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Cellular regulation of the iron-responsive element binding protein: Disassembly of the cubane iron-sulfur cluster results in high-affinity RNA binding

[aconitase (in)activation/substrate protection/thiol effect]

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ABSTRACT **The translation of ferritin mRNA and degradation of transferrin receptor mRNA are regulated by the interaction of an RNA-binding protein, the iron-responsive element binding protein (IRE-BP), with RNA stem–loop structures known as iron-responsive elements (IREs) contained within these transcripts. IRE-BP produced in iron-replete cells has aconitase (EC 4.2.1.3) activity. The protein shows extensive sequence homology with mitochondrial aconitase, and sequences of peptides prepared from cytosolic aconitase are identical with peptides of IRE-BP. As an active aconitase, IRE-BP is expected to have an Fe-S cluster, in analogy to other aconitases. This Fe-S cluster has been implicated as the region of the protein that senses intracellular iron levels and accordingly modifies the ability of the IRE-BP to interact with IREs. Expression of the IRE-BP in cultured cells has revealed that the IRE-BP functions either as an active aconitase, when the cells are iron-replete, or as an active RNA-binding protein, when the cells are iron-depleted. We compare properties of purified authentic cytosolic aconitase from beef liver with those of IRE-BP from tissue culture cells and establish that characteristics of the physiologically relevant form of the protein from iron-depleted cells resemble those of cytosolic aconitase apoprotein. We demonstrate that loss of the labile fourth iron atom of the Fe-S cluster results in loss of aconitase activity, but that more extensive cluster alteration is required before the IRE-BP acquires the capacity to bind RNA with the affinity seen *in vivo*. These results are consistent with a model in which the cubane Fe-S cluster is disassembled when intracellular iron is depleted.**

The regulation of proteins of iron metabolism in complex eukaryotes has provided a model system for the study of the role of specific RNA binding proteins in posttranscriptional gene regulation. Ferritin is translationally regulated in response to changes in iron availability (1–4). Translational regulation of ferritin requires the presence of a specific stem–loop structure referred to as an iron-responsive element (IRE) located near the 5′ end of the 5′ untranslated region (5). IREs have also been shown to have functional importance in a region of the 3′ untranslated region that mediates the iron-dependent control of the half-life of transferrin receptor mRNA (6, 7). The IREs in both of these mRNAs function as recognition sites for binding of a cytosolic protein known as the IRE binding protein (IRE-BP) (8, 9). The RNA-binding capacity of the IRE-BP is altered in response to changes in iron availability (10), implicating this cytosolic protein as an essential iron sensor/regulator in the cell. The IRE-BP has high affinity for IREs when the cell is depleted of iron, and this RNA-binding capacity is reduced when the cell is iron-replete (10).

The IRE-BP was purified (11–13), and cDNAs encoding the protein were cloned (14–18). An impressive similarity between the sequence of this protein and that of mitochondrial aconitase suggested a possible mechanism by which the IRE-BP could sense iron availability (19, 20). Aconitase contains a labile Fe-S cluster that has been shown to be readily interconverted *in vitro* between cubane [4Fe-4S] and [3Fe-4S] forms (21). By analogy, the IRE-BP might contain a labile Fe-S cluster that could provide a reversible binding site for iron. That the IRE-BP is an active aconitase was suggested by the observation that, although the overall level of sequence identity was only 30%, active-site residues of mitochondrial aconitase were identical in the IRE-BP (19). Recent work has provided evidence that the protein cycles between an iron-replete functional aconitase, which does not bind IREs, and a form in which the Fe-S cluster is altered, and enzymatic activity is lost while RNA-binding ability is acquired (22). However, we were not able to determine whether the induction of the RNA-binding state of the IRE-BP was due to the loss of the labile fourth iron (Fe_a) or to more extensive cluster disassembly. Here we present evidence that the removal of Fe_a is not sufficient to reproduce the characteristics of the RNA-binding form of the protein isolated from cells depleted of iron or produced by manipulation of the cluster *in vitro*. Rather, cytosolic aconitase apoprotein most closely approximates defined characteristics of the RNA-binding form of the protein isolated from iron-depleted cells, thus suggesting a more extensive structural change of this Fe-S cluster than has previously been suspected (19, 20). These results imply that the apoprotein is the physiologically relevant form of the protein in iron-depleted cells.

