Hemerythrin-like Domain within F-box and Leucine-rich Repeat Protein 5 (FBXL5) Communicates Cellular Iron and Oxygen Availability by Distinct Mechanisms

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Background: The hemerythrin-like domain of FBXL5 (FBXL5-Hr) governs its stability.

Results: The ability of FBXL5-Hr to sense iron and oxygen availability is restricted within cells and is mediated by distinct conformations.

Conclusion: FBXL5-Hr employs different mechanisms to signal changes in iron and oxygen availability.

Significance: This study provides new mechanistic insights into the maintenance of iron homeostasis.

Iron regulatory proteins play a principal role in maintaining cellular iron homeostasis by post-transcriptionally regulating factors responsible for iron uptake, utilization, and storage. An E3 ubiquitin ligase complex containing FBXL5 targets IRP2 for proteasomal degradation under iron- and oxygen-replete conditions, whereas FBXL5 itself is degraded when iron and oxygen availability decreases. FBXL5 contains a hemerythrin-like (Hr) domain at its N terminus that mediates its own differential stability. Here, we investigated the iron- and oxygen-dependent conformational changes within FBXL5-Hr that underlie its role as a cellular sensor. As predicted, FBXL5-Hr undergoes substantive structural changes when iron becomes limiting, accounting for its switch-like behavior. However, these same changes are not observed in response to oxygen depletion, indicating that this domain accommodates two distinct sensing mechanisms. Moreover, FBXL5-Hr does not behave as a dynamic sensor that continuously samples the cellular environment, assuming conformations in equilibrium with ever-changing cellular iron levels. Instead, the isolated domain appears competent to incorporate iron only at or near the time of its own synthesis. These observations have important implications for mechanisms by which these metabolites are sensed within mammalian cells.

Although iron and oxygen are essential micronutrients for virtually all forms of life, they can be very toxic to cells if present in excess (1–3). Both prokaryotes and eukaryotes have evolved efficient mechanisms for tightly regulating the bioavailable levels of these metabolites (4–6). In mammals, cellular iron homeostasis is achieved by maintaining a delicate balance between iron import, utilization, and export (7–9). Coordinate expression of the gene products mediating these processes is regulated by iron regulatory proteins (IRPs)4 that bind to iron-responsive elements located within their cognate mRNAs, influencing translation or transcript stability (8, 10). Both IRPs are competent to bind mRNAs under low iron conditions. This ability is lost when bioavailable iron levels are high, largely due to the assembly of an iron-sulfur cluster within IRP1 (11–14) and the degradation of IRP2 by the proteasome (15–17).

The differential stability of IRP2 is mediated through the actions of an E3 ubiquitin ligase complex, SCFFBXL5, composed of SKP1, Cullin-1, RBX1, and the F-box protein FBXL5 (F-box and leucine-rich repeat protein) (18, 19). Under iron-replete conditions, FBXL5 recruits IRP2 to the SCF complex, where it is polyubiquitinated. Under iron-deplete conditions, however, FBXL5 itself is targeted for ubiquitination and degraded in a proteasome-dependent manner. As a result, IRP2 is subsequently stabilized and binds iron-responsive elements (18, 19). In addition to iron, the stability of IRP2 is also responsive to cellular oxygen availability, as IRP2 degradation is attenuated under hypoxic conditions (20, 21). These effects are thought to be mediated through FBXL5, which is preferentially destabilized in cells exposed to a low oxygen environment (18, 19).

Regulatory elements conferring iron and oxygen responsiveness reside within the N-terminal FBXL5 hemerythrin-like domain (FBXL5-Hr) (18, 22). Hr domains, previously reported in a small number of marine invertebrates and bacteria, are typically composed of a bundle of α-helices enveloping a non-heme diiron center capable of reversible O2 binding (23, 24). Although FBXL5-Hr features several atypical structural elements, it nevertheless assembles into an extended bundle of five helices held in place by its diiron center (22). Characterization of FBXL5-Hr suggests a model in which a degron residing within this domain becomes preferentially accessible to an as-yet-unknown E3 ubiquitin ligase under iron-deplete conditions.

