The activity of the transcription factor hypoxia-inducible factor (HIF) is regulated by oxygen-dependent hydroxylation. Under normoxic conditions, hydroxylation of proline residues triggers destruction of its o-subunit while hydroxylation of Asn803 in the C-terminal transactivation domain of HIF-1α (CAD) prevents its interaction with p300. Here we report crystal structures of the asparagine hydroxylase (factor-inhibiting HIF, FIH) complexed with Fe(II), 2-oxoglutarate co-substrate, and CAD fragments, which reveal the structural basis of HIF modification. CAD binding to FIH occurs via an induced fit process at two distinct interaction sites. At the hydroxylation site CAD adopts a loop conformation, contrasting with a helical conformation for the same residues when bound to p300. Asn803 of CAD is buried and precisely orientated in the active site such that hydroxylation occurs at its β-carbon. Together with structures with the inhibitors Zn(II) and N-oxaloylglycine, analysis of the FIH-CAD complexes will assist design of hydroxylase inhibitors with proangiogenic properties. Conserved structural motifs within FIH imply it is one of an extended family of Fe(II) oxygenases involved in gene regulation.

In hypoxic cells, activation of the HIF transcriptional cascade directs a series of adaptive responses that enhance oxygen delivery or limit oxygen demand (1). Activation of HIF in cancer and ischemic/hypoxic vascular diseases has indicated a central role in hypoxia response (1). The transcriptional complex is composed of an αβ heterodimer, HIF-β being a constitutive nuclear protein that dimerizes with oxygen regulated HIF-α subunits (2). The activity of the HIF system is regulated by a series of Fe(II) and 2OG-dependent dioxygenases that catalyze hydroxylation of specific HIF-α residues. In normoxia, 4-hydroxylation of human HIF-1α at Pro402 or Pro564 by a set of HIF prolyl hydroxylase isoforms (PHD1–3) (3, 4) mediates HIF-1α recognition by the von Hippel-Lindau ubiquitin ligase complex leading to its proteasomal destruction (5–8). In a complementary mechanism FIH (9) catalyzes hydroxylation of HIF-1α Asn803 (10, 11), which blocks interaction with the transcriptional coactivator p300 (12, 13). In hypoxia, lack of hydroxylase activity enables HIF-α to escape destruction and become transcriptionally active. Inhibition of HIF hydroxylases by Fe(II) chelators and 2OG analogues activates the HIF transcriptional cascade even in normoxia (3, 5, 14). The HIF hydroxylases therefore provide a focus for understanding cellular responses to hypoxia and a target for therapeutic manipulation. Here we report crystal structures for the HIF asparagine hydroxylase (FIH) alone and complexed with CAD polypeptides, cosubstrates, and inhibitors.

EXPERIMENTAL PROCEDURES

Protein Expression, Purification, and Crystallization—FIH, CAD775–826, and CAD786–826 were prepared as described (10). Selenomethionine (SeMet) substituted FIH was produced using a metabolic inhibition protocol and LeMaster media supplemented with 50 μg/liter t-selenomethionine. SeMet incorporation was >95% by electrospray ionization-mass spectrometry. Aerobic crystallization of SeMet FIH (at 11 mg/ml) was accomplished by hanging-drop vapor diffusion at 17 °C. The mother liquor consisted of 1.2 M ammonium sulfate, 4% PEG 400 and 0.1 M Hepes pH 7.5. Crystallization of FIH-Fe-CAD fragment complexes was accomplished under an anaerobic atmosphere of argon in a Bello Technology glove box (0.3–0.4 ppm O2) using the same mother liquor and a solution containing FIH (at 11 mg/ml), Fe2+ (1 mM), 2OG/NOG (2 mM), and CAD fragment (1 mM). Crystallization of FIH-Zn-CAD fragment was accomplished aerobically under similar conditions. Peptides were either synthesized by solid phase peptide synthesis or purchased from Biopeptide Co. (San Diego, CA).

Crystallographic Data Collection and Structure Solution—Crystals were cryocooled by plunging into liquid nitrogen and x-ray data were collected at 100 K using a nitrogen stream. Cryoprotection was accomplished by sequential transfer into a solution containing 1.2 M ammonium sulfate, 3% PEG 400, 0.1 M Hepes, pH 7.5, and 10% followed by 24% glycerol. A three-wavelength multiple anomalous dispersion data set was collected to 2.9 Å resolution on beamline 14.2 of the Synchrotron Radiation Source, Daresbury, UK. Data from crystals of FIH-CAD complexes were collected on beamlines 14.2, 9.6, or 9.5 using ADSC Quantum 4 (14.2 and 9.6) or MarCCD detectors (9.5). All data were processed with MOSFLM and the CCP4 suite (15). The crystals belonged to space group P412121. The crystallographic asymmetric unit contains one FIH molecule. Six selenium positions were located and increased the figure of merit from 0.56 to 0.66, was performed using RESOLVE (17).