MATERIALS AND METHODS

Cells and Protein Purification. K-562 cells (human erythroleukemia cell line) and H/IRE-BP-1 cells (mouse fibroblasts expressing a Myc epitope-tagged human–mouse chimeric IRE-BP) used in this study were maintained and treated with hemin and desferrioxamine (Df) (6, 22), and cytosolic lysates were prepared as described (6). The protein so derived will be referred to as IRE-BP. Cytosolic aconitase was purified from beef liver and characterized as described in the preceding paper (23), and the different forms of the enzyme (4Fe, 3Fe, and apo) were prepared as described (21, 24).

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Abbreviations: IRE, iron-responsive element; IRE-BP, IRE binding protein; Df, desferrioxamine.

Gel Retardation Assays: Effects of Substrate and Reductant. Radiolabeled RNA was synthesized and the gel retardation assay with cell lysates was done as described previously (10). Purified cytosolic aconitase from bovine liver was dissolved at a final concentration of 3.5 ng/20 μl in 25 mM Tris·HCl, pH 7.4/40 mM KCl/0.05% bovine serum albumin. IRE RNA (0.5–1 ng) was added to 20 μl of this solution prior to the gel retardation assay. The amount of RNA bound per mole of IRE-BP (3.5 ng per point, or 36 fmol) (23) was quantitated by scintillation counting of the band representing the IRE/IRE-BP complex in a nondenaturing polyacrylamide gel. The number of moles of IRE present was determined after calculation of the specific activity of the IRE and direct scintillation counting. The effects of aconitase substrates on RNA binding of the IRE-BP were studied by addition of substrates to lysates for 5 min at room temperature prior to addition of radiolabeled RNA and/or 2-mercaptoethanol.

Methods for Effecting Cluster Disassembly. For ferricyanide titrations, cytosolic lysate (10 mg) was immunoprecipitated (22) in the presence of 1 mM sodium citrate. Immunoprecipitated IRE-BP bound to Sepharose through binding of a monoclonal antibody to a Myc epitope present at the 3′ terminus of the protein (22) was washed four times with 25 mM Tris·HCl, pH 7.4/40 mM KCl. The Sepharose resin was divided into 15 equal portions of ≈100 μl of packed beads each and treated for 5 min on ice with potassium ferricyanide at concentrations ranging from 0.1 nM to 5 mM in buffer (final volume, 200 μl) containing 100 μM EDTA in a 200-μl volume. The beads were subsequently washed three times with buffer containing 100 μM EDTA. The first wash also had 0.01% 2-mercaptoethanol added to the buffer. RNA binding and aconitase activity were measured as described (22).

RESULTS

RNA-Binding Properties of the 4Fe, 3Fe, and Apo IRE-BP. The purification of bovine liver cytosolic aconitase, using spectroscopy and aconitase assays to evaluate purification steps, is described in the preceding paper (23). Partial peptide sequencing of the purified protein (23) confirmed that bovine liver cytosolic aconitase and the human liver IRE-BP are essentially identical. The purification allowed the preparation of three different forms of the protein, one containing a [4Fe-4S] cluster (4Fe), one containing a [3Fe-4S] cluster (3Fe), or a protein entirely lacking iron (apoprotein). The 4Fe, 3Fe, and apo forms of the cytosolic aconitase were examined for enzymatic activity and by spectroscopy. The purity of the protein was affirmed by SDS/PAGE (23). All three forms of the protein had low RNA-binding capacity after purification (Fig. 1). RNA binding could be induced in all three by 2% 2-mercaptoethanol, and there was no discernible difference in the affinity or specificity of RNA binding among the three forms of purified aconitase when treated with 2-mercaptoethanol. Quantitative gel retardation assays led to an estimate of 0.3–0.5 mol of RNA bound per mol of protein, suggesting that either 30–50% of the protein in the preparation was functional or that the protein may be a dimer with a single RNA binding site, although there is no evidence indicating that the IRE-BP functions as a dimer. When the IRE-BP was assayed in cell lysates, only the protein derived from iron-replete cells required reducing agents to activate RNA binding, whereas the IRE-BP derived from iron-deprived cells bound RNA without *in vitro* activation. Thus, none of the three forms of purified cytosolic aconitase appeared to mimic the RNA-binding characteristics of IRE-BP in lysates of iron-deficient cells. We have previously observed that the RNA-binding form of the IRE-BP from iron-depleted cells can be reversibly inactivated for RNA binding *in vitro* by exposure to oxidizing agents (25), and therefore suspected that oxidation, which was used in the preparation of the 3Fe and apo forms, might eliminate RNA-binding ability in these forms of the protein. If this were the case, it would clearly point to a site, other than the cluster, the oxidation of which abrogates RNA-binding ability.

