the arrangements of helices are quite different and cannot be aligned to each other well. Other differences in the structure of FIH-1 include the long insertions in N- and C-terminal regions (Fig. 2). Substantial differences are also found in several loops and helices in the middle of the protein.

Active Site Conformation—Previous investigations have demonstrated a strict requirement of FIH-1 for ferrous ion and 2OG (13, 14). At the center of the $\beta$-barrel structure of FIH-1, there is a big cavity that is lined with the residues implicated in coordinating a ferrous ion (His-199, Asp-201, and His-279), termed the 2-His-1-carboxylate facial triad (Fig. 3, a and b; Fig. 4a). In one side of the facial triad, there is an opening that is spacious enough to accommodate the cofactor, 2OG. Side chains of the facial triad residues are aligned as if they were coordinating a ferrous ion, despite that the FIH-1 structure is in apo state (Fig. 3a). Positions of the triad residues in FIH-1 match well with those of CAS (Fig. 3a). Consistent with the crystal structure, mutation of either His-199 or Asp-201 to Ala impaired the FIH-1 activity (14). The facial triad residues are
highly exposed to the solvent due to the wide opening of the pocket entrance (−15 Å wide, Fig 4a). In CAS the entrance of pocket is blocked by three extended loops (residues 111–117, 131–135, and 202–205 in CAS), allowing only small molecule substrates to enter the active site. The wide-opened pocket entrance of FIH-1 indicates that the substrate may have a helical conformation rather than an extended strand (see below).

Whereas conformations of the facial triad residues are highly conserved between FIH-1 and other 2OG-dependent dioxygenases, the putative binding site for 2OG in FIH-1 is substantively different from that in other 2OG-dependent dioxygenases. In CAS, 5-carboxylate of 2OG is bound to the charged side chain of Arg-293 that is highly conserved in 2OG-dependent dioxygenases (2, 37). Arg-281 and Arg-297 also are positioned near the 5-carboxylate, contributing to the binding of 2OG. However, in FIH-1, those arginines are replaced with hydrophobic or non-ionic residues such as Thr-290, Ile-281, and Asn-294 (Fig. 2). Instead, side chain of Lys-214 protruding from different secondary structural element (strand β10 of sheet 1) appears to be involved in the binding of 2OG. Although the Cα position of Lys-214 is different from that of Arg-293 of CAS, the charged moiety of Lys-214 is located at the similar position as that of CAS (Fig. 3a). Lys-214 is completely conserved in the subfamily members of FIH-1 from various species (14), supporting its possible role in the FIH-1 activity by coordinating 2OG.

The Putative Binding Sites for HIF-α CAD and VHL—The most prominent feature in the FIH-1 structure is a distinctive groove that extends from the active site toward the interconnecting loop between domains I and II (loop D1–D2) (Fig. 4, a and b). The groove, being −15 Å wide and −40 Å long, is lined mainly by residues from helix α7, loop D1–D2, and strands β7 and β14. In CAS, the region corresponding to the FIH-1 groove is occupied by several loop structures (residues 111–117, residues 148–153, residues 202–205, and residues 317–324 in CAS). The groove surface consists of many hydrophobic residues and is wide enough to accommodate a single α-helix (Fig. 4, a and b). Recently, NMR structures for the complex of the CH1 domain of P300 with the HIF-1α CA were reported (38, 39). In the complex structures, residues preceding the hydroxylation site, Asn-803 has an α-helical conformation. Thus, we propose that the long groove found in the FIH-1 structure serves as the binding site for HIF-1α CAD. Previous analyses (12) suggested that the HIF-binding site is located at the C-terminal region of FIH-1, consistent with the location of the putative helix-binding groove revealed from the structure. The VHL-binding site is located N-terminal (until residue 126) to the HIF-binding site (12). In the FIH-1 structure, the N-terminal 126 residues are divided into two structural segments that overlap the core of the protein from opposite sides (Fig. 4b). The first segment comprising the N-terminal 89 residues lies at the opposite side of the active site, whereas the second segment (residues 90–126) is positioned at the entrance of active site. The second segment is close to the putative binding site for HIF-1α CAD (Fig. 4b), indicating that there may be a steric hindrance if VHL were to bind to the second segment. Thus, VHL is likely to bind FIH-1 by utilizing the first segment, and FIH-1 may act as a bridge in the interaction between VHL and HIF1α CAD (Fig. 5). Consistent with this structural implication, VHL interacts with HIF-1α CAD in the presence but not in the absence of FIH-1 (12).

Implications for the Regulation of HIF-1 Activity—The FIH-1 structure suggests a mechanism for the physiological regulation of the HIF-1 activity by revealing three-dimensional locations of regions in FIH-1 that are implicated in the interactions with HIF-1α CAD and VHL. The distinctive binding sites for HIF-1α CAD and VHL on the surface of FIH-1 support the possibility of the ternary complex and indicate that the cooperative binding of the three proteins may be required in the hydroxylation of HIF-1α CAD by FIH-1 (Fig. 5). Previous analyses reported that FIH-1 was active under hypoxic conditions despite its expected role as an oxygen sensor, whereas the activity of HIF-1-PH was suppressed in hypoxia (2, 12, 14). This implies that the FIH-1 active site itself may not act as a direct oxygen sensor. Instead, the oxygen dependence of the hydroxylation by FIH-1 may be due to its substrate recognition mechanism that requires a complex formation with VHL and HIF-1α. In normoxia, VHL binds to the HIF-1α ODD domain through the hydroxypoline and then recruits FIH-1 around HIF-1α CAD for the hydroxylation of Asn-803, whereas in hypoxia, FIH-1 cannot bind the substrate due to the absence of the HIF-1α-bound VHL. The dimerization of FIH-1 may lead to the formation of a large complex that includes two molecules of HIF-1α and VHL as well as various histone deacetylases (Fig. 5). The multimerization-dependent regulation of FIH-1 activity may have an advantage for the HIF-1α regulation by providing sequential checks for the cellular oxygen level by using different mechanisms.

Appropriate hypoxia responses are important in overcoming oxygen and nutrient deficiency during hypoxic states in ischemic diseases such as stroke and cardiovascular diseases. The crystal structure of FIH-1 reveals critical information about the active site of the enzyme such as the wide-opened entrance for the catalytic site and a distinctive 2OG-binding site. These characteristic features would be an aid in designing specific drugs that can inhibit the enzyme activity and promote the hypoxia-inducible responses in ischemic diseases. The prominent groove near the active site, which is likely the HIF-1α CAD-binding site, may also be exploited for the design of inhibitors.

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