RESEARCH COMMUNICATION

Hypoxia-inducible factor asparaginyl hydroxylase (FIH-1) catalyses hydroxylation at the *β*-carbon of asparagine-803

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Asparagine-803 in the C-terminal transactivation domain of human hypoxia-inducible factor (HIF)-1 α-subunit is hydroxylated by factor inhibiting HIF-1 (FIH-1) under normoxic conditions causing abrogation of the HIF-1α/p300 interaction. NMR and other analyses of a hydroxylated HIF fragment produced *in vitro* demonstrate that hydroxylation occurs at the β-carbon of Asn-803 and imply production of the *threo*-isomer, in contrast with other known aspartic acid/asparagine hydroxylases that produce the *erythro*-isomer.

Key words: asparagine hydroxylation, factor inhibiting hypoxia-inducible factor-1 (FIH-1), hypoxia, oxygenase, oxygen sensing.

INTRODUCTION

Hypoxia-inducible factor (HIF) is a heterodimeric $\alpha\beta$-transcription factor present in multicellular organisms. In mammalian cells at least two isoforms of the α-subunit (HIF-1α and HIF-2α) exist, each of which is regulated by cellular levels of dioxygen, and contains a basic helix-loop-helix domain [1,2]. The transcriptional activity of HIF-α is modulated by hydroxylation at at least three defined sites: Pro-402, Pro-564 and Asn-803 in human HIF-1α [3,4]. 4-Hydroxylation at the two prolyl residues by the recently characterized iron(II)- and 2-oxoglutarate-dependent dioxygenases [5–9] causes binding of the von Hippel–Lindau tumour suppressor protein (pVHL), which triggers ubiquitylation by the E3 ligase complex and consequent destruction of HIF-α in the proteasome. Crystal structures of the hydroxylated peptide, containing Pro-564 complexed with the pVHL–Elongin B–Elongin C complex, have revealed that two hydrogen bonds to the introduced oxygen atom mediate binding to pVHL [10,11].

In a complementary regulatory mechanism, hydroxylation of the Asn-803 of HIF-1α abrogates the interaction between HIF-1α and p300 [3], a nuclear co-activator protein that forms part of the transcriptional response. Under hypoxic conditions HIF-α is not degraded, and is available to dimerize with constitutively present HIF-β and interact with p300 in the nucleus, leading to the transcriptional activation of genes such as those for encoding for erythropoietin and vascular endothelial growth factor [1].

Like prolyl hydroxylation, the post-translational hydroxylation of Asn-803 in human HIF-1α is catalysed by an iron(II)- and a 2-oxoglutarate-dependent dioxygenase [3]. Recently, this has been shown to be identical to factor inhibiting HIF-1 (FIH-1) [12,13], a protein first identified through association with HIF-1α in yeast two-hybrid assays [14]. Although the position of the hydroxylated residue in the HIF-1α sequence has been established, the site of hydroxylation on Asn-803 has not been defined. NMR studies on the interaction between fragments of HIF-1α and p300 [15,16], indicate that the C-terminal activation domain (CAD) of HIF-1α is unstructured when free in solution.

However, on binding to a transcriptional adapter zinc-binding motif from the CH1 (cysteine/histidine-rich) domain of p300, Asn-803 becomes buried in a hydrophobic pocket, and the side-chain amide participates in the maintenance of a short α-helical region adopted by HIF-1α. The NMR analyses indicate that the conformation of the Asn-803 side chain in the pocket is such that hydroxylation either at the side-chain amide or at the β-carbon would destabilize the HIF-1α/p300 complex.

Mammalian hydroxylases that catalyse the post-translational modification of aspartic acid/asparagine residues have been described previously [17,18]. These enzymes act on aspartic acid/asparagine residues in the conserved sequence CX(D/N)XXXX(F/Y)XCXC in epidermal growth factor (EGF)-like domains in several proteins, including Protein S, Protein K, Factor IX and Factor X [19], and hydroxylate the β-carbon to give the *erythro* (2*S*,3*R*) product isomer [20]. Secondary structure predictions suggest that an α-helical membrane-spanning region locates these 'EGF-hydroxylases' in the lumen of the endoplasmic reticulum [21]. In the present study, we report analyses that define the position of CAD hydroxylation by FIH-1.

