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MINI-REVIEW

An iron–sulfur cluster plays a novel regulatory role in the iron-responsive element binding protein

Tracey A. Rouault, David J. Haile, William E. Downey, Caroline C. Philpott, Careen Tang, Felipe Samaniego, Jean Chin, Ian Paul, David Orloff, Joe B. Harford & Richard D. Klausner

Cell Biology and Metabolism Branch, National Institute of Child Health & Human Development, National Institutes of Health, Bethesda, MD, USA

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Post-transcriptional regulation of genes important in iron metabolism, ferritin and the transferrin receptor (TfR), is achieved through regulated binding of a cytosolic protein, the iron-responsive element binding protein (IRE-BP), to RNA stem–loop motifs known as iron-responsive elements (IREs). Binding of the IRE-BP represses ferritin translation and represses degradation of the TfR mRNA. The IRE-BP senses iron levels and accordingly modifies binding to IREs through a novel sensing mechanism. An iron–sulfur cluster of the IRE-BP reversibly binds iron; when cytosolic iron levels are depleted, the cluster becomes depleted of iron and the IRE-BP acquires the capacity to bind IREs. When cytosolic iron levels are replete, the IRE-BP loses RNA binding capacity, but acquires enzymatic activity as a functional aconitase. RNA binding and aconitase activity are mutually exclusive activities of the IRE-BP, and the state of the iron–sulfur cluster determines how the IRE-BP will function.

Keywords: aconitase, ferritin, iron-responsive element binding protein, iron-responsive elements, iron–sulfur clusters, RNA binding

Introduction and background

Iron–sulfur proteins were first described as a distinct class of proteins in the 1960s. Biochemical characterization of iron–sulfur clusters was late in coming, even though these clusters are likely to be among the most ancient of protein modifications. Iron–sulfur clusters assemble spontaneously when elemental iron and sulfur are permitted to interact in an anaerobic environment such as that which was present early in the Earth's history. It has become clear that iron–sulfur clusters are important post-translational modifications that confer critical functions on the proteins with which they are associated. Because of the flexible redox states of iron, iron–sulfur clusters can undergo redox reactions and play important roles in electron transport (Beinert & Kennedy 1989, Beinert 1990). In addition, a class of reactions involving the iron–sulfur proteins has been defined in which redox reactions are not the feature of importance; rather, the importance stems from other roles of iron–sulfur clusters including direct liganding of enzymatic substrates (Beinert & Kennedy 1989, Switzer 1989).

Recent studies of a protein critical in iron metabolism, the iron-responsive element binding protein (IRE-BP), have revealed a novel regulatory role for iron–sulfur clusters (Haile *et al.*, 1992). The iron–sulfur cluster of the IRE-BP serves as a sensor of cellular iron levels and this information is transduced into regulation of binding of the IRE-BP to its intracellular ligand, the iron-responsive element (IRE). IREs are RNA stem–loop structures that

Address for correspondance: T. A. Rouault, Cell Biology and Metabolism Branch, National Institute of Child Health & Human Development, National Institutes of Health, Building 18, Bethesda, MD 20892, USA.