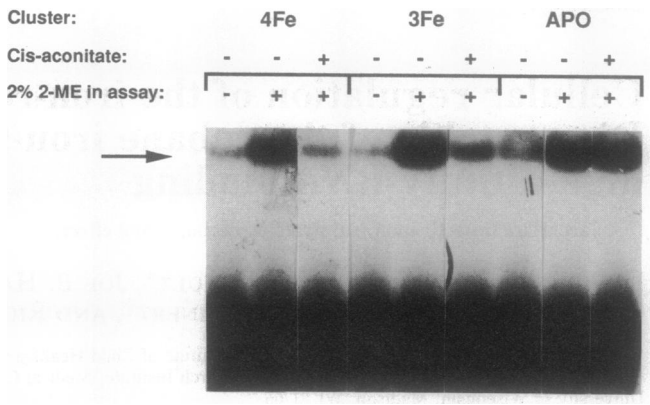

FIG. 1. Effect of aconitase substrates on induction of RNA binding to 4Fe, 3Fe, and apo forms of the protein with 2% 2-mercaptoethanol (2-ME). Purified cytosolic aconitase (5 ng) was assayed by gel retardation using 1 ng of radiolabeled IRE RNA in the presence or absence of 5 mM *cis*-aconitate and/or 2% 2-ME as indicated. Arrow indicates the position of the RNA–protein complex. Unbound RNA migrates to the bottom of the gel.

Ability of Apo, 3Fe, 4Fe Forms of Liver Aconitase to Interact with RNA: Role of Substrate and Reductant. To correlate the characteristics of spectroscopically characterized cytosolic aconitase with properties of the IRE-BP obtained from lysates of cells subjected to iron manipulations, we examined a different defining characteristic of the RNA-binding properties of the IRE-BP that we have recently described (22): the ability of aconitase substrates to inhibit the *in vitro* activation of the RNA binding of the [4Fe-4S] form of the IRE-BP isolated from iron-replete cells by treatment with high concentrations of reductants. We therefore asked whether the presence of substrates would inhibit the induction of RNA binding of the three forms of the purified protein, in order to determine which form of the cytosolic aconitase shared features of the IRE-BP from iron-depleted cells (Fig. 1). The induction of RNA-binding activity of the [4Fe-4S] form was completely abolished by the inclusion of either citrate or *cis*-aconitate. Similar effects were observed for the [3Fe-4S] form, consistent with the fact that cytosolic aconitase binds substrate tightly when the cluster has either three or four Fe atoms (23). In contrast, there was no effect of substrate on the 2-mercaptoethanol-induced activation of RNA binding of the apoprotein.