EXPERIMENTAL

L-Aspartic acid β-hydroxamate (36 % overall yield in two steps from L-aspartic acid) was synthesized by following the procedure of Charvillon and Amouroux [22], as reported for the preparation of D-aspartic acid β-hydroxamate. Thus, selective methyl esterification of the side chain carboxylic acid function [$SOCl_2$ (1 mol), methanol in excess at 0 °C for 15 min then 20 °C for 90 min], followed by evaporation gave crude monoester that was used directly in the next step. A solution of hydroxylamine hydrochloride (1 mol) in the presence of triethylamine (1 mol) in methanol was added to a solution of the monoester in methanol and the mixture stirred for 12 h. Recrystallization from methanol gave a purified product (> 95 % by ^{1}H-NMR analysis).

Racemic *threo*-β-hydroxy-asparagine (44 % overall yield) was synthesized in in two steps from racemic *threo*-β-hydroxyaspartic

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acid by selective methyl esterification of the side chain carboxylic acid function [$SOCl_2$ (1 mol), excess methanol at 0 °C for 15 min then 20 °C for 90 min] as described above [22]. The monoester was then converted to the desired racemic *threo*-β-hydroxyasparagine via treatment with 25 % aqueous ammonia following the procedure of Sendai et al. [23], as reported for the *erythro* isomer of *threo*-β-hydroxy-D-asparagine. Evaporation *in vacuo* followed by washing of the resultant solid with water and recrystallization from aqueous ethanol gave a purified product (> 95 % by ^{1}H-NMR analysis).

A 19-residue peptide, DESGLPQLTSYDCEVNAPI (Biopeptide Co., San Diego, CA, U.S.A.), corresponding to residues 788–806 of HIF-1α was prepared for NMR analysis in neat [2]H$_2$O containing 1,4-dioxan as the internal chemical shift reference ([1]H 3.750, [13]C 67.80 p.p.m.). All experiments were performed with sample temperatures of 298 K on a Bruker Avance DRX500 equipped with an inverse-broadband pulsed-field gradient probe. Proton spectra were acquired with solvent presaturation. Proton correlations were identified in two-dimensional (2D) COSY and 2D TOCSY experiments (with a mixing time of 80 ms). [13]C chemical shifts were measured in 2D ^{1}H-^{13}C HSQC (heteronuclear single quantum correlation) spectra recorded with pulsed field gradients and processed with linear prediction in F$_1$. ^{13}C decoupling within the HSQC was via the globally optimized alternating-phase rectangular pulses ('GARP') sequence.

cDNA sequences encoding FIH-1 were cloned into the pET28a (+) vector (Novagen, Madison, WI, U.S.A.), as described previously [12], to yield FIH-1 protein with an N-terminal His-tag to facilitate purification. Purification of crude material by nickel affinity chromatography, followed by thrombin cleavage of the His-tag and size exclusion chromatography (Superdex S75), yielded > 95 % pure protein by SDS/PAGE analysis. MS confirmed the identity of the isolated species.

The 19-residue peptide comprising amino acids 788–806 of human HIF-1α was modified by aerobic incubation with FIH-1 in the presence of ascorbate, dithiothreitol, catalase, 2-oxoglutarate, and iron(II) for 30 min at 37 °C. The reaction was quenched by cooling to 4 °C and addition of an equal volume of methanol. Precipitate was removed by centrifugation and the supernatant purified by HPLC using a Jupiter C4 column (15 cm × 4.6 mm). Peptide was eluted using a gradient of acetonitrile in 0.1 % trifluoroacetic acid, freeze-dried from the HPLC solvent for amino acid and MS analyses. The sample was freeze-dried a second time from [2]H$_2$O in preparation for NMR analysis.

[15]N-labelled N-terminal oxygen dependent degradation domain (NODD; HIF-1α 344–503) and C-terminal oxygen dependent degradation domain (CODD; HIF-1α 530–698) fragments of HIF-1α were produced as His-tagged proteins using the pET28a(+) vector from Novagen [5] and fermentation in M9 minimal media including ^{15}N-labelled ammonium chloride as the nitrogen source. Isolation by nickel affinity chromatography was as described previously [5]. Samples of protein at 1 mM in phosphate buffer were diluted with 5–10 % [2]H$_2$O and ^{15}N HSQC (^{1}H-^{15}N HSQC) NMR experiments were performed on a spectrometer with a ^{1}H operating frequency of 500 MHz.

