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The functional duality of iron regulatory protein 1

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Summary

Iron homeostasis in animal cells is controlled post-transcriptionally by the iron regulatory proteins IRP1 and IRP2. IRP1 can assume two different functions in the cell, depending on conditions. During iron scarcity or oxidative stress, IRP1 binds to mRNA stem-loop structures called iron responsive elements (IREs) to modulate the translation of iron metabolism genes. In iron-rich conditions, IRP1 binds an iron-sulfur cluster to function as a cytosolic aconitase. This functional duality of IRP1 connects the translational control of iron metabolizing proteins to cellular iron levels. The recently determined structures of IRP1 in both functional states reveal the large-scale conformational changes required for these mutually exclusive roles, providing new insights into the mechanisms of IRP1 interconversion and ligand binding.

Introduction

Iron is an essential element for most life forms, but because it can be toxic at elevated cellular levels, elaborate regulatory systems have evolved to maintain it at sufficient yet safe concentrations. In metazoans, the primary controllers of iron transport and storage are the two iron regulatory proteins IRP1 and IRP2. In conditions of low iron availability, the IRPs modulate the expression of proteins involved in iron metabolism by binding to conserved stem-loop structures called iron responsive elements (IREs) in the untranslated regions (UTRs) of their mRNAs (Figure 1, left). The regulatory outcome depends upon the position and context of the IRE in the mRNA: an IRP bound to a 5' UTR IRE represses translation, whereas an IRP bound to an IRE in the 3' end can indirectly activate translation through suppression of mRNA degradation. In conditions of high iron concentrations, the IRP inventory largely changes, where IRP2 is degraded, and IRP1 binds a 4Fe-4S cluster to become a functioning cytosolic (c-) aconitase enzyme (Figure 1, right). The two roles of IRP1 link gene regulation in iron homeostasis to the sensing of intracellular iron levels and oxidative stress. Coordinated regulation of the IRP/IRE network enables cells to respond to the multiple signals of iron availability and demand in a balanced manner.

Many of the recent advances in the molecular and cellular control of iron homeostasis pertain to IRP responses to phosphorylation, heme compounds and reactive oxygen and nitrogen species, effectors that destabilize the Fe-S cluster of c-aconitase. These topics have been summarized recently [1]. This review will focus on the structural biology results concerning the two functional states of IRP1 (Figure 2), and also discuss the implications of the nucleotide sequence variations among the IREs.

IRPs and the aconitase family

IRPs are members of the aconitase family, a five-branched tree of Fe-S cluster-dependent hydrolases that catalyze the isomerization of β -hydroxy-acid metabolites [2–4]. True aconitases are committed to citrate-isocitrate isomerization, while two minor branches of the family adapted to the isomerization of the related compounds isopropylmalate and homocitrate (in alternate biosynthetic pathways for leucine and lysine, respectively). Active aconitases require a 4Fe-4S cubane cluster, where the first three irons are ligated by cysteine side chains, and the remaining most labile iron is available for participation in the isomerization reaction. The structural prototype of the family is mitochondrial aconitase, but most eukaryotes also possess a cytoplasmic version of the enzyme that has ~30% amino acid sequence identity. IRP1 with a complete Fe-S cluster is the cytoplasmic aconitase.

All members of the aconitase family have a four-domain organization, with some interesting variations in arrangement. The first three domains of typical mitochondrial aconitases are in tight association to nestle the Fe-S cluster, while the fourth, although spatially close, is tethered through a long (~60 amino acids) linker. In a subgroup of isopropylmalate isomerases, domain 4 is a separate, unlinked peptide chain, whereas in the aconitase B group, the four domains are contiguous but cyclically permuted in the order 4-1-2-3. These oddities suggest a certain structural, functional, and evolutionary lability for domain 4.

The fold of IRP1 in the c-aconitase form is closest to that of mammalian mitochondrial aconitase [5 $\lambda\lambda$ –6]. The Fe-S cluster and substrate binding residues are strictly conserved, so the active sites are basically the same. The notable differences are side chains and structural elements that participate in the conformational transition for binding of the IRE.

