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Backbone Resonance Assignment of a Proteolysis-Resistant Fragment in the Oxygen-Dependent Degradation Domain of the Hypoxia Inducible Factor 1α

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Hypoxia-inducible factor 1α (HIF1α) is a transcription factor that plays a key role in the adaptation of cells to low oxygen stress and oxygen homeostasis. The oxygen-dependent degradation (ODD) domain of HIF1α responsible for the negative regulation of HIF1α in normoxia is intrinsically unfolded. Here, we carried out the backbone ^{1}H, ^{15}N, and ^{13}C resonance assignment of a proteolysis-resistant fragment (residues 404-477) in the HIF1α ODD domain using NMR spectroscopy. About 98% (344/352) of all the ^{1}HN, ^{15}N, ^{13}Cα, ^{13}Cβ, and ^{13}CO resonances were unambiguously assigned. The results will be useful for further investigation of the structural and dynamic states of the HIF1α ODD domain and its interaction with binding partners.

INTRODUCTION

Hypoxia-inducible factor 1α (HIF1α) is a master regulator in oxygen homeostasis and the adaptation of cells to low oxygen stress (Bruick and McKnight, 2002; Semenza, 2001). HIF1α is a ubiquitous basic helix-loop-helix/PAS (Per, Arnt, and Sim protein) transcription factor that activates the expression of genes involved in angiogenesis, erythropoiesis, energy metabolism, apoptosis, and proliferation in response to hypoxia (Semenza, 2003). HIF1α is stable in hypoxia, yet in normoxia, it is subject to ubiquitination by von Hippel-Lindau tumor suppressor (pVHL) and subsequent proteasomal degradation. This oxygen level sensing is mediated by the oxygen-dependent degradation (ODD) domain of HIF1α (Fig. 1). The binding of pVHL to HIF1α ODD is dependent on the hydroxylation of two functionally independent proline residues, Pro^{402} and Pro^{564}, within N- and C-terminal ODD motifs (Ivan et al., 2001; Jaakkola et al., 2001). The HIF1α ODD domain also exerts a transcriptional activation function through the interaction of its C-terminal part (residues 531-575, N-TAD) with basal transcription machinery (Jiang et al., 1996). Recently, interactions of the HIF1α ODD domain with multiple target proteins such as ARD1 (Jeong et al., 2002), PHD (Schofield and Ratcliffe, 2004), and p53 (Hansson et al., 2005) have been reported. The structural basis for such promiscuous binding characteristics of the HIF1α ODD domain is not understood yet. Thus, characterization of the structural states of HIF1α ODD and its binding to target proteins is likely to shed some insight into the binding promiscuity of HIF1α ODD. In this study, we have identified a proteolysis-resistant fragment in the HIF1α ODD domain and completed the backbone ^{1}H, ^{15}N, and ^{13}C resonance assignment.

MATERIALS AND METHODS

The full-length HIF1α ODD and ODD_N constructs, corresponding to residues 403-603 and 404-477, respectively, were subcloned into the pET-21a vector. Transformed *Escherchia coli* BL21(DE3) cells were grown at 37°C to an A_{600} of 0.5 and induced with 0.5 mM isopropyl thio-β-D-thiogalactopyranoside (IPTG). Next, the cells were cultivated at 37°C for 6 h. The harvested cell suspension was sonicated in 50 mM TrisHCl (pH 7.5), 50 mM NaCl, 1 mM PMSF, 2 mM DTT, and centrifuged for 30 min at 30,000 × *g*. An unlabeled or ^{15}N-labeled full-length HIF1α ODD as well as a ^{13}C, ^{15}N-labeled proteolysis-resistant N-termial sub-domain in HIF1α ODD (ODD_N hereafter) were purified by a Q-sepharose column, DEAE-sepharose column, and Hiprep 26/60 Sephacryl S-200 FPLC column (Amersham Pharmacia Biotech, Sweden). The molecular weights of the purified proteins were measured by MALDI-TOF mass spectrometry. The final nuclear magnetic resonance (NMR) buffer was 90% H_2O/10% $^{2}H_2O$ containing 20 mM sodium phosphate (pH 6.0), 0.1 mM PMSF, 0.1 mM EDTA, and 0.1 mM benzamidine. The final protein concentrations of ^{15}N-labeled HIF1α ODD and ^{13}C, ^{15}N-labeled HIF1α ODD_N were 0.4 mM and 1 mM, respectively.