RESULTS

### MS and N-terminal sequence analysis of a hydroxylated CAD fragment

Catalytic FIH-1 mediated hydroxylation of a synthetic 19-residue peptide corresponding to residues 788–806 of HIF-1α was confirmed by MS analysis of HPLC purified material: native peptide 19mer $[M+2H]^{2+} = 1026.67$ Da, modified peptide 19mer

$[M+2H]^{2+} = 1034.61$ Da; a mass difference of +8 Da in the doubly charged ions corresponded with addition of oxygen (+16 Da) to the peptide. N-terminal Edman degradation of the product peptide gave the following sequence: DESGLPQLTSYDCEVxA, where x was not asparagine. The peak from this (16th) cycle of Edman degradation ran to a similar position as the β-hydroxyasparagine standard. Acid hydrolysis of the modified peptide followed by amino acid analysis showed the presence of β-hydroxyaspartic acid only. Hydrolysis of the primary amide of β-hydroxyasparagine was anticipated, as a similar observation was made during previous work on uromodulin and the plasma complement proteins C1r and C1s [20].

It has been shown previously that incubation of peptides containing the sequence NHyn-Ala, where NHyn is asparagine hydroxylated at the side-chain amide nitrogen, with molar concentrations of hydroxylamine at high temperatures and pH 9.5, results in the cleavage of that peptide via (it is thought) an imide intermediate [24]. The 19mer synthetic peptide DESG-LPQLTSYDCEVNAPI, modified by FIH-1 as above, was incubated at 60 °C for 5 h in the presence of 1 M hydroxylamine at pH 9.5, and the mixture re-analysed by MS. Only the full-length peptide was observed, suggesting that no cleavage had occurred, consistent with lack of modification of the side-chain amide nitrogen.

NMR analysis of a hydroxylated CAD fragment

Both ^{1}H and ^{13}C chemical shift changes between the 19mer peptide substrate and the HPLC purified incubation product were assessed by 2D ^{1}H-^{13}C HSQC experiments. In the substrate, a grouping of four β-CH$_2$ resonances were assigned as belonging to Asp-1, Tyr-11, Asp-12 and Asn-16 according to their ^{1}H and ^{13}C shifts (Figure 1) [25]. In the product, it was clear from both the 2D HSQC and the one-dimensional proton spectra that only three of these four resonances were present. Comparison of the two spectra indicated that the signal assigned to the Asn-16 β-carbon (at δH 2.813 and 2.695 p.p.m. and δC 37.40 p.p.m. in the substrate) had disappeared, consistent with hydroxylation of the asparagine residue at its β-carbon. The resonances due to the two aspartic acid residues had shifted slightly, presumably due to changes in the protonation state, and were then at a similar ^{1}H chemical shift as the β-protons of the asparagine in the substrate. A difference in the oxidation state of the cysteine between the two samples was unlikely given the near identical chemical shifts for the cysteinyl β-carbon and hydrogens. The change from a double doublet to a single doublet for the β-hydrogen of the hydroxylated residue also ruled out any possibility that the observed alterations in the NMR spectrum were due to aggregation. Two new resonances appeared in the product spectrum at δH 4.913 p.p.m. and δC 56.26 p.p.m. and at δH 4.654 p.p.m. and δC 72.22 p.p.m. (Figure 2). These resonances correlated with one another in the 2D COSY spectrum and shared a [1]H–[1]H coupling constant of 2.4 Hz and were therefore assigned as the CH$^{\alpha}$–CH$^{\beta}$ of the hydroxylated asparagine. The appearance of these resonances also coincided with the disappearance of the δH 4.706 p.p.m. and δC 51.43 p.p.m. resonances observed in the substrate spectra, which was assigned, therefore, as the CH$^{\alpha}$ of the parent asparagine prior to modification (Table 1). Comparison of the CH$^{\alpha}$–CH$^{\beta}$ coupling constant of 2.4 Hz, observed for the hydroxylated Asn-803, with literature values implies that the *threo* isomer is produced. Thus, for example, for β-hydroxyaspartic acid, *threo*, 2.1 Hz (present study) and *erythro*, 3.0 Hz [26]; for β-hydroxyaspartic acid β-hydroxamate, *threo*, 2.7 Hz, *erythro*, 5.0 Hz [22]; for β-hydroxyasparagine, *threo*, 2.5 Hz (present
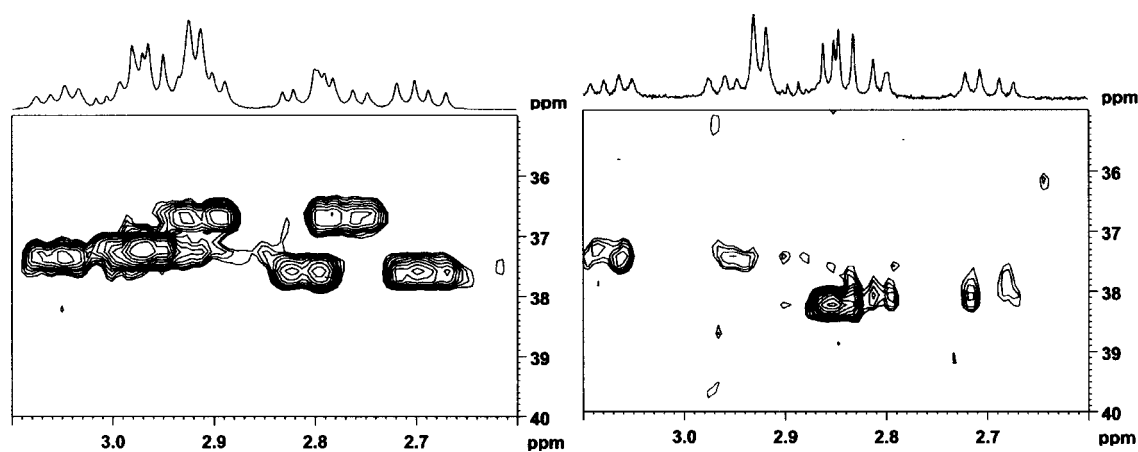
Figure 1   Amino acid β-carbon region of the 2D ^{1}H-^{13}C HSQC spectrum for the synthetic peptide DESGLPQLTSYDCEVNAPI (left-hand panel) and for the same peptide after modification with FIH-1 followed by HPLC isolation (right-hand panel)