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(22). This hypothesized switch-like behavior of the Hr domain could contribute to the iron sensing and responsiveness needed to maintain cellular iron homeostasis (18, 19, 22).

Nevertheless, several outstanding questions remain. The manner in which O2 levels are sensed by FBXL5-Hr remains unresolved. Although the oxidation state of the diiron center clearly changes as a function of O2 availability, no evidence of direct O2 binding to the diiron center was observed in any of the reported the FBXL5-Hr crystal structures (22). Crowded side chain packing at the canonical O2-binding site would appear to prevent FBXL5-Hr from reversibly binding molecular oxygen unless accompanied by substantial conformational rearrangements (22). Such changes would, however, be expected to significantly alter the accessibility of the degron to an E3 ligase. In addition, the static models of FBXL5-Hr do not address the dynamic responsiveness of the switch to changes in iron availability within cells.

Here, we employed a range of molecular and biophysical analyses to show that FBXL5-Hr resides in two very distinct conformations as a function of cellular iron availability. In contrast, oxygen availability does not appear to induce the same gross structural rearrangements used to communicate cellular iron status. Importantly, the ability of FBXL5-Hr to “sense” an elevation in bioavailable iron levels appears to be restricted to a period at or near the time of its biosynthesis, as accumulated pools of apoHr fail to assemble a diiron center in the presence of iron. These observations have important implications for the nature of both cellular iron and oxygen sensing and the maintenance of iron homeostasis.

EXPERIMENTAL PROCEDURES

Recombinant Protein Expression and Purification—Expression and purification of human FBXL5-Hr were performed as described previously (18). The iron content of FBXL5-Hr was determined by the method of Beinert (25) with modifications (18). A 2:1 molar ratio of iron to FBXL5-Hr was obtained at the end of the purification, indicating that the preparation comprised almost entirely of the holoHr state. 13C labeling was performed as described previously (18, 22, 26).

To remove iron from the holoHr preparation, purified FBXL5-Hr was incubated with 10 molar eq of defereroxamine mesylate (DFO), followed by isolation of apoHr using a desalting column. To reduce holoHr, 10 molar eq of Na2S2O4 were added to purified FBXL5-Hr under a nitrogen gas blanket, followed by flushing with N2 for an additional 30 min. Limited trypsin proteolysis experiments on reduced holoHr were carried out in an anoxic chamber (Coy Laboratory Products Inc.) in which O2 was maintained at <1 ppm.

CD Spectrometry—Recombinant FBXL5-Hr (26 µM) in buffer containing 20 mM HEPES (pH 7.4) and 100 mM NaCl was used for both CD and thermal denaturation experiments. Measurements for both experiments were performed on an AVIV 62 DS instrument with a 1-mm path length quartz cuvette. CD measurements were taken with an averaging time of 3 s with five repeats. Thermal denaturation data were recorded by measuring ellipticity at 222 nm from 15 to 95 °C in 1 °C increments over equilibration times of 90 s. Melting curves measured in reverse from 95 to 15 ºC did not overlap, demonstrating that denaturation is not reversible (data not shown). All data are reported in mean residue ellipticity (θ).

NMR—Constant time 13C/1H heteronuclear single quantum coherence (HSQC) spectra (27) were recorded on uniformly 13C-labeled FBXL5-Hr (100 µM in phosphate-buffered saline) on a cryoprobe-equipped Varian Inova 600-MHz NMR spectrometer at 25 ºC. Spectra were processed using NMRPipe (28) and analyzed using NMRView (29).