IRP2 has ~60% sequence identity to IRP1, so it is anticipated to have the same fold. But IRP2 is not known to bind an Fe-S cluster, and does not display aconitase activity [7]. A partial explanation is that although IRP2 has retained its equivalents of the three iron-ligating cysteines (437, 503, and 506, IRP1 numbering), a number of amino acid substitutions in the active site could possibly interfere with the cluster assembly [5 $\lambda\lambda$]. IRP2 also differs from IRP1 by having a 73 amino acid insertion of unusual composition (high cysteine, glycine, lysine, and proline content) in domain 1. The IRP1 structure suggests that this insertion is on the exterior but in the same general region as the ligand-binding residues. Early reports proposed that the IRP2 insertion is the site for iron- and/or heme-mediated degradation [8–9]. More recent work argues that it is a poorly ordered, protease-sensitive region [10]. Finally, IRP2 exhibits a different pattern of affinities to the IRE family than IRP1, having in general weaker binding to the non-ferritin types [11].

Fe-S cluster assembly and disassembly in IRP1

An obvious directional influence on the functional pathway for IRP1 is the process of Fe-S cluster assembly, committing IRP1 to its aconitase state. In normal tissues with abundant iron, most IRP1 is in the c-aconitase form. Although Fe-S clusters can be inserted into IRP1 in non-physiological conditions *in vitro*, [12], these are always assisted processes *in vivo*. Cluster biogenesis in eukaryotes is mediated by complex assembly systems in the mitochondria and cytosol. A number of cytosolic cluster assembly factors have been identified [13–14 λ], but their roles in IRP1 regulation have not been conclusively defined.

Fe-S cluster disassembly directs IRP1 back to the high IRE-affinity apo form (Figure 2) (c-aconitase does not bind RNA with high affinity). The factors that facilitate this process are low iron/heme availability, phosphorylation, and reactive oxygen and nitrogen species. The primary means of IRP1 ‘switching’ is the iron-dependent pathway. Phosphorylation of the protein can be considered an indirect manner of cluster destabilization, while the small

molecule oxides and other reactive species that appear during oxidative stress are thought to act through direct attack on the cluster. The different types of cluster perturbations can happen concurrently, and beyond just the release of apo-IRP1, can ultimately lead to IRP1 degradation [1λ].

Iron responsive elements

IRPs exert translational control by binding to IREs. IREs are conserved, ~30 nucleotide stem-loops found in the 5' or 3' UTRs of mRNA transcripts of genes related to iron metabolism. ~150 IRE homologues have been identified [15λ] that belong to the eleven unique types of IREs known to be regulated by IRPs (Figure 3a). Sequence conservation is higher within IRE types rather than within species [16]. IREs regulate the translation of key proteins involved in iron uptake, utilization, and storage [17]. Recent studies extend the link of iron metabolism to varied cellular processes such as cytoskeletal maintenance (MRCKα) [18], cell cycle control (CDC14a) [19], and oxygen sensing (EPAS1) [20]. Control of the IRP/IRE network is based on selectivity in IRP1:IRE recognition, determined by the sequence and structural variations within the IRE family.

The canonical IRE structure is an apical loop of six residues, a semi-rigid five base-pair upper helix, an interhelical junction, and a variable lower helix (Figure 3b). The highly conserved signature sequence is the unpaired C8 at the junction, followed six bases later by the CAGUGX (X = U, C, or A) residues (14–19) of the loop. The apical residues form a pseudotri-loop [21], subdivided into a terminal AGU triplet pinched off by a pairing of the C14 and G18 bases, leaving the variable position 19 unpaired (Figure 3) [22–23].

The IRE upper helix is thought to serve as a spacer between the unpaired C8 and the apical loop [24–26]. Its secondary structure is highly conserved, in spite of many base-pair interconversions. Any base pair is acceptable as long as the A-form helix is maintained [24–26]. The only deviation is the additional unpaired uracil following position 21 in EPAS1 and DMT1 (Figure 3a), which would not necessarily disrupt the helix. Modelling shows that this position is far from the IRP1 protein, and unlikely to interact.