The signals associated with the β-carbon of Asn-16 (Table 1) disappear from this region of the spectrum on hydroxylation.
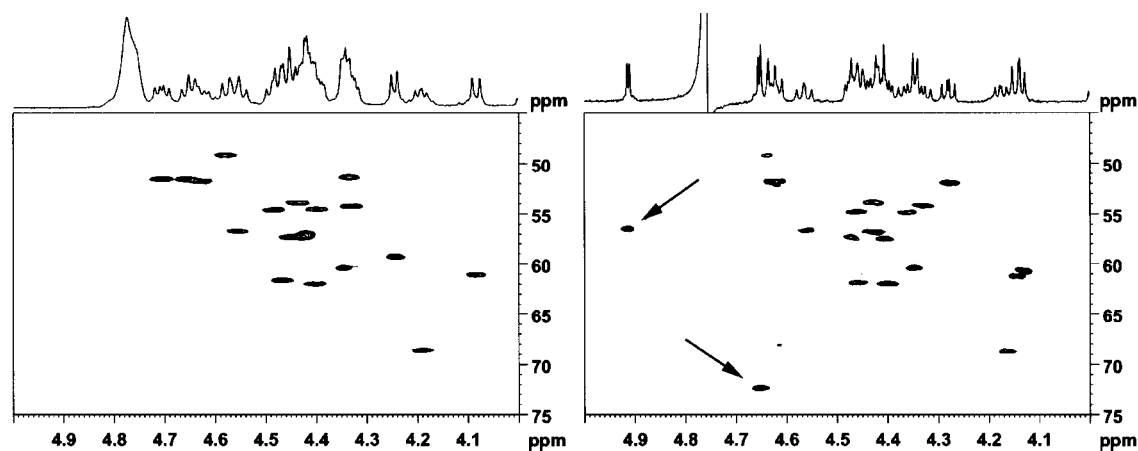
Figure 2   Amino acid α-proton region of the 2D ^{1}H-^{13}C HSQC spectrum for the synthetic peptide DESGLPQLTSYDCEVNAPI (left-hand panel) and for the same peptide after modification with FIH-1 followed by HPLC isolation (right-hand panel)

The new positions of two resonances, assigned as CH$^\alpha$ and CH$^\beta$ of β-hydroxy-Asn-16 (equivalent to Asn-803 of HIF-1α), are indicated by arrows.

study) and *erythro*, 3.0 Hz [23]; for protected β-hydroxyaspartic acid, *threo*, 2.0 Hz, *erythro*, 4.0 Hz [27]; and for protected β-hydroxyasparagine, *threo* 1.8–2.2 Hz [28].