Comparison of the Spectroscopically Characterized Cytosolic Aconitase with IRE-BP from Iron-Manipulated Cells. To compare the different forms of the c-aconitase with the IRE-BP derived from iron-depleted cells, whose cluster status had not been determined, we added substrate to lysates of Df- or hemin-treated cells and evaluated RNA binding. The RNA binding of the IRE-BP in lysates from cells treated with Df did not show inhibition by substrate in the absence (Fig. 2) or presence (data not shown) of 2% 2-mercaptoethanol. In contrast, IRE-BP in lysates from cells that had been treated with hemin could be induced to bind RNA only in the presence of 2% 2-mercaptoethanol, and induction of RNA binding was blocked by the presence of substrate (Fig. 2). To compare the binding characteristics of the IRE-BP from Df-treated cells and the forms of the protein from the bovine liver preparation, the lysate was treated with high concentrations (5 mM) of ferricyanide before the RNA-binding activity of the IRE-BP was assessed (Fig. 3). Ferricyanide treatment abolished the IRE-binding activity of the

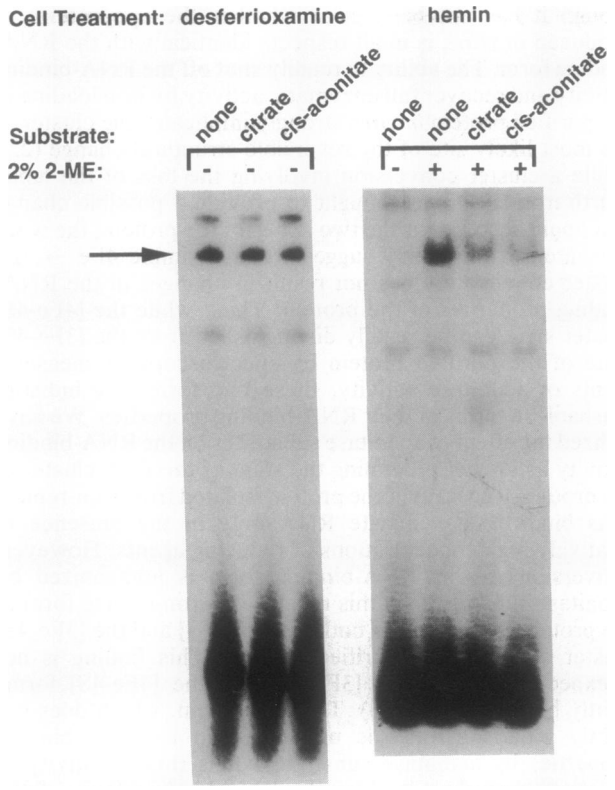

FIG. 2. Effect of aconitase substrates on RNA binding of the IRE-BP from hemin- and Df-treated cells. Cytosolic lysate (5 μg) was used in the gel retardation assay with 0.25 ng of IRE RNA probe. Citrate or *cis*-aconitate (5 mM) was added as indicated to the lysate prior to addition of 2-mercaptoethanol (2-ME) and/or radiolabeled IRE RNA.

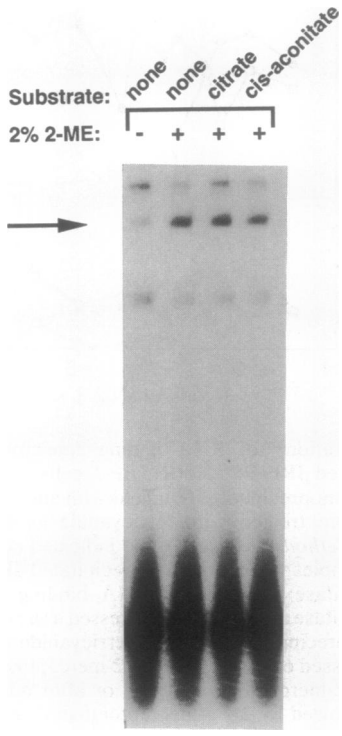

FIG. 3. Effect of oxidation of IRE-BP by ferricyanide on the inhibition by aconitase substrates of RNA binding by IRE-BP from Df-treated cells. Cytosolic lysate (100 μg) from Df-treated cells was treated with 10 mM potassium ferricyanide for 10 min at room temperature. The sample was subsequently desalted on a Sephadex G-50 spin column and 5 μg of this preparation was assayed with or without 2% 2-mercaptoethanol (2-ME) by gel retardation using IRE RNA in the presence or absence of the indicated aconitase substrates (5 mM).