Cell Culture and Reagents—HEK293 cells were stably transfected with the FLAG-Hr-HA or FLAG-FBXL5-HA expression construct (18) and isolated through two rounds of clonal selection in the presence of 400 µg/ml G418 (Research Products Inc.). Cells were grown and maintained in DMEM (HyClone) supplemented with 10% fetal bovine serum (Atlanta Biologicals). Low O2 experiments were performed in a hypoxic incubator (Coy Laboratory Products Inc.) containing 1% O2, 5% CO2, and balance N2. The temperature-sensitive ts20 Balb/c 3T3 clone A31 fibroblast cells (30, 31), kindly donated by Dr. H. L. Ozer, were cultured in DMEM supplemented with 10% fetal bovine serum. Immunoblot analyses were performed as described (18) with the respective mouse antibodies: anti-FLAG (Sigma F3165), anti-IRP2 (Santa Cruz Biotechnology sc-33682), anti-tubulin (Sigma T6199), anti-ubiquitin (Santa Cruz Biotechnology sc-8017) antibodies and a mouse monoclonal antibody raised against the human FBXL5 protein (Neoclon Biotechnology, LLC) (supplemental Fig. S1).

Limited Proteolysis—Cells were incubated with either 50 µM ferric ammonium citrate (FAC) or 50 µM DFO under high (21%) or low (1%) oxygen conditions for 6 h. Where noted, cells were pretreated with 35 µM MG132 (Boston Biochem). Cells were then washed with cold phosphate-buffered saline and lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% (v/v) Triton X-100, 0.5 mM PMSF, 1 mM DTT, and 1× protease inhibitor mixture (Sigma) at 4 ºC for 10 min. Lysates were centrifuged at 16,000 × g for 20 min, and the protein concentration of the lysates was measured using the Bradford assay. Trypsin (3 µg; Promega) was then added to lysates (100 µg of total protein), and proteolysis was carried out at 37 ºC for 1 h. The reaction was quenched by the addition of 0.2 mM PMSF, and the extent of FBXL5-Hr digestion was assessed by immunoblot analysis using an anti-FLAG antibody. For recombinant FBXL5-Hr, limited proteolysis was performed in a similar manner using 100 µg of purified protein/sample.

RESULTS

Iron- and Oxygen-dependent Regulation of FBXL5-Hr—Previous reports have shown that the stability of FBXL5 is dependent on both iron and oxygen availability and that its N-terminal Hr domain is sufficient to confer this regulation (18, 19, 22). Consistent with these observations, the stability of FBXL5-Hr alone (Fig. 1A) mimicked that of endogenous FBXL5 (Fig. 1B).

In these stably transfected cells, FBXL5-Hr had a substantially longer half-life (~7.3 h) under iron-replete conditions compared with iron-deplete conditions (~1.9 h). Oxygen was also required for full Hr stabilization, as its half-life dropped to ~3 h in cells incubated under low oxygen conditions, even in the presence of excess iron (Fig. 1C). In both instances, decreased
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protein stability was accompanied by a corresponding increase in polyubiquitination of the domain (Fig. 2A).

FBXL5-Hr is particularly responsive to cellular iron levels in comparison with other metals. As shown in Fig. 2B, introduction of even low levels of iron to cells preincubated with the metal chelator DFO promoted accumulation of FBXL5-Hr. In contrast to iron, cobalt and zinc treatment induced FBXL5-Hr accumulation only at much higher concentrations. Manganese appeared to induce intermediate accumulation of the Hr domain, whereas nickel, copper, and magnesium treatment had almost no effect. Thus, cellular FBXL5-Hr accumulation is most responsive to exogenous iron incubation, consistent with its purported function as an iron-binding and iron-sensing domain.

Responses of FBXL5-Hr to Iron and Oxygen Are Not Mediated by Analogous Conformational Changes—We previously hypothesized that the apoHr and holoHr states of FBXL5-Hr are defined by distinct tertiary conformations (18). In fact, the spectra observed by CD spectrometry of the purified Hr domain remain virtually unchanged following removal of iron (Fig. 3A, ApoHr) or oxygen (Fig. 3A, Reduced HoloHr), indicating that all three preparations retained roughly equivalent overall content of helical secondary structure. However, removal of iron from the domain resulted in a significantly destabilized protein as reflected by the decreased melting temperature (Fig. 3B). Somewhat surprisingly, reducing the sample with sodium dithionite yielded no apparent change in the overall integrity of the domain as assessed by T_m (Fig. 3B), even though such treatment is sufficient to alter the oxidation state of the diiron center (22).