Refinement—An initial model was built using O (18) and refined against the SeMet data (remote wavelength) using CNS (19). One cycle

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The atomic coordinates and structure factors (code 1H2K, 1H2L, 1H2M, and 1H2N) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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9 The abbreviations used are: HIF, hypoxia-inducible factor; CAD, C-terminal transactivation domain; FIH, factor inhibiting HIF; 2OG, 2-oxoglutarate; NOG, N-oxaloylglycine; SeMet, selenomethionine; PEG, polyethylene glycol; DSBH, double-stranded β-helix; CBP, CREB-binding protein (where CREB is cAMP-response element-binding protein).
of simulated annealing followed by grouped B-factor refinement brought the $R_{BBO}$ to 36.2%. Following further rebuilding and refinement, which brought the $R_{BBO}$ to 32.3%, the model was transferred to the 2.15-Å data set. Rebuilding and refinement using REFMAC5 (20), including addition of iron, substrate and solvent molecules, and refinement of TLS parameters brought the conventional R-factor to 17.8% and the $R_{BBO}$ to 21.3%. The following residues are missing in the current refinement. Significant positive difference electron density was visible throughout refinement. Significant positive difference electron density was observed between the iron and the CAD Asn$^{803}$ –carbon. Since 20G/NOG was visible throughout refinement, this may represent an alternative binding mode for the 20G 1-carboxylate in the absence of substrate, although it could also be due to a ligating water molecule, again in the absence of substrate.

Other structures were solved by molecular replacement using the coordinates from the 2.15-Å data and refinement using REFMAC5. In all structures electron density for the iron and 20G/NOG was visible throughout refinement. Significant positive difference electron density was observed between the iron and the CAD Asn$^{803}$ –carbon. Since B-factor differences between FIH and CAD imply that the CAD is not at 100% occupancy, this may represent an alternative binding mode for the 20G 1-carboxylate in the absence of substrate, although it could also be due to a ligating water molecule, again in the absence of substrate.

Figures were prepared with Molscript (22), Bobscript (23), Raster3D (24), and Pymol (www.pymol.org).

**RESULTS AND DISCUSSION**

To obtain an FIH-CAD complex without oxidation of the CAD or the Fe$^{II}$, anaerobic conditions were employed to crystallize FIH in the presence of Fe$^{II}$, 20G, and various CAD polypeptides from 7–52 residues. Crystals were also obtained anaerobically for FIH complexed with Fe$^{II}$ and the FIH inhibitor NOG and aerobically for FIH complexed with Zn$^{II}$ and NOG. The structures were solved by molecular replacement using a model obtained by multiple anomalous dispersion on selenomethionine-substituted apo-FIH.

Crystalline FIH-CAD complexes were also obtained with Fe$^{II}$, HIF-1α 775–786, and 20G or NOG (data not shown, since no additional CAD residues were resolved over the structures with HIF-1α 786–826). r.m.s.d., root mean square deviation.

**Table I**

Summary of FIH-CAD-fragment complex structures

| Structure No. | Resolution | Metal | Cosubstrate/ inhibitor | Corcrystallization CAD peptide | Site 1 CAD residues resolved | Site 2 CAD residues resolved | $R_{BBO}$ | r.m.s.d. from structure | Protein Data Bank ID |
|---------------|------------|-------|-------------------------|--------------------------------|----------------------------|----------------------------|----------|-----------------------|----------------------|
| 1             | 2.15       | Fe$^{II}$ | NOG                     | HIF-1α 786–826                  | 795–806                    | 812–823                     | 17.8/21.3 | 0.149                 | 1H2K                 |
| 2             | 2.25       | Fe$^{II}$ | 20G                     | HIF-1α 786–826                  | 795–806                    | 813–822                     | 18.3/21.3 | 0.136                 | 1H2L                 |
| 3             | 2.50       | Zn$^{II}$ | NOG                     | HIF-1α 775–826                  | 795–806                    | 813–822                     | 19.2/22.5 | 0.136                 | 1H2M                 |
| 4             | 2.84       | Fe$^{II}$ | 20G                     | HIF-2α 850–862                  | None                       | None                       | 23.1/25.7 | 0.226                 | 1H2N                 |