DISCUSSION

The HSQC experiments give direct evidence for hydroxylation occurring at the β-carbon of the target asparagine, with the hydroxylated β-carbon appearing significantly deshielded (at 72.22 p.p.m.) and the adjacent α-carbon deshielded to a lesser extent (at 56.26 p.p.m.) relative to the parent asparagine. Changes of these magnitudes in the ^{13}C chemical shifts are inconsistent with hydroxylation of the side-chain nitrogen, but consistent with hydroxylation at the β-carbon. Furthermore, the ^{13}C spectrum of free D,L-*threo*-β-hydroxyasparagine (present study), has resonances at 58.63 p.p.m. and 73.85 p.p.m., corresponding to α- and β-carbons. The product assignment is also consistent with ^{1}H-NMR chemical shifts of the α- and β-hydrogens in the β-hydroxyaspartyl residues in EGF-like domains, which are

Table 1   Chemical shift values for the α- and β-position of the asparagine and hydroxyasparagine residues in 19-residue (corresponding to residues 788–806 of HIF-1α) substrate and product peptides

		Resonance chemical shifts (p.p.m.)	
		Substrate	Product
β-position	^{1}H	2.813, 2.695	4.703
	^{13}C	37.40	72.22
α-position	^{1}H	4.706	4.913
	^{13}C	51.43	56.26

4.48 p.p.m. and 4.36 p.p.m. respectively (with respect to water at 4.75 p.p.m.) when calcium is absent [29]. The analysis of the coupling constant reported in the present study suggests that the *threo*-isomer is the one formed on hydroxylation of Asn-803 by FIH-1.
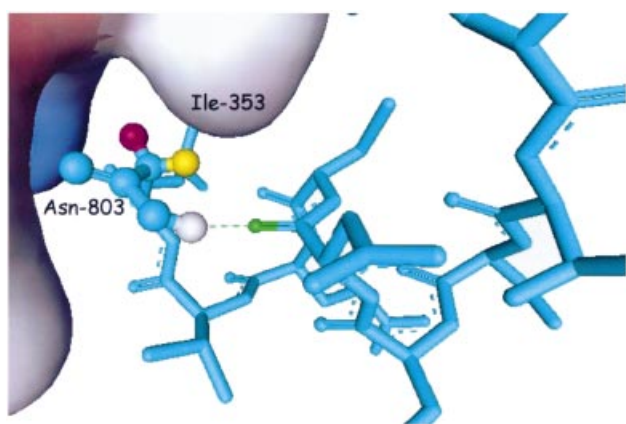
Figure 3   Interface between HIF-1α and p300 (PDB 1L86, model number 2 of 4)

HIF-1α is shown in cyan, and p300 is represented by the surface, where grey represents hydrophobic, blue represents basic, and red represents acidic areas. The hydrogens of the β-carbon of Asn-803 are shown in magenta (pro-R) and yellow (pro-S). Substitution of the pro-S β-hydrogen with a hydroxy group will create an unfavourable steric clash with Ile-353 of p300, and may, as proposed, disrupt a helix stabilizing hydrogen bond between the asparagine side-chain amide (white) and the carbonyl of Asp-799 (green). See Dames et al. [15] and Freedman et al. [16] for NMR structures. This figure was prepared using WebLab Viewer Lite version 3.10 from Molecular Simulations Inc. (Cambridge, U.K.).

Two reports [15,16] show how β-hydroxylation of Asn-803 of HIF-1α would be damaging to complex formation with p300. Although the position of hydroxylation was not identified in either report, both imply that hydroxylation at the pro-S position of the β-carbon, i.e. to give the threo (2S,3S)-isomer, would interfere with the hydrogen bonding that maintains the α-helical conformation adopted by this part of HIF-1α, and also create a need for the energetically unfavourable desolvation of the hydroxy group. Figure 3 also indicates how a steric clash between the inserted pro-S hydroxy group and Ile-353 (num-bering from Dames et al. [15]) of p300 would disrupt the interaction of the two proteins. Presumably the same mechanism is also used to abrogate the interaction of HIF-2α and p300.