IRE-BP in lysates of cells treated with Df. RNA binding was restored by treatment with 2-mercaptoethanol, and inclusion of aconitase substrates did not inhibit the activation of RNA-binding activity. Only the aconitase apoprotein shared this property (Table 1). Oxidative inactivation of RNA binding by 5 mM ferricyanide could be reversed by 0.02% 2-mercaptoethanol, 100-fold less than was required to activate the IRE-BP derived from hemin-treated cells (data not shown).

Removal of the Fourth Iron of the IRE-BP Cluster Does Not Produce the RNA-Binding Form of the Protein. We have recently demonstrated that removal of iron from the fully assembled cluster can result in activation of the RNA-binding capacity *in vitro*. Disassembly of the Fe-S cluster was accomplished by treatment with high concentrations of reducing agents at pH 8.9. To assess whether the removal of only the fourth cluster iron, Fe_a (24), was sufficient for induction of RNA binding, we gradually degraded the 4Fe cluster of the IRE-BP by titration with ferricyanide (Fig. 4). Previous work had shown that at low concentrations of ferricyanide, Fe_a was removed, and only with much higher levels of ferricyanide was the 3Fe cluster also lost with formation of the apoprotein. The source of the [4Fe-4S] IRE-BP was cells treated with hemin. As expected, this IRE-BP had no RNA-binding capacity and was an active aconitase. Addition of increasing concentrations of ferricyanide led to the complete loss of aconitase activity between 1 nM and 100 nM ferricyanide. That this was due to the loss of cluster iron was supported by the fact that the enzyme activity could be restored by established iron loading procedures (data not shown). Samples were also assessed for RNA-binding ability under three conditions: (*i*) 0.02% 2-mercaptoethanol, which will induce binding of the ferricyanide-treated IRE-BP derived from Df-treated cells, but not from hemin-treated cells; (*ii*) 2% 2-mercaptoethanol, which will induce RNA binding of all forms of the IRE-BP; and (*iii*) 2% 2-mercaptoethanol in the presence of 1 mM *cis*-aconitate, which will inhibit the induction of RNA binding of the 4Fe and 3Fe forms of the IRE-BP but not of the apoprotein (see Table 1 for summary). The exact effect of 2% 2-mercaptoethanol on the cluster has not been determined, although it is clear that this treatment does not result in cluster extrusion (22). The inactivation of the IRE-BP aconitase activity with 100 nM ferricyanide produced IRE-BP that did not bind RNA and could not be

Table 1. Comparison of the properties of purified cytosolic aconitase with cellular forms of the IRE-BP

Protein	Aconitase activity	RNA binding in presence of 2% 2-ME	
		− substrate	+ substrate
Pure aconitase			
Apoprotein	−	+	+
[3Fe-4S]	−	+	−
[4Fe-4S]	+	+	−
Cytosol			
Df-treated cells	−	+*	+
Hemin-treated cells	+	+	−

The spectroscopically characterized forms of the protein are shown with accompanying features. Following Df or hemin treatment of H/IRE-BP-1 cells, the IRE-BP was immunoprecipitated as described under *Materials and Methods*. Aconitase activity was measured in all preparations as described (21, 22). RNA binding in the presence of 2% 2-mercaptoethanol (2-ME) was assessed by gel retardation assays (10) with or without substrate (5 mM *cis*-aconitate).
*Sample does not require 2% 2-ME for RNA binding.