All the NMR data were collected at 5°C on Varian Unity INOVA 600 and Bruker Avance II 900 spectrometers equipped with cryogenic probes. The ^{13}C chemical shifts are referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), and ^{15}N chemical

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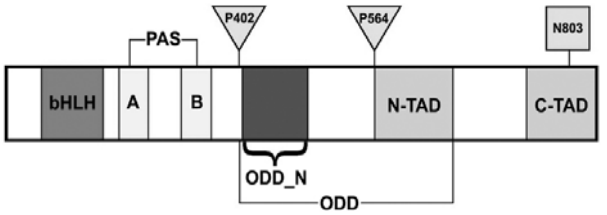

Fig. 1. Functional domains in HIF1α and the location of ODD_N under NMR investigation

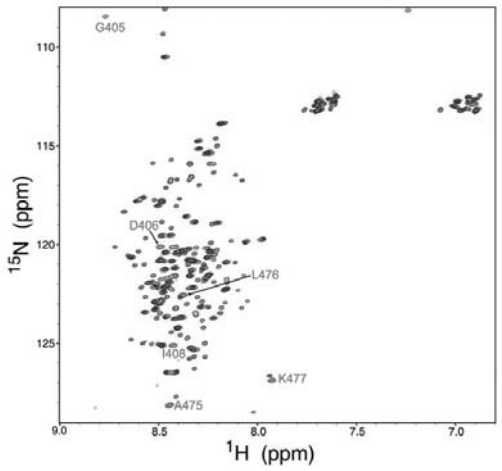

Fig. 2. Overlaid 2D ^{15}N-^{1}H HSQC spectra of the full-length HIF1α ODD and ODD_N domains. The spectrum shown in black and red is for the full-length HIF1α ODD and ODD_N, respectively. The resonances that display the significant chemical shift difference between the two spectra are labeled with assigned amino acid residues.

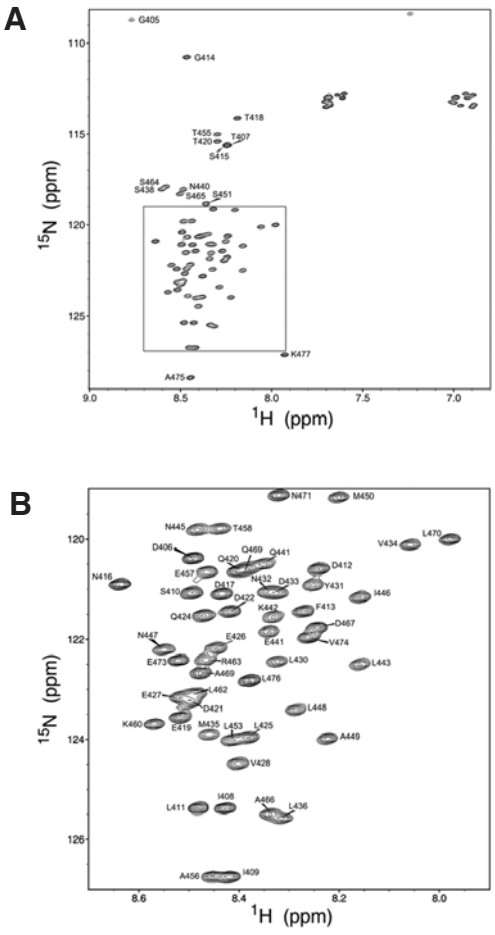

Fig. 3. 2D ^{15}N-^{1}H HSQC spectrum of HIF1α ODD_N domain. The each resonance in the spectrum is labeled with the assigned amino acid residues. In (B), the boxed region in (A) is expanded for clarity.