**Fig. 1.** The FIH-CAD complex (a–c, structure 1; d, structure 2). a, FIH monomer. The CAD peptide is shown as a ball-and-stick representation in red and the DSBH motif in green. b, FIH dimer. The two molecules of FIH are in dark and light blue, the DSBH motif is in green, and the CAD peptide is in red. c, the 2OG binding site with bound NOG is shown in yellow. The Fe$^{II}$ is colored pink, and the 2mF$_{o}$ – DF$_{c}$ electron density map is contoured at 1.5 σ. d, orientation of CAD Asn$^{803}$ at the FIH active site. The 20G and CAD peptide are shown in yellow.
The crystal structure of FIH was determined at 2.4 Å resolution. The structure comprises two closely juxtaposed molecules, designated FIH1 and FIH2, connected by disulfide bonds at Cys58 and Cys112. The structures unexpectedly reveal the existence of two distinct HIF-CAD interaction sites, one involving the hydroxylation site itself (CAD795-806, site 1) and a second lying to the C-terminal side of this site (CAD813-823, site 2). The binding sites involve contact surface areas of 1640 Å^2 and 1080 Å^2, respectively, and CAD residues in these regions are highly conserved in all known HIF-1α and HIF-2α sequences. Kinetic analyses were employed to investigate the relative importance of sites 1 and 2. CAD fragments shorter than 20 residues are not efficient in vitro substrates (Table II). Those containing site 1 only are hydroxylated by FIH but less efficiently than those containing both sites, consistent with the crystallographic data. Electron density for site 1 is of good quality, with only the side chain of Tyr798 poorly defined, while that for site 2 is at a lower level and quality, probably reflecting weaker binding at this site (Fig. 2c). CAD804–806, and presumably also CAD807–811 for which density was not observed, do not form direct interactions with FIH.

At site 1, HIF-CAD795–803 residues are bound in a groove (Fig. 2b) and adopt a largely extended conformation linked to FIH by ten hydrogen bonds. Asn803 of CAD is completely buried at the active site and lies directly adjacent to the Fe(II). CAD Asn803 and Ala804 form a tight turn, stabilized by a hydrogen bond between the backbone carbonyl of Val802 and NH of Ala804, which projects the side chain of Asn803 toward the Fe(II). This side chain is precisely orientated by three hydrogen bonds to enable hydroxylation at the pro-S position of the β-carbon (Fig. 1d), consistent with NMR assignment of hydroxylation at this site. The primary amide of CAD Asn803 is sandwiched between FIH residue Tyr102 and the Fe(II). It forms hydrogen bonds with the side chains of FIH residues Gln239 and Arg263 (Fig. 1d), residues located on the insert to the DSBH motif, rationalizing the unusual selectivity of FIH for asparagine over aspartate (10). Interestingly, the substrate and Fe(II) binding sites are directly linked, since the backbone nitrogen of CAD Asn803 also forms a hydrogen bond (~3 Å) with the carboxylate oxygen of Asp203 that is not complexed to the iron. Six additional hydrogen bonds stabilize the binding of FIH to

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L. A. McNeill, unpublished results.
In contrast with site 1, site 2 is bound on the FIH surface and involves only two hydrogen bonds (Fig. 2a). CAD816 of site 2 form an α-helix, in exact agreement with the structure of this region in complex with CBP/p300 (12, 13). As in that complex, the highly conserved Leu418, Leu419 and Leu422 sit in a hydrophobic pocket on the surface of FIH (Fig. 2a); it is not possible for CAD to bind simultaneously to CBP/p300 and FIH.

The extended loop conformation adopted by the CAD residues at site 1 contrasts with the α-helical conformation adopted by the same residues when complexed with the 1st transcrip
tional adaptor zinc-binding domain (TAZ1) of CBP/p300 (12, 13). The disordered structure observed for the CAD, and other HIF-α residues (7), when free in solution may thus reflect a requirement to adopt more than one conformation for complex formation with different proteins. The changes in the conformation of CAD on binding are complemented by changes in FIH revealing an induced fit binding process. Most strikingly, the unusual hydrogen bond between Asp201 and CAD Asn803 (the Fe(II) and Asn803 Fe(II) from the active site of HIF hydroxylases. FIH-CAD structure is indicated above the alignment. Selected 2OG binding residues found in FIH are indicated by red triangles –806 Å apart) that is effects accommodation of a dioxygen ligand (or an alternate 2OG conformation) opposite His279 may require disruption of the unusual hydrogen bond between Asp201 and CAD Asn803 (the Fe(II) and Asn803 Fe(II) –4.9 Å apart) that is observed in the anaerobic enzyme substrate complex (Fig. 1d). This hydrogen bond may have energetic consequences for the binding of dioxygen that are relevant to the oxygen sensing function of the enzyme.