To further investigate the potential differences underlying iron and oxygen sensing by FBXL5-Hr, we employed limited proteolysis to reveal conformational changes. Purified recombinant FBXL5-Hr quantitatively depleted of iron (apoHr) could be digested by trypsin in a dose-dependent manner. However, holoHr remained relatively resistant to proteolysis in comparison with apoHr (Fig. 3C), consistent with the expected destabilization of the domain following iron removal. In contrast, reduction of oxidized holoHr and subsequent maintenance within an anoxic environment again failed to elicit a difference in protease susceptibility (Fig. 3C). An identical result was observed using crude cellular extracts from cells expressing FLAG-Hr-HA, as the addition of an iron chelator to the culture medium rendered the domain sensitive to proteolysis (Fig. 3D). In contrast, incubation of the cells under a low oxygen environment (1% O_2, 6 h) capable of promoting FBXL5 degradation and IRP2 induction (Fig. 1B) did not have a significant effect on trypsin sensitivity (Fig. 3D).

To determine whether this Hr behavior might differ in the context of the entire FBXL5 protein, we repeated the experiment with a cell line expressing the full-length FBXL5 protein featuring an N-terminal FLAG tag immediately adjacent to the Hr domain. A trypsin-resistant N-terminal fragment with an apparent molecular weight slightly larger than that of the 161-amino acid Hr domain (denoted by the arrow in Fig. 3E) accumulated in trypsinized crude lysates from iron-replete cells incubated under either high or low oxygen conditions. As observed for the isolated Hr domain (Fig. 3D), this species did not appreciably accumulate only in trypsinized lysates prepared from iron-depleted cells (Fig. 3E).

Although both the T_m and limited proteolysis data indicated that FBXL5-Hr responds differently to iron and oxygen depletion, it remains possible that these methods lack the sensitivity to detect relevant but localized O_2-dependent conformational changes. To further validate these results and to examine the effects of metal binding and oxidation state on the structure of FBXL5-Hr at a higher resolution, we used solution NMR spectroscopy to study the conformations of the apoHr, reduced

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**FIGURE 1. Responses of ectopically expressed FBXL5-Hr to iron and oxygen mimic those of endogenous FBXL5.** A, immunoblot analysis of FLAG-Hr-HA accumulation in HEK293 cells following incubation for 6 h under iron-replete (FAC) or iron-deplete (DFO) conditions in the presence of high (21%) or low (1%) O_2. B, immunoblot analysis of endogenous human FBXL5 accumulation in HEK293 cells in response to changes in iron and oxygen levels. As indicated, protein degradation was blocked by the addition of the proteasome inhibitor MG132. C, upper panel, the half-life of isolated FBXL5-Hr is dependent on both cellular iron and oxygen availability. HEK293 cells were incubated overnight in the presence of 50 μM FAC and 21% O_2. Following a 30-min pretreatment with 10 μM cycloheximide, cells were moved to the indicated incubation conditions and lysed at various time points. FLAG-Hr-HA expression from the stably transfected transgene was assessed by immunoblot analysis. Lower panel, the -fold change in total protein used to calculate t_{1/2} is quantitated.
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holoHr, and oxidized holoHr forms of the protein. Constant time $^{13}$C/$^1$H HSQC spectra of uniformly $^{13}$C-labeled protein provided such an overview of the environments around the protein side chains, particularly those containing methyl groups that can typically be observed with high sensitivity and resolution. The spectra of the apoHr preparation (Fig. 4A) show considerably fewer signals than expected for the number of methyl-containing residues in the protein (52 of 84 total methyl groups). The signals that were observed had significant signal intensity heterogeneity and poor $^1$H chemical shift dispersion, consistent with the protein being unfolded, as predicted from limited proteolysis. The presence of metal at the diiron center significantly improved both intensity and chemical shift dispersion (Fig. 4A), reaffirming the importance of the two coordinated iron ions for establishing protein tertiary structure. However, a change in the oxidation state (22) did not significantly alter the spectra between the oxidized and reduced forms (Fig. 4B), suggesting that the protein does not undergo major structural changes upon $O_2$ removal. Together, these data suggest that distinct molecular mechanisms distinguish iron and oxygen sensing by FBXL5-Hr.