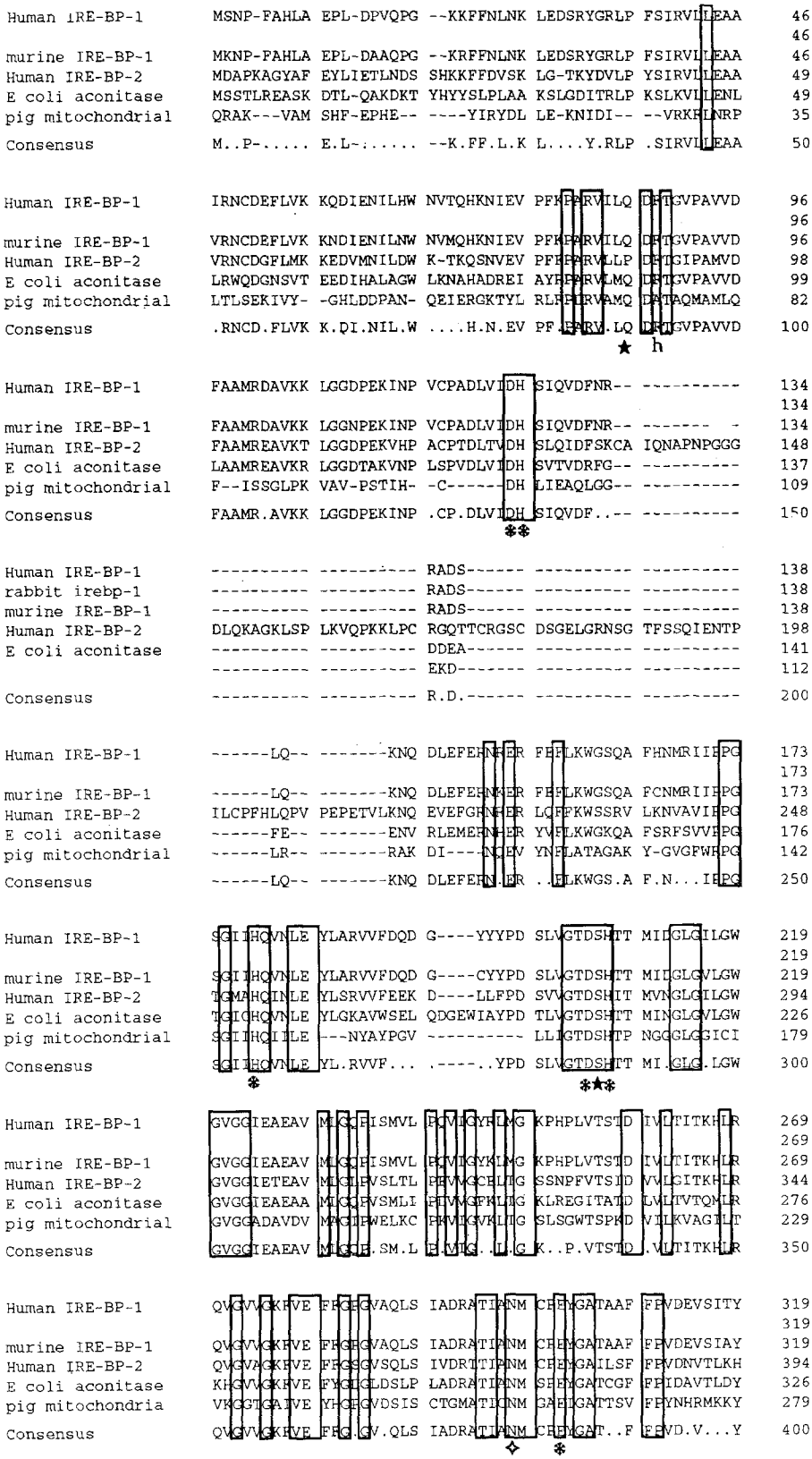

Figure 1. Sequence comparison of human IRE-BP-1 with the sequences of porcine mitochondrial aconitase, murine IRE-BP-1, IRE-BP-2 (described in text) and *E. coli* aconitase. Active site residues as identified by crystallography are denoted by symbols indicating function.