shifts were referenced indirectly using the gyromagnetic ratio of ^{15}N/^{1}H (Wishart et al., 1995). The sequence-specific resonance assignment of the HIF1α ODD_N domain was carried out using standard triple resonance NMR techniques, as previously described (Bax and Grzesiek, 1993). NMR data were processed and analyzed on a Sun SPARCstation (Sun Microsystems, Inc., USA) using Varian Vnmr and nmrPipe/nmr Draw software.

RESULTS AND DISCUSSION

The resonances in a 2D ^{15}N-^{1}H heteronuclear single quantum coherence (HSQC) spectrum of a full-length HIF1α ODD domain (residues 403-603) are clustered within a narrow proton chemical shift window of ~0.6 ppm (Fig. 2), indicating that this domain is, under the experimental conditions employed, lacking a globular structure or largely unfolded like the σ^{28} binding domain of FlgM (Daughdrill et al., 1997; 1998), c-Myb TAD (Parker et al., 1999), p53 TAD (Chi et al., 2005; Lee et al., 2000), ribosomal protein S4 (Sayers et al., 2000), amyloid precursor protein (Ramelot et al., 2000), GCN4-p1 (Zitzewitz et al., 2000), p27^{Kip1} (Bienkiewicz et al., 2002), thymosin β4 (Domanski et al., 2004), and HBV preS1 (Chi et al., 2007; Kim et al., 2008). This corroborates the previously reported circular dichroism (CD) results (Sanchez-Puig et al., 2005). To test for presence of any structurally stable subdomains, we have carried out limited protease digestion of the full-length HIF1α ODD domain using trypsin and V8 and identified a protease-resistant fragment, HIF1α ODD_N weight, of ~8 kDa. An N-terminal amino acid sequencing and a mass spectral analysis of the proteolytic fragment allowed us to confirm that this domain corresponds to residues 404-477 in HIF1α ODD (Fig. 1). Based on this result, we have sub-cloned expressed and purified this ODD_N construct. Comparisons of the ^{15}N-^{1}H HSQC spectra between the full-length HIF1α ODD and the ODD_N domain (Fig. 2) show that the resonances from the two domains mostly overlap, except a few N- and C-terminal residues (residues 405, 406, 408, and 475-477), indicating that the structural state of HIF1α ODD_N is not significantly influenced by residues 478-603. The sharp resonance linewidth in the NMR spectrum indicates that ODD_N exists as a monomer. Figure 3 shows an ^{15}N-^{1}H HSQC spectrum of the HIF1α ODD_N with assigned resonances. We performed the NMR resonance assignment for the HIF1α ODD_N by standard triple resonance experiments (Bax and Grzesiek, 1993). The assignment of the backbone ^{1}H, ^{15}N, and ^{13}C resonances is 98% (344/352) complete (Table 1). The missing resonance is mainly due to the presence of eight prolines. Conventional secondary structure predictions by the chemical shift index (CSI) and TALOS did not suggest the presence of any stable secondary structure in the HIF1α ODD_N domain.

To determine if there is any residual local structural elements in the unfolded HIF1α ODD_N, we have calculated the second-

Table 1. Chemical shifts of ^{1}HN, ^{15}N, ^{13}CO, ^{13}Cβ and ^{13}Cα of HIF1a ODD_N domain