Ability of FBXL5-Hr to Sense Increased Iron Availability Is Restricted within Cells—With respect to its role as a metal sensor, FBXL5-Hr is strongly selective for iron binding, can be competed for iron binding by metal chelators such as DFO, and undergoes a substantial conformational change upon loss of its diiron center both in vitro and in cultured cells. Theoretically, a metal sensor should have an affinity for iron within the physiological range of iron concentrations. The $K_m$ for iron has been difficult to measure in vitro, as we have yet to identify conditions in which purified apoHr assembles a diiron center de novo. This difficulty raises an interesting question. Is FBXL5-Hr in constant equilibrium with the cellular pool of bioavailable iron, reverting back and forth between conformations? We have shown that FBXL5-Hr can convert from a folded to less folded state, consistent with putative disassembly of its diiron center, when intracellular levels are depleted. However, it remains to be demonstrated that apoHr can reassemble its diiron center.

To address this question, HEK293 cells stably transfected with a FLAG-Hr-HA expression construct were depleted of iron by the addition of DFO. The failure of these cells to accumulate FLAG-Hr-HA (Fig. 5A, first lane) could be reversed by the addition of excess iron to the medium (second lane). FLAG-Hr-HA accumulation required new protein synthesis (Fig. 5A, third lane), and the majority of the protein contained a diiron center as reflected by its resistance to trypsin digestion (second lane). Alternatively, a pool of FLAG-Hr-HA could accumulate under low iron conditions by blocking degradation with a proteasome inhibitor, although this pool remained sensitive to proteolysis (Fig. 5A, fourth lane) as expected for an apoHr species. Subsequent reintroduction of iron to these cells resulted in the accumulation of protease-resistant holoHr (Fig. 5A, fifth lane). However, holoHr accumulation still required de novo protein synthesis (Fig. 5A, sixth lane). When translation was blocked by cycloheximide, the remaining pool of apoHr, which accumulated in the presence of DFO, remained equally sensitive to trypsin despite the reintroduction of iron (Fig. 5A, fourth and sixth lanes). Moreover, even in the context of the full-length FBXL5 protein, the accumulated apoHr domain similarly failed to adopt a trypsin-resistant conformation following iron addition (denoted by the arrow in Fig. 5B). These results suggest that once FBXL5-Hr adopts an unfolded conformation, it is subsequently unable to bind iron in cells.

However, the pool of apoHr that accumulated under low iron conditions in the presence of proteasome inhibitor was also subject to polyubiquitination (Fig. 2A). To eliminate the possibility that ubiquitination precludes a reversion to the holoHr conformation and the ability to sense the replenishment of cellular iron levels, we used a temperature-sensitive fibroblast cell line that only expresses functional E1 enzyme when incubated at 35 °C (30, 31). At the restrictive temperature (39 °C), a lack of E1 inhibits activation of ubiquitin and its subsequent addition to substrates such as transiently transfected FLAG-Hr-HA (22). As with MG132 addition (Fig. 5A), incubation at 39 °C allowed accumulation of a pool of trypsin-sensitive apoHr under iron-
FIGURE 3. FBXL5-Hr responses to iron and oxygen are accompanied by different conformational states. A, CD spectra of the helical content of oxidized holoHr, reduced holoHr, and iron-depleted apoHr. deg, degrees. B, measurement of the mean molar residual ellipticity at 222 nm as a function of the thermal denaturation of oxidized holoHr, reduced holoHr, and apoHr. C, upper panel, Coomassie Blue staining of recombinant oxidized holoHr, reduced holoHr under anoxia, and iron-depleted apoHr following digestion with increasing amounts of trypsin. Lower panel, the -fold change in undigested protein is quantitated. D, upper panel, the protein levels of stably transfected FBXL5-Hr-HA were assessed by immunoblot analysis before (-T) or after (+T) incubation in the presence of trypsin for 1, 4, or 8 h. Lower panel, the -fold change in undigested protein is quantitated. E, lysates (100 µg) were incubated in the presence of 0, 1, or 2 µg of trypsin, and the remaining fragments containing the N-terminal FLAG tag adjacent to the Hr domain were assessed by immunoblot analysis. The arrow indicates a trypsin-resistant N-terminal fragment with an apparent molecular weight slightly larger than that of the 161-amino acid Hr domain.
Deplete conditions (Fig. 5C, fourth lane). Even though this accumulated apoHr was not ubiquitinated, it still failed to adopt a trypsin-resistant holoHr conformation upon readdition of iron (Fig. 5C, sixth lane). Together, these data suggest that FBXL5 is not a “dynamic” sensor in constant equilibrium with the cellular iron environment. Instead, the ability of FBXL5-Hr to sense an elevation in bioavailable iron levels appears to be restricted to a period coinciding with its own synthesis and initial folding.