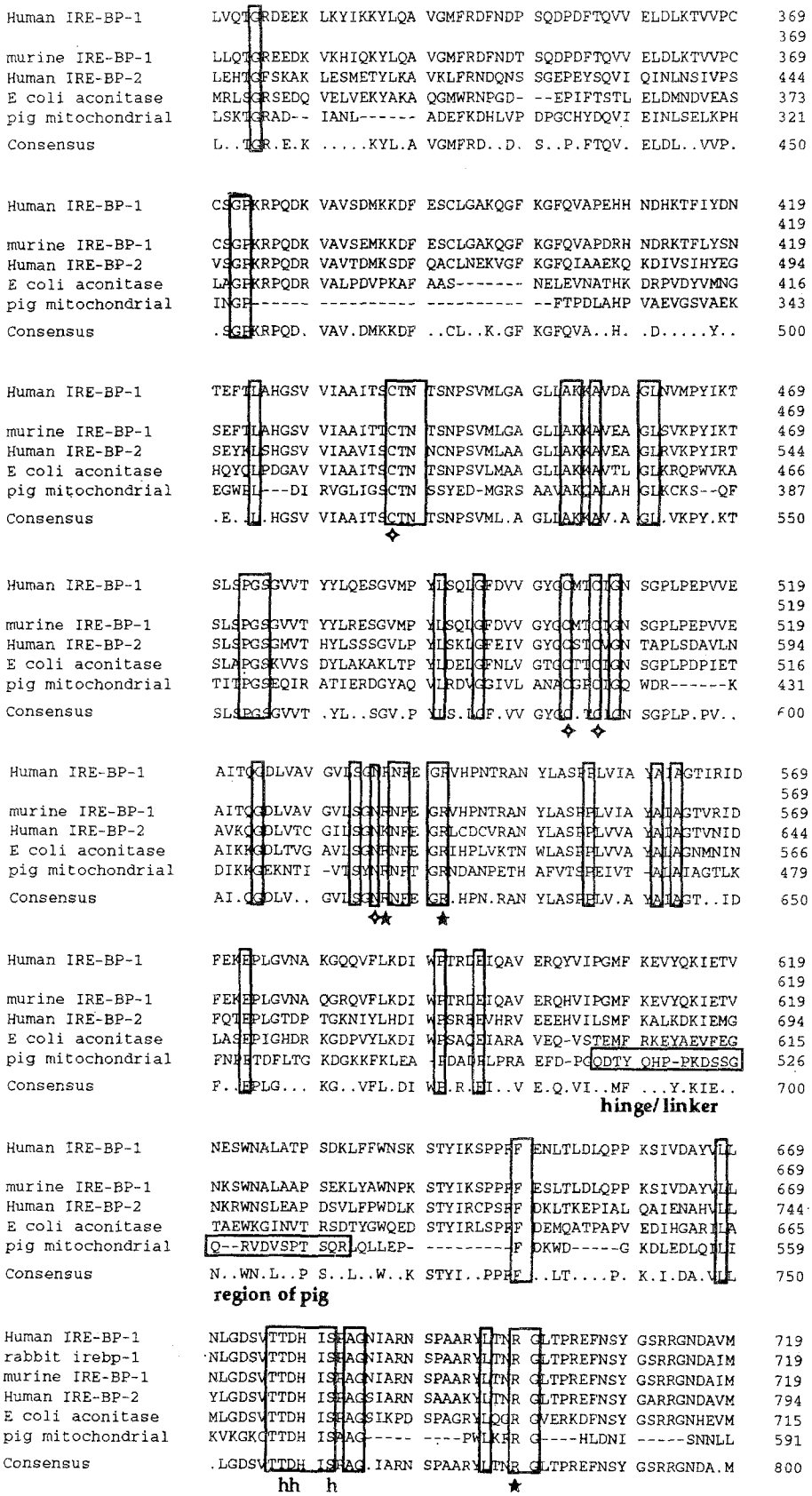

Figure 1. *Continued.*

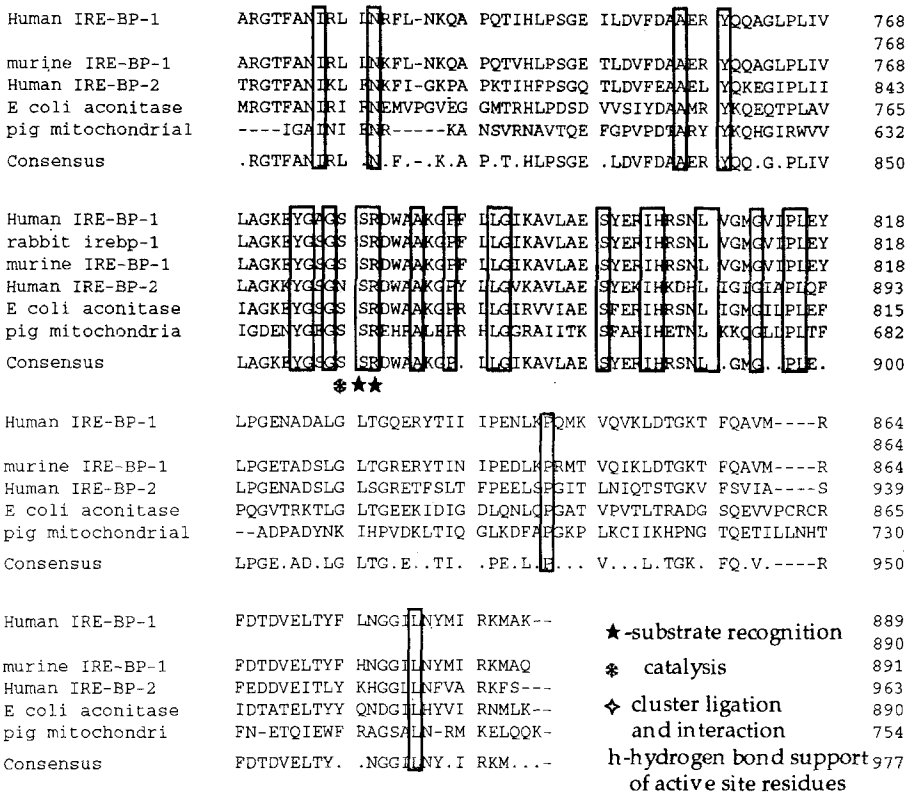

Figure 1. *Continued.*

have been identified in the mRNAs of ferritin (Aziz *et al.* 1987, Hentze *et al.* 1987), the transferrin receptor (Casey *et al.* 1988) and more recently in the mRNAs of porcine aconitase and erythrocyte 5-aminolevulinate synthase (ALA synthase) (Cox *et al.* 1991, Dandeker *et al.* 1991). The IRE in the ferritin mRNA is in the 5′ untranslated region (UTR) and its proximity to the mRNA cap site is responsible for regulation of the translation of ferritin (Hentze *et al.* 1987, Caughman *et al.* 1988, Goossen *et al.* 1990). When the cell is iron replete, there is a decrease in the amount of IRE-BP that binds IREs with high affinity, though the amount of total IRE-BP as assessed by western blotting does not change significantly (unpublished results). Furthermore, treatment with high concentrations of reducing agents *in vitro* fully restores the residual IRE binding activity in the lysate, indicating that there has not been a total loss of IRE-BP, but rather a change in the capacity of the IRE-BP to bind to RNA (Hentze *et al.* 1987, Haile *et al.* 1989, Barton *et al.* 1990). When the cell is iron-replete, translation of ferritin proceeds, an appropriate response since ferritin is a repository in the cell for excess iron and sequestration of iron within the ferritin molecule serves to protect the cell from oxidative damage caused by free iron (for review, see Theil 1990). When the cell is iron-starved the amount of IRE-BP that binds to IREs with high affinity increases and translation of ferritin is inhibited. IREs are also present in the 3' UTR of the transferrin receptor (TfR) and function in stabilization of the mRNA of the TfR when the cell is iron-starved, leading to increased expression of the TfR and increased acquisition of iron from the extracellular milieu (Casey *et al.* 1989). IREs are the elements that permit regulation of expression of transcripts in which they are present, but the IRE-BP is the critical determinant of regulation (for review, see Klausner & Harford 1989).