Residue	^{1}HN	^{15}N	^{13}CO	^{13}Cα	^{13}Cβ	Residue	^{1}HN	^{15}N	^{13}CO	^{13}Cα	^{13}Cβ
A404	*ND	ND	ND	ND	ND	E441	8.34	121.85	176.69	57.19	30.16
G405	8.77	108.72	173.79	45.29		K442	8.33	121.56	176.85	56.67	32.68
D406	8.49	120.38	176.32	54.31	41.16	L443	8.16	122.51	177.46	55.30	42.12
T407	8.24	115.63	174.22	62.23	69.78	Q444	8.35	120.05	175.73	55.83	29.43
I408	8.43	125.38	176.12	61.03	38.53	N445	8.48	119.81	175.34	53.34	38.68
I409	8.42	126.75	176.01	60.85	38.40	I446	8.16	121.16	175.88	61.56	38.63
S410	8.49	121.07	174.37	57.88	63.77	N447	8.55	122.21	175.33	53.22	38.55
L411	8.48	125.38	176.81	55.04	42.24	L448	8.29	123.42	177.25	55.37	42.23
D412	8.24	120.60	175.89	54.04	41.15	A449	8.22	123.99	177.68	52.61	18.97
F413	8.27	121.45	176.43	58.05	39.38	M450	8.20	119.16	176.14	55.15	33.07
G414	8.47	110.77	174.09	45.31		S451	8.36	118.83	ND	56.49	63.23
S415	8.25	115.63	174.36	58.28	63.86	P452			176.72	62.77	31.97
N416	8.64	120.90	174.92	53.24	39.06	L453	8.41	124.02	175.37	53.15	41.48
D417	8.44	121.09	176.40	54.57	40.99	P454			177.02	62.95	31.96
T418	8.19	114.14	174.69	62.03	69.82	T455	8.30	115.01	174.35	61.72	69.91
E419	8.52	123.56	176.61	56.58	30.18	A456	8.45	126.74	177.56	52.47	19.38
T420	8.30	115.41	174.29	61.71	69.91	E457	8.46	120.66	176.45	56.17	30.54
D421	8.50	123.22	176.10	54.33	41.10	T458	8.44	119.79	172.62	60.20	69.64
D422	8.42	121.44	175.05	54.40	40.90	P459			176.62	62.97	32.05
Q423	8.40	120.64	176.02	ND	41.48	K460	8.57	123.69	174.58	54.24	32.14
Q424	8.47	121.53	175.88	55.76	29.24	P461			176.74	62.79	32.01
L425	8.38	123.97	177.38	55.09	42.48	L462	8.49	123.08	177.53	55.18	42.41
E426	8.45	122.18	176.17	56.23	30.47	R463	8.47	122.42	176.16	55.93	31.04
E427	8.51	123.18	176.15	56.20	30.34	S464	8.58	117.90	174.76	58.13	63.94
V428	8.40	124.49	174.25	59.95	32.62	S465	8.51	118.29	174.05	58.48	63.78
P429			176.30	62.81	32.04	A466	8.34	125.50	177.13	52.17	19.32
L430	8.32	122.44	177.11	54.98	42.61	D467	8.24	121.77	175.04	52.19	41.03
Y431	8.25	120.91	175.31	57.53	38.83	P468			177.12	63.64	32.02
N432	8.34	121.07	174.36	52.98	42.31	A469	8.48	122.68	178.19	52.79	18.86
D433	8.33	121.08	176.15	54.36	41.08	L470	7.98	120.00	177.25	55.12	42.29
V434	8.06	120.12	176.02	62.43	32.66	N471	8.32	119.12	176.44	53.39	38.75
M435	8.46	123.91	175.87	55.04	32.46	Q472	8.38	120.59	175.89	55.91	29.50
L436	8.32	125.58	174.93	52.94	41.69	E473	8.52	122.43	176.47	56.65	30.15
P437			176.72	62.55	31.99	V474	8.26	121.96	175.72	62.18	32.85
S438	8.60	118.04	173.30	56.34	63.31	A475	8.44	128.39	177.52	52.29	19.30
P439			176.95	63.74	31.93	L476	8.38	122.83	176.45	55.17	42.24
N440	8.49	118.04	175.59	53.56	38.71	K477	7.93	127.15	ND	57.52	33.80