NMR structures of two full-length IRE motifs have been determined [26–28]. The results are in general agreement with the x-ray structure of IRP1-bound ferritin H IRE [29λλ], showing the C14-G18 base pairing in the terminal loop, and A-form helical geometry for both the upper and lower helices. The solution conformations show variable relative orientations between the upper and lower helices, suggesting that the intervening junction may function as a flexible hinge [26]. Finally, note that the greatest sequence and structural variability in the IRE family is in the lower helix (Figure 3a), a possible basis for differential recognition (see below).

IRP1 as an RNA binding protein

Extensive conformational changes occur in IRP1 when it binds an IRE, compared to its closed, c-aconitase conformation (Figure 2) [29λλ]. The IRE-bound IRP1 molecule adopts an open “L” shaped conformation, approximately 25 Å wider than c-aconitase, making a two-point contact with the IRE: one centered on the extra-helical C8 of the stem, and the other on the apical loop (Figure 3b). The IRE binding sites generally coincide with the Fe-S cluster-binding region in aconitase (Figure 4), utilizing many of the same amino acids, which explains the mutually exclusive nature of the two functionalities.

The opening of the IRP1 molecule creates two new spaces in which localized rearrangements permit extensive base-specific IRE binding. Domain 4 rotates out to expose a newly formed pocket for binding the extrahelical C8, and domain 3 rotates to create a large cavity between it and domain 2 to accommodate the exposed nucleotides A15, G16, and U17 of the apical loop

(Figures 3b and 4). Each of the two sites contains about a dozen protein-RNA interactions, consistent with the picomolar affinities of IRPs for IREs.

As usual in specific protein:RNA recognition, the exposed nucleotides of the IRE stem-loop mediate most of the contacts with the protein, and helices the least. The tight upper helix of the IRE contributes only one hydrogen bond, from its backbone near the apical loop. In slight contrast, the lower helix of the IRE is more open and has more intimate contacts with IRP1, through domain 4. Since the lower helix exhibits the greatest sequence variation and departure from A-form helical geometry in terms of wobble pairs, insertions, and deletions (Figure 3a), it is likely to be a major locus for selectivity in IRE recognition.

In summary, the general points about IRP1:IRE recognition are that 1) the invariant C8 is a critical binding anchor for specificity, 2) the five base-pair upper helix is a semi-rigid spacer between C8 and the loop, 3), the apical loop is the second specificity determinant, likely to be in the same conformation for all IRP-bound IREs, 4) the variability in composition and conformation of the lower helix confers binding selectivity, and 5) the interhelix junction centered on the G7:C25 joint may provide flexibility between the upper and lower helices to adjust the interactions.

Conclusions and perspectives

IRP1 undergoes multiple modes of post-translational regulation, including cluster assembly/disassembly, functional switching, and modifications by iron, heme, phosphorylation, and reactive oxygen and nitrogen species [1λ]. This complex combination of factors signals the iron balance and stress level within the eukaryotic cell to determine the functional fate of IRP1. How the latter modifications affect IRP structure remains to be determined.

A number of questions arise concerning the molecular mechanism of the IRP1 structural transformation. What determines which conformation to assume? The large influence of the IRE RNA on the conformation of IRP1 is obvious, but does the Fe-S cluster constrain the conformation of IRP1 to the c-aconitase form? Is the apo form of IRP1 dynamic and flexible, or does it have a restricted conformation that could influence the direction of the structural transition? Neutron scattering studies [30] give radii of gyration of c-aconitase and complexed IRP1:IRE commensurate with their crystal structures, but that for apo-IRP1 was the largest of the three. Results from sedimentation velocity experiments [31] agree, showing that apo-IRP1 is less compact than the IRP1:IRE complex. Together these results suggest that a crystal structure of apo-IRP1, if obtainable, would reveal an open conformation. If so, what would be the local conformations in the ligand binding regions?