The IRE-BP is a cytosolic iron–sulfur cluster protein that is highly related to mitochondrial aconitase

While the dependance of the IRE/IRE-BP interactions on cellular iron status was known from gel-retardation assays (Rouault *et al.* 1988, Haile *et al.* 1989), the mechanism of iron-sensing was unclear. Initial clues came from a remarkable sequence similarity between the IRE-BP and mitochondrial aconitase (Hentze & Argos 1991, Rouault *et al.* 1991), a Krebs cycle enzyme that has been purified,

cloned (Gangloff *et al.* 1990, Zheng *et al.* 1990) and crystallized (Robbins & Stout 1989a,b, Lauble *et al.* 1992). Biochemical studies and spectroscopic analyses have demonstrated that mitochondrial aconitase contains a [4Fe–4S] cluster which is critical to its enzymatic activity. A key feature in the function of mitochondrial aconitase is that the fourth iron of a cubane iron–sulfur cluster provides two covalent bonds to citrate through binding of carboxyl and hydroxyl groups of the substrate (Beinert & Kennedy 1989, Lauble *et al.* 1992). The enzymatic stereospecific dehydration and hydration of citrate to form isocitrate is negligible in the absence of the fourth iron (Surerus *et al.* 1989, Beinert & Kennedy 1990).

Thus, presence of full aconitase function implies that the iron–sulfur cluster contains four irons. Sequence comparison of the IRE-BP to the sequence of mitochondrial aconitase strongly implied that the IRE-BP would be a functional aconitase because, although the amino acid homology was only 30%, 18 active site residues were identical (Rouault *et al.* 1991) (Figure 1). After cloning and expression of the IRE-BP, it is now clear that the IRE-BP is a functional aconitase (Kaptain *et al.* 1991). For many years it was known that there was also a form of aconitase present in human cytoplasm (Shows & Brown 1977). It migrated differently from the mitochondrial form on starch gels and was known to be a marker for chromosome 9, whereas the mitochondrial aconitase gene was localized to chromosome 22 (Shows & Brown, 1977). The IRE-BP has been localized to chromosome 9 (Rouault *et al.* 1990) and probably represents the predominant, if not sole, previously described cytosolic aconitase.