**DISCUSSION**

Recent studies (18, 19) have identified SCFFBXL5 as the E3 ligase responsible for decreasing the cellular levels of IRP2 protein under iron-replete conditions. Conversely, when iron is limiting, the FBXL5 subunit itself is ubiquitinated and targeted for proteasomal degradation. This interplay between the stabilities of FBXL5 and IRP2 plays a critical role in maintaining cellular iron homeostasis. Indicative of the pivotal role of FBXL5 in the physiological development of mammals, mice lacking FBXL5 die during early embryogenesis due to unregulated IRP2 induction and excessive iron accumulation in the developing fetus (32). Even adult animals in which the Fbxl5 gene was selectively inactivated in the liver fail to sense iron levels properly and die when fed a high iron diet (32).

FBXL5 is a critical factor in the maintenance of cellular iron homeostasis because the Hr domain at its N terminus functions as a sensor of both iron and oxygen availability (18, 19, 22). FBXL5-Hr directly incorporates iron within a diiron center held together by conserved histidine and glutamic acid side chains (22). As shown in Fig. 2B, this domain is exquisitely responsive to iron. These results are generally consistent with previous investigations of IRP2 responses to various metals. For example, nickel has virtually no effect on FBXL5-Hr or IRP2 activity (33), and only high levels of zinc antagonize IRP2 induction (34, 35), in accordance with the reciprocal effects observed for FBXL5-Hr. In contrast, despite promoting some modest FBXL5-Hr accumulation, cobalt and manganese have been reported to up-regulate IRP2 levels, but only at much higher concentrations (20, 36). It is likely that incubation with metals at superphysiological concentrations induces many additional cellular responses that complicate subsequent interpretation. Nevertheless, it is clear that FBXL5-Hr selectively senses iron, in agreement with its preferential role in regulating IRP2.
As we have shown, the ability of FBXL5-Hr to communicate cellular iron availability is dependent on its ability to switch between two distinctly different conformational states. The holoHr domain folds into a compact tertiary structure (both in vitro and in extracts prepared from iron-replete cells) that is resistant to limited proteolysis. Although the $^{13}$C/$^1$H HSQC spectra for holoHr show good chemical shift dispersion of the aliphatic methyl groups, characteristic of an ordered tertiary structure, apoHr exhibited poor chemical shift dispersion indicative of an unfolded protein with a low degree of ordered structure. Likewise, depletion of iron from cultured cells induces a less ordered apoHr conformation reflected by increased protease sensitivity. These two conformational states are accompanied by corresponding differences in the extent of polyubiquitination (Fig. 2A) likely due in part to the differential accessibility of a putative degron within an extended loop between the second and third helices (22).