To understand the mechanisms of action of hydroxylase inhibitors, structures were also obtained for FIH complexed with NOG and with Zn(II). FIH was demonstrated to bind Zn(II) in an identical manner to Fe(III) (structure 3), consistent with the metal-mediated mimic of hypoxia being due to displacement of Fe(II) from the active site of HIF hydroxylases. FIH-CAD structures with NOG reveal that like 2OG it is ligated to Fe(II) in a bidentate manner (Fig. 1c) and imply that it is an inhibitor due to decreased susceptibility to attack by an iron bound (su)peroxide. Kinetic analyses of a series of inhibitors based upon N-oxaloyl amino acids demonstrated that the R-enantiomer (IC50 <0.4 mM) of N-oxalylalanine was significantly more.

### Crystal Structure of FIH

**Table II**

| Peptide sequence | Length (residues) | Equivalent residues | Activity relative to HIF-1a 775–826 % |
|------------------|-------------------|---------------------|--------------------------------------|
| HIF-1a 52-mer    | 52                | HIF-1a 775–826      | 100                                  |
| HIF-1a 41-mer    | 41                | HIF-1a 786–826      | 100                                  |
| MDESLPQLTSYDCEVNAPI | 20              | HIF-1a 787–806      | ~30                                  |
| VNVPLGSTMILQ     | 13                | HIF-2a 850–862      | 0.10                                 |
| CEVNAPI          | 7                 | HIF-1a 800–806      | 0.05                                 |
| DCEVNVPLG        | 10                | HIF-2a 847–856      | <0.05                                |
| TSYDCEVNAPIQSRNL | 13                | HIF-1a 796–811      | 40                                   |
| DESGLPQKTSYDCEVNAPI | 19              | HIF-1a 788–806      |                                      |

**Fig. 3. Mechanism for FIH and sequence alignment of FIH with JmjC domain proteins.** a, part of the FIH catalytic cycle, showing the role of 2OG and the mechanism of inhibition by NOG. b, partial sequence alignment of FIH with a selection of JmjC domain containing proteins. FIH secondary structure is indicated above the alignment. Selected 2OG binding residues found in FIH are indicated by red triangles under the alignment and the two iron binding residues by green triangles. SWALL accession numbers are indicated on the lower left of the alignment.
potent than the S-enantiomer (IC_{50} 2.5 mM). Analysis of the 2OG binding pocket in FIH suggests that the binding of the S-enantiomer is disfavored by interactions with Thr^{196} and Ile^{281} in the 2OG binding pocket. Since a reversed selectivity (i.e., the S-enantiomer was more observed) is observed both for procollagen prolyl hydroxylase and the PHD isozymes (5), it should be possible to develop selective inhibitors for individual types of PHD hydroxylase based on such structural constraints. The unusual and precise structural determinants of both CAD and 2OG binding to FIH may aid inhibitor design via linkage of the 2OG and CAD binding sites and development of heterocyclic compounds that mimic the tight turn adopted by the CAD<sub>802–804</sub> when complexed to FIH.

Recognition of post-translational hydroxylation as a major mode of regulation of the HIF pathway raises an important question as to its general role in biological signaling. Sequence analyses based on the FIH structure indicate that the 2OG oxygenase superfamily extends further than has previously been foreseen (Fig. 3b). Of particular interest are similarities with the JmjC homology region of the junonji transcription factors (10, 29). These proteins are predicted to have a DSBH core and have been implicated in diverse biological processes such as cell growth and heart development. Conserved Hx(D/E) residues had been identified in some JmjC domains but not assigned as an iron binding motif (29). In the light of the FIH structure it is clear that many JmjC proteins have conserved residues including both this motif and the newly defined 2OG 5-carboxylate binding site involving FIH residues Lys<sup>214</sup> and Thr<sup>196</sup> on the fourth strand of the DSBH (Fig. 3b).

The structure therefore implies that FIH is a one of a large family of iron- and 2OG-dependent oxygenases that are involved in the regulation of gene expression.

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