Iron-dependant changes in aconitase activity of the IRE-BP are inversely correlated with changes in RNA binding activity

Aconitase activity of the IRE-BP serves as a signature of the [4Fe–4S] state of the iron–sulfur cluster of the IRE-BP. In order to use this information to gain insight into *in vivo* regulatory mechanisms, a murine fibroblast cell line was developed that stably expressed the human IRE-BP (Haile *et al.* 1992). The human IRE-BP was tagged with an epitope such that it could be readily immunoaffinity purified (Kaptain *et al.* 1991). Since the human IRE-BP/IRE complex migrates differently from murine IRE-BP/IRE complexes in gel-retardation assays, correlations could be made between RNA binding activity of the human IRE-BP and aconitase function after *in vivo* manipulation of cells that were either iron loaded or iron starved. These experiments demonstrated that treatment with iron produced IRE-BP that was a fully active aconitase, but which bound IREs poorly (Figures 2–4). Conversely, iron starvation produced protein that bound IREs with high affinity, but which lacked aconitase activity. Thus, the iron switch that promotes concomitant repression of ferritin translation and decrease in TfR degradation apparently depends on the state of the iron-sulfur cluster of the IRE-BP.

Mechanisms by which alterations in the iron–sulfur cluster may alter functions of the IRE-BP

How might a change in the iron–sulfur cluster potentiate RNA binding activity? One option might involve a local conformational change in the region

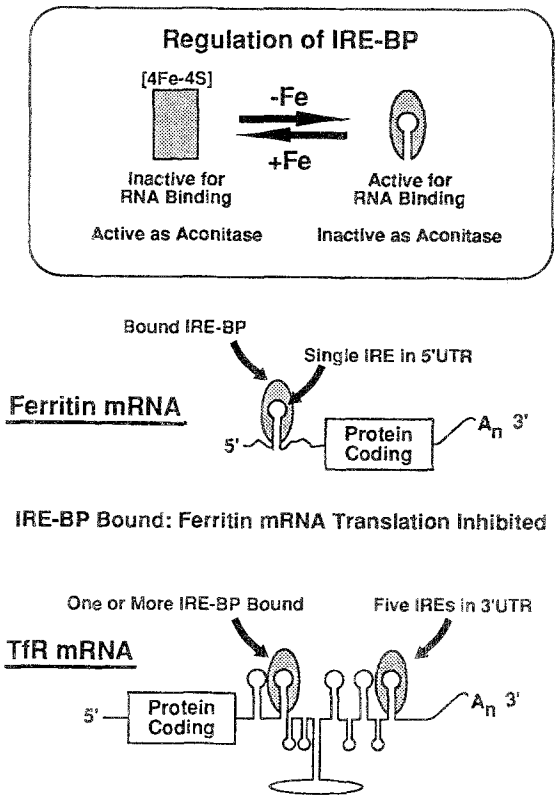

Figure 2. Coordinate regulation of ferritin and TfR biosynthesis is achieved through alteration of the iron–sulfur cluster of the IRE-BP. IRE-BP that is active for RNA binding simultaneously represses translation of ferritin and degradation of the TfR.

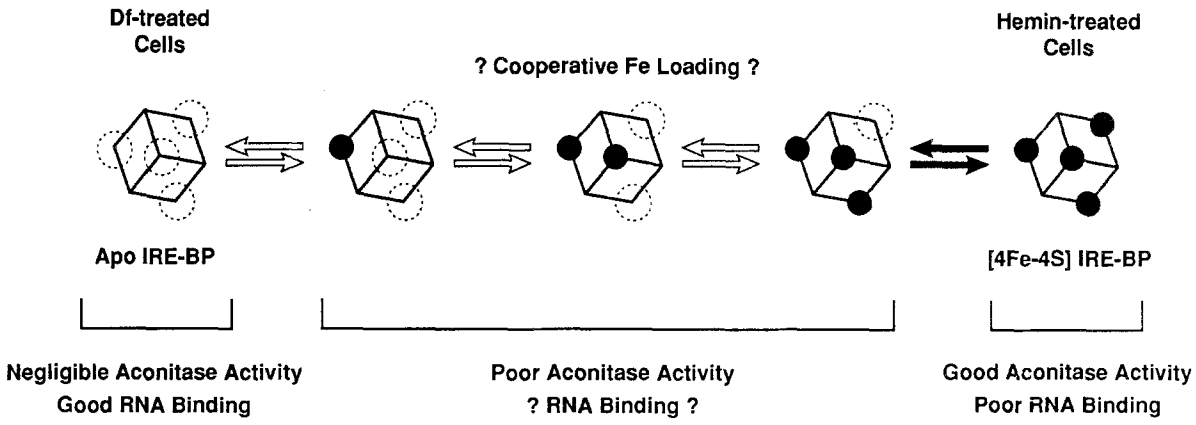

Figure 3. Aconitase activity/RNA binding model.