Somewhat surprisingly, the ability of FBXL5-Hr to sense and respond to changes in oxygen availability is not mediated through analogous conformational gymnastics. Although low oxygen conditions promote increased FBXL5-Hr ubiquitination and degradation (Figs. 1A and 2A), there is no corresponding change in the overall stability (Fig. 3B) of the domain or its susceptibility to protease digestion (Fig. 3, C–E), nor are there overt differences between the oxidized and reduced holoHr $^{13}$C/$^1$H HSQC spectra suggestive of even localized conformational changes that could account for increased degron accessibility.

Shu et al. (37) recently reported $^{15}$N/$^1$H HSQC NMR spectra of FBXL5-Hr containing a C159S mutation. This variant appears to be well folded when prepared under aerobic conditions, with multiple peaks observed for several residues, suggesting an equilibrium of two stable conformations (37). Our spectra in Fig. 4 are also consistent with multiple states for wild-type holoHr in solution with minor variability in the occupancy of these two states between experiments (data not shown). However, we did not observe a change in the relative occupancy of the two conformational states following reduction (Fig. 4B) of wild-type holoHr, unlike the C159S protein, which was reported to reside in a single conformational state when reduced (37). It remains possible that these different results reflect changes in the oxygen-responsive properties of the C159S variant.

Unlike canonical Hr domains that are known to bind molecular oxygen, the O$_2$-binding site within FBXL5-Hr is otherwise occupied by amino acid side chains (22). If FBXL5-Hr were to directly bind O$_2$, a substantial rearrangement of residues would be required, yet no such changes are indicated by protease sensitivity or NMR. In addition, studies quantifying the changes in absorption spectra at ~500 nm also indicate the absence of oxygen at the diiron center of oxidized FBXL5-Hr (22, 37). Together, these data clearly suggest that oxygen sensing is not mediated by direct oxygen binding to the diiron center. It was recently reported that the crystal structure of a “reduced” FBXL5-Hr C159S variant lacks a bridging oxygen atom between the two iron atoms (37). Such destabilization of the diiron center might be expected to result in iron loss and domain unfolding in hypoxic cells. However, closer examination of the electron density maps for the reduced structures does reveal density where the $\mu$-hydroxyl should be.

Nevertheless, the oxidation state of the diiron center remains sensitive to the oxygen environment, which could subtly alter the accessibility of the degron, which already resides within a relatively disordered region of the protein (22). The extent of ubiquitination observed in response to hypoxia is significantly less than that observed upon iron depletion, also consistent with the possibility of a modest increase in degron accessibility. Alternatively, oxygen sensing may be mediated by a completely different mechanism uncoupled from changes at the diiron center, e.g. post-translational modifications on the protein involving additional factors. Together, these results suggest that degradation of FBXL5-Hr in response to oxygen depletion is mechanistically distinct from that induced upon iron depletion.

Previous in vitro studies on Hr domains have shown that some have the ability to interconvert between their apo and holo forms in equilibrium with metal concentrations (24, 38). Such behavior would suggest a dynamic sensor, constantly sampling the cellular environment and adopting a ratio of conformational states commensurate with iron bioavailability. However, pools of apoHr forced to accumulate under iron-deplete conditions could not revert to the holoHr conformation when cellular iron levels were re-established (Fig. 5). These results suggest that FBXL5-Hr is only competent to sense high iron levels at or near the time of its own synthesis. Rather than serving as a dynamic iron sensor, this domain more accurately captures the cellular iron status at the moment in time during which it is synthesized. Any subsequent loss of iron irrevocably leads to a conformation destined for degradation.

The mechanism by which diiron center assembly is restricted remains undetermined. It is possible that a localized metallochaperone activity may be required to facilitate holoHr formation. Alternatively, an open conformation may associate with cellular factor(s) that preclude iron binding and refolding. Nevertheless, these results establish that this mammalian Hr domain can sense multiple cellular cues through distinct molecular mechanisms. Although both iron and oxygen sensing are regulated by ubiquitination, only changes in iron are accompanied by large structural changes. These distinct conformations are not freely interconvertible. Rather, competency for iron binding is limited for this sensor, necessitating new protein synthesis to convey increases in cellular iron bioavailability.

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