IRP2 is now an attractive target for structural investigation. As mentioned above, IRP2 exhibits an IRE selectivity profile different than IRP1, having reduced affinity for the non-ferritin-type IREs [11]. Is this due to differences in recognition of the lower stems and/or interhelical junctions of the IREs? Does the 73 amino acid insertion of IRP2 change those recognition regions? Structures of both IRPs in complex with a series of IREs could address these questions.

The remaining questions also concern IRP selectivity in IRE recognition. While IRPs bind their target IREs in the 10–100 picomolar range, translational outcomes in the cell vary up to 50-fold [32]. Is this based on structural selectivity in IRP:IRE binding? Variations in the IRE stem-loops are presumably responsible, but differences in RNA structure in the flanking regions beyond the stem-loops may also play a role, reinforcing the point that context is important in IRE function [33–34]. A prime example is the higher degree of secondary structure possible in the transferrin receptor mRNA that contain multiple IREs [34–36], which suggests that additional layers of macromolecular assembly may be at work. This may include the action of other regulators and as yet unidentified binding proteins.

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Abbreviations

c-aconitase	cytosolic aconitase
IRP	iron regulatory protein
IRE	iron responsive element
UTR	untranslated region

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Figure 1.

Schematic of translational regulation by iron and IRPs. Left: conditions of low iron permit the IRPs to bind stem-loop IREs, resulting in either translational repression (top) or activation (bottom), depending on the position and context of the IREs. Right: high iron conditions cause release of the IRPs, reversing the effects on translation.

Figure 2.

Structures of the two functional conformations of IRP1. Right: c-aconitase (PDB 2B3X and 2B3Y) [5 λ]. The Fe-S cluster is in the center of the molecule, hidden from view, accessible by solvent channels between domain 4 and the other domains. Center: cartoon of apo-IRP1, suggesting a dynamic, open conformation [30–31]. Left: IRP1:ferritin H IRE RNA complex (PDB 2IPY) [29 λ]. Domains 3 and 4 open up by ~ 25 Å relative to c-aconitase to bind the IRE. Left and right figures done with PyMOL [44].

Figure 3.

Figure 3a. Primary sequence alignment of known functional human IREs. Conventional base pairs are in regular upper case, lower case denotes unpaired bases, and italic denotes non-Watson-Crick base pairing. Numbering is for ferritin H IRE, and may differ for the other IREs due to deletions and/or insertions. Dashes represent true gaps in the alignment, while spaces are for formatting. The color scheme highlights the separate structure elements of the IRE motif: violet, lower helix; green, interhelical junction; red, highly conserved nucleotides; blue, upper helix; yellow, variable position 19 of the loop. Ftn H, ferritin H [37]; ftn L, ferritin L [38]; eALAS, erythroid aminolevulinate synthase [39]; mAco, mitochondrial aconitase [40]; FPN, ferroportin [41]; EPAS1, endothelial PAS domain protein 1 [20]; TfR C, transferrin receptor C [33]; DMT1, divalent metal ion transporter [42]; CDC14A, cell division cycle 14A [19]; and MRCK α , myotonic dystrophy kinase-related Cdc42-binding kinase α [18]. The

functional succinate dehydrogenase IRE (not shown) [43] appears restricted to the *Drosophila* genus [15λ]. The first six IREs are located in the 5' region of their mRNA transcripts; the bottom four have 3' locations.

Figure 3b. Secondary structure schematic and three-dimensional structure of ferritin H IRE (bullfrog) as bound to IRP1. Color scheme as above. Center figure done with PyMOL [44]. View is from the same side as figure 2, left.

Figure 4. Overlap of the two ligand-binding sites of the IRP1 protein. The solvent-accessible-surface is of IRP1 with bound IRE RNA, and the IRE is shown as a stick model. The 'footprint' of the IRE is colored green, while the active site residues of c-aconitase are colored red. Most of the active site residues are within the IRE footprint. Orientation approximately the same as figure 3b. Figure done with Grasp [45].