of the iron–sulfur cluster that unmasks previously unavailable RNA contact sites. Another might be that in the absence of ligand, a large conformational change takes place that opens an entirely new binding site for IREs. The crystal structure of mitochondrial aconitase is interesting in this regard. Aconitase consists of four domains, three of which are intimately associated and a fourth that is connected to the others by a hinge/linker. Large conformational changes have been previously shown to occur as a result of motion in the region of hinge/linkers (Dobson, 1990, Faber & Matthews, 1990). In fact, a closely related bacterial protein, the isopropylmalate isomerase of *Salmonella typhimurium*, consists of two subunits encoded by two separate genes, the first of which encodes the first three domains and the second of which encodes the fourth domain which then associates as a part of a heterodimeric enzyme complex (Rosenthal & Calvo 1990, Prodromou *et al.* 1992). Clearly, the fourth domain is a stable entity in the absence of the first three domains in the isopropylmalate isomerase of *S. typhimurium*. Studies of interactions of the first three domains of mitochondrial aconitase and the fourth domain suggest that interactions between the fourth domain and the first three are concentrated largely in the region of substrate binding and an immediately adjacent site (C. D. Stout, unpublished observations). The contact surfaces between the fourth domain and the rest of the molecule contain dozens of trapped water molecules in mitochondrial aconitase and the same basic structure is probably also a feature of the IRE-BP, although the IRE-BP has not yet been crystallized. Thus, the opening of the cleft by a motion about the hinge region and complete exposure of the residues in the cleft to solvent would be energetically favorable. It might be that in the absence of substrate, the fourth domain would no longer be in close apposition to the first three domains as it is in the crystal structure, and regions along the cleft might be available for binding of the IRE. Such a mechanism would be compatible with the observation that aconitase activity and IRE-binding are mutually exclusive properties of the IRE-BP (Haile *et al.*, 1992).

Aconitases and IRE-BPs from other species

The recently cloned aconitase from *Escherichia coli* (Prodromou *et al.* 1992) is 53% identical to the IRE-BP and 30% identical to porcine mitochondrial aconitase, suggesting that it represents the progenitor of both the eukaryotic mitochondrial aconitase and the IRE-BP (Figure 1). The mitochondrial aconitase does not appear to bind IREs (unpublished observations) and it will be interesting to determine whether the *E. coli* aconitase can bind IREs.

The human and rabbit forms of the IRE-BP have been purified (Rouault *et al.* 1989, Walden *et al.* 1989, Neupert *et al.* 1990) and cloned (Rouault *et al.* 1990, Philpott *et al.* 1991, Hirling *et al.* 1992, Walden *et al.*, in press) along with the bacterial aconitase from *Bacillus subtilis* (Dingman & Sonenshein 1987). Thus far, attempts to find a counterpart to the IRE-BP in yeast have been unsuccessful. Specific binding of protein to IREs has been observed in numerous diverse species including *Drosophila melanogaster*, *Xenopus laevis* and the worm *Tubifex tubifex* (Rothenburger *et al.* 1990). The IRE-BP has also been referred to by the names ferritin repressor protein (Walden *et al.* 1989) and the iron regulatory factor (Mullner *et al.* 1989).

A protein related to IRE-BP termed IRE-BP-2 was cloned incidentally when IRE-BP (IRE-BP-1)