*ND, Not detected

dary shifts of $^{13}C_{\alpha}$, ^{13}CO, and H_{α} using the sequence-corrected random coil chemical shift values (Schwarzinger et al., 2001). Figure 4 shows actual deviation of the $^{13}C_{\alpha}$, ^{13}CO, and H_{α} chemical shifts from the random coil values (ΔC_{α}, ΔCO, and ΔH_{α}). The chemical shift deviations in $^{13}C_{\alpha}$ and ^{13}CO indicate that the residues 438-449 and 467-477 have a weak propensity to form the α-helix (Figs. 4A and 4B). The tendency for the local structural order for residues 438-449 and 467-477 is similar. The helical propensity found here is consistent with the previous CD data, showing that the HIF1α ODD domain is natively unfolded, but may contain a small amount of poly(L-proline) helix type II (PPII) (Sanchez-Puig et al., 2005). The presence of local structural order may explain, in part, the resistance of HIF1α ODD_N to protease digestion. There has been ample evidence that local structural elements or pre-structured motifs in intrinsically unfolded proteins play a crucial functional role (Chi et al., 2005; 2007; Fuxreiter et al., 2004; Kim et al., 2008; Lee et al., 2000). Therefore, our NMR data on HIF1α ODD_N should be valuable for the future study of protein-protein interactions involving HIF1α ODD_N. The presence of two dissected HIF1α ODD domains seems to explain, in part, why two functionally independent N- and C-terminal HIF1α ODD subdomains exist within HIF1α and interact with target proteins such as pVHL and p53.

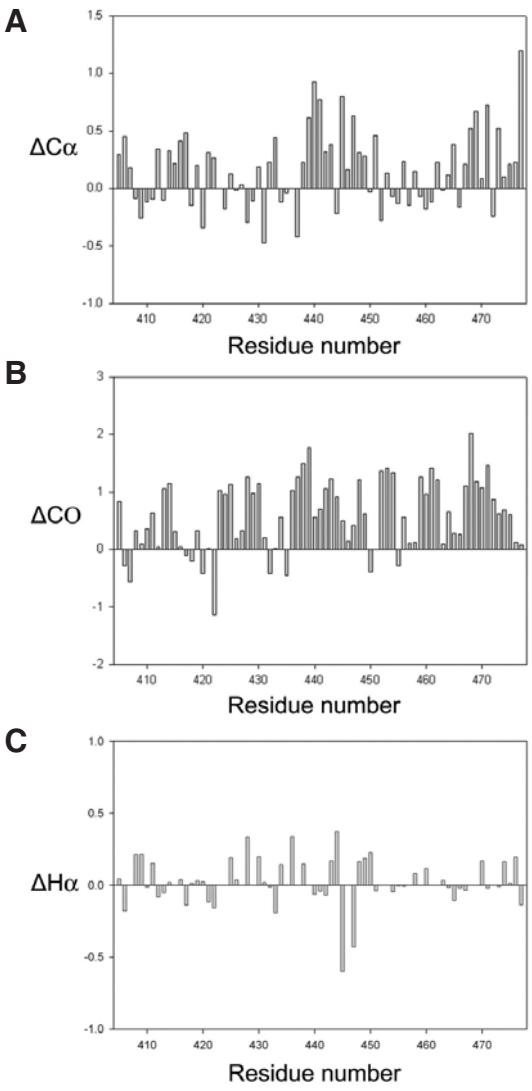

Fig. 4. The chemical shift deviations in $^{13}C\alpha$, ^{13}CO, and H_α for HIF1α ODD_N domain. The ΔCα (A), ΔCO (B), and ΔHα (C) values of backbone resonances to random coil chemical shifts were plotted. (A) $\Delta C_\alpha = C_\alpha$ (measured)-C_α (random). (B) ΔCO = CO (measured)-CO (random). (C) $\Delta H_\alpha = H_\alpha$ (measured) - H_α (random).

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