The way in which the addition of an appropriately placed single oxygen atom to HIF-1α, a 93 kDa protein, can either cause (in the case of pVHL) or abrogate (in the case of p300) binding interactions is an exquisite example of natural molecular precision. It contrasts with structural studies on HIF-α itself. NMR analyses have revealed that the HIF-1α CAD domain is largely unstructured in the absence of the p300 CH1 domain [15,16]. NMR studies on the CODD and NODD domains similarly imply they are largely unstructured when free in solution (Figure 4). Furthermore, the basic helix-loop-helix N-terminal domain of HIF-1 has so far resisted structural analysis, with the only reported 'structural' study employing a modelling approach [30]. Together, the work to date suggests that when free in solution a significant part of HIF-1α may be unstructured in the absence of other proteins; this may reflect the presumed rapid turnover of HIF-α under normoxic conditions.

The results of the present study and elsewhere [12,13] imply that FIH-1 is part of a new sub-family of human enzymes catalysing the post-translational hydroxylation of asparagine residues. FIH-1 is characterized by production of the threo-(2S,3S) isomer of β-hydroxyasparagine and the observation that it is selective for asparagine over aspartic acid residues [12]. In contrast, the available evidence implies that the EGF-hydroxylase enzyme accepts both the aspartic acid and asparagine residues in appropriate recognition sequences [31].

The different product stereoselectivities of the two enzymes may reflect a need to maintain a division between the roles of β-hydroxyasparagine residues in different pathways. The enzymes are also distinct in the fact that FIH-1 has no predicted α-helical membrane-spanning region, whereas those aspartic acid/asparagine hydroxylases reported from bovine, murine and human sources do possess such regions and consequently localize their activity in the endoplasmic reticulum. On the basis of sequence comparisons with the JmjC (a C-terminal domain that is similar to the mammalian protein jumonji) proteins, FIH-1 has been recently proposed as the first identified member of family of iron(II) oxygenases involved in transcription [12] and it will
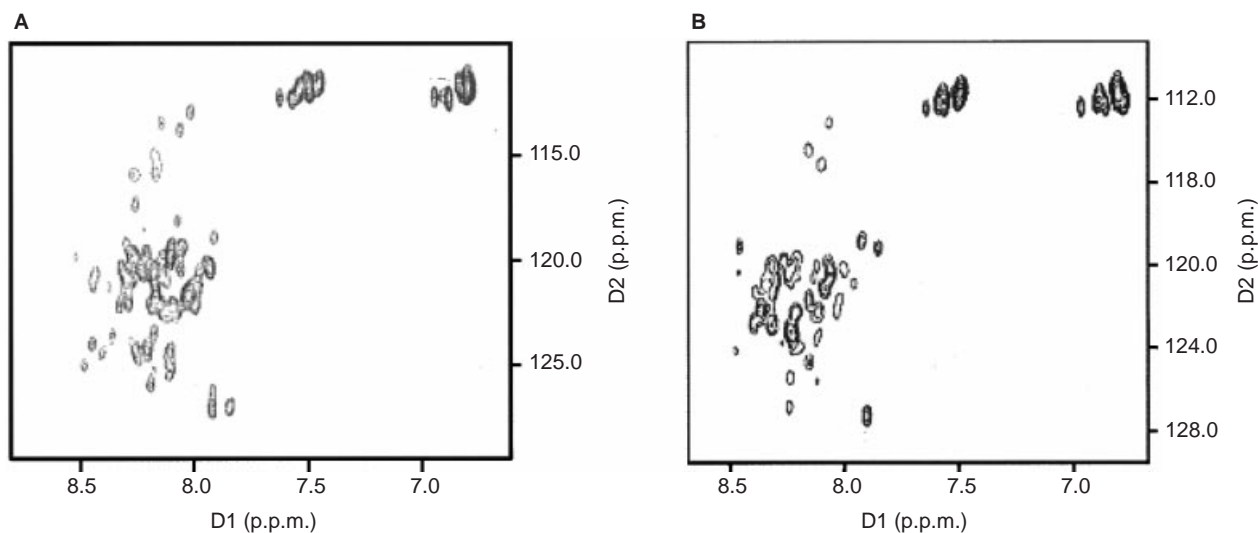
Figure 4 ^{1}H-^{15}N HSQC spectra of the isolated oxygen dependent degradation domains of HIF-1α

(A) NODD; HIF-1α (344–503) and **(B)** CODD; HIF-1α (530–698). In both cases, the spectra indicate a lack of secondary structure.

be of interest to see how well the selectivity of FIH-1 is conserved in the related enzymes.

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