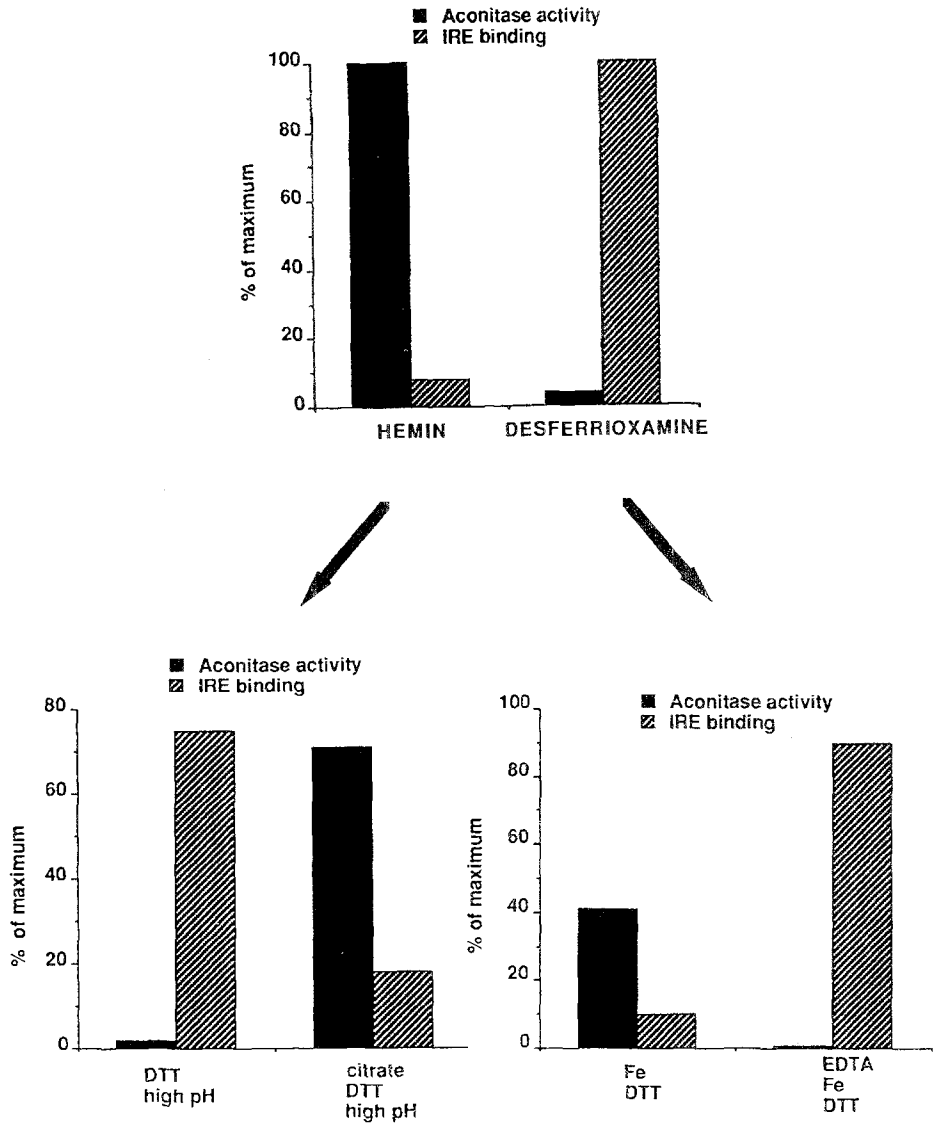

Figure 4. Iron loading of cells versus treatment with iron chelators. There is reciprocal regulation of RNA binding and aconitase activity in IRE-BP purified from treated cells. When IRE-BP from iron-treated cells is subjected to *in vitro* manipulations thought to disrupt iron–sulfur clusters, the aconitase activity is lost and RNA binding activity is gained. This change in phenotype is largely inhibited when aconitase substrate is present before manipulation. When IRE-BP from iron-starved cells is treated with reducing agents and iron, the IRE-BP becomes an active aconitase and loses RNA binding. This change in phenotype is prevented if the iron chelator EDTA is present during treatment, thus showing that iron is critical to the transition in becoming an active aconitase.

was cloned (Rouault *et al.* 1990) and the protein also binds IREs with high affinity (unpublished observations). The mRNA for IRE-BP-2 is distributed throughout all tissue types studied to date and the role the IRE-BP-2 plays in iron regulation is not known.

Speculations and future directions

An interesting feature of the IRE-BP is that it is a relatively abundant protein with approximately 100 000 copies per cell (Haile *et al.* 1989). It is not clear how many target IREs are present in eukaryotic cells at this time, though the list of potential binding targets is growing (Cox *et al.* 1991, Dandekar *et al.* 1991). A relatively abundant iron–sulfur protein might offer an answer to a long standing question as to the major source of chelatable iron in the cytoplasm. It has long been known that iron enters an undefined pool after it has traversed the membrane of the endosome into the cytosol of the cell (Rouault *et al.* 1985). Low

molecular weight compounds have been considered to be candidate binding compounds (Weaver & Pollack, 1989), but it is not clear if these compounds can maintain a sufficient repository of iron in a soluble available state (Crichton, 1991). Perhaps the relatively abundant IRE-BP serves as a major source of chelatable iron as a result of its reversible capacity for binding iron. Increased cellular utilization of iron would result in iron loss from the IRE-BP. Thus, the IRE-BP would be uniquely poised to sense iron levels, since it would donate iron from the iron–sulfur cluster and the iron depleted IRE-BP would then meditate appropriate compensatory changes in iron metabolism.