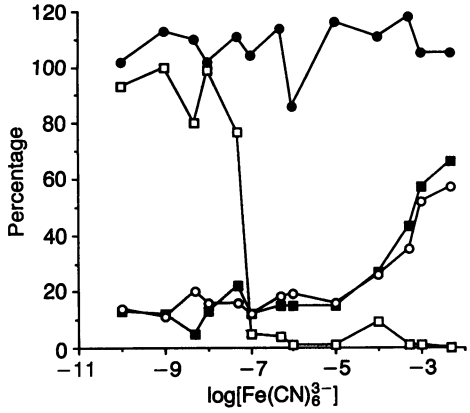

FIG. 4. "Titration" of RNA-binding capacity and aconitase activity of purified IRE-BP. H/IRE-BP-1 cells were treated with hemin, and immunoprecipitates obtained with anti-Myc plus protein A-Sepharose were treated with ferricyanide as described under *Materials and Methods*. At each of the indicated concentrations of ferricyanide, samples of the immunoprecipitated IRE-BP were assessed for aconitase activity and RNA binding under different conditions. Aconitase activity (□) is expressed as a percentage of that seen in immunoprecipitations without ferricyanide treatment. RNA binding was assessed either with 0.02% 2-mercaptoethanol (○), after addition of 2% 2-mercaptoethanol (●), or after addition of 1 mM *cis*-aconitate followed by 2% 2-mercaptoethanol (■). The full RNA binding across the ferricyanide titration is expressed as a percentage of the average of these values. Values for RNA binding with 0.02% 2-mercaptoethanol addition or after addition of *cis*-aconitate plus 2% 2-mercaptoethanol are expressed as a percentage of the full RNA binding seen with 2% 2-mercaptoethanol alone.

induced to bind RNA by treatment with 0.02% 2-mercaptoethanol. RNA-binding capacity of the same preparation of IRE-BP was induced by treatment with 2% 2-mercaptoethanol, but the induction of RNA binding was completely blocked by *cis*-aconitate. At ferricyanide concentrations > 0.1 mM, induction of RNA-binding activity in the presence of 0.02% 2-mercaptoethanol was observed. After treatment with 0.1 mM ferricyanide, substrate did not antagonize RNA binding in the presence of 2% 2-mercaptoethanol. As with the recovery of aconitase activity after inactivation with low concentrations of ferricyanide, the effects of these high concentrations of ferricyanide were reversed by the addition of iron plus reducing agents to reconstitute the Fe-S cluster (data not shown). The data of the ferricyanide titration experiment are consistent with a loss of aconitase activity after removal of the fourth iron atom, and acquisition of RNA-binding ability only after more extensive cluster disassembly.

DISCUSSION

Recent evidence has implicated the Fe-S cluster of the IRE-BP as the site that responds to changes in the amount of iron available to the cell (22, 26, 27). The form of the protein isolated from iron-replete cells possesses a [4Fe-4S] cluster as indicated by the presence of aconitase activity. The IRE-BP from iron-replete cells has not been directly characterized by spectroscopy because of limitations in the amount that can be obtained, but the presence of aconitase activity has been shown to depend on the presence of the fourth iron atom in the [4Fe-4S] clusters (21). *In vitro* manipulations of IRE-BP under conditions that are known to affect the Fe-S cluster of aconitase modulate RNA binding (22, 27). The reciprocal modulation of aconitase activity and RNA binding *in vivo* and *in vitro* has recently been directly demonstrated (22). Several studies of IRE binding have already suggested that apoprotein can bind RNA (12, 16), though it has not been clear whether the apoprotein, as produced *in vitro*, is in all respects identical with the RNA-binding form. The ability to readily shut off the RNA-binding activity and recover full enzymatic activity by iron loading of the purified protein *in vitro* strongly implicates the cluster as the most likely site of the reversible structural change (22). While a cluster conversion involving the loss of the labile fourth iron (Fe_a) was thought to provide a possible change that could account for the two states of the protein, the work presented in this study suggests that a simple 4Fe → 3Fe cluster conversion does not result in changes in the RNA-binding properties of the protein. Thus, while the [4Fe-4S] cluster state can be readily distinguished from the [3Fe-4S] state of the purified protein by spectroscopy or measurements of aconitase activity, these two forms are indistinguishable in terms of their RNA-binding properties. We have utilized the effect of aconitase substrates on the RNA-binding activity as an aid to defining the state of the Fe-S cluster of the protein. The form of the protein isolated from iron-replete cells binds to its cognate RNA only in the presence of relatively high concentrations of reducing agents. However, conversion to this RNA-binding form is antagonized by aconitase