The aconitase of *E. coli* is inactivated by oxidation and it has been postulated that it is among the enzymes most sensitive to the damage of oxidative stress (Gardner & Fridovich, 1991). Dysfunction of aconitase slows generation of electrons by the Krebs cycle and may slow generation of damaging superoxides. Thus, as a sensor of oxidative stress, the iron–sulfur cluster of aconitase may help protect the cell from oxidative damage.

Many questions remain. Are the IREs present in the 5′ UTR of porcine aconitase and erythrocyte ALA synthase important in the regulation of the expression of the proteins? What is the role of aconitase activity in the regulatory function of the IRE-BP? Is enzymatic function crucial to regulation of RNA binding? These questions remain to be answered.

There are conflicting data relating to the question of whether heme plays a significant role in the *in vivo* regulation of IRE-BP function (Eisenstein *et al.* 1991, Haile *et al.* 1990, Lin *et al.* 1990, 1991), although pulse–chase experiments followed by immunoprecipitation of the epitope tagged IRE-BP (unpublished observations) are not in agreement with the recent report (Goessling *et al.* 1992) that there is significantly increased degradation of the IRE-BP in response to treatment of cells with heme compounds.

Another important question involves definition of the RNA binding site and identification of the underlying mechanisms that lead to changes in the iron–sulfur cluster from [4Fe–4S] to disruption of the full cluster in iron-deprived cells. Previously defined RNA consensus binding motifs (Mattaj 1989, Query *et al.* 1989) are not present in the sequence. To date, an enzyme that facilitates assembly of iron–sulfur clusters has not been identified. The mechanism of iron–sulfur cluster assembly remains a major mystery. Through manipulations of pH, reducing agents and iron salts, it is clear that the

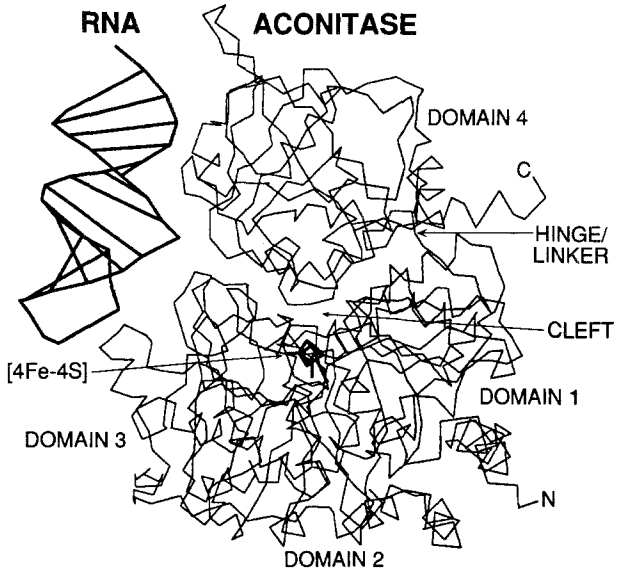

Figure 5. Crystal structure of aconitase compared with a model for the IRE drawn to scale to illustrate the relative sizes of the IRE and the IRE-BP, which is 15 kDa larger than the 83 kDa mitochondrial aconitase depicted here. The hinge/linker that connects domain 4 to the first three domains is shown, along with the iron–sulfur cluster. Only the phosphorous atoms of the IRE are shown and the orientation relative to the IRE-BP is random.

binding state and aconitase activity of the IRE-BP can be stably interconverted (Haile *et al.*, 1992) (Figure 4). However, these manipulations involve conditions and/or concentrations of reagents that are unlikely to be achieved within the cell and it is likely that the cell uses considerably more subtle means to effect changes in the protein. For this reason, an important goal is to recreate regulatory events in an *in vitro* system using purified protein and cellular components.

What are the broad implications of this research? Foremost is the implication that the iron–sulfur clusters will prove to be regulatory switches in diverse and as yet undescribed systems (Thomson 1990). Perhaps this system will be the vehicle through which insight is gained into iron–sulfur cluster formation. Further insights may be gained into the important issues of regulated mRNA degradation and translation. Perhaps, the intracellular distribution system for iron will also be more fully defined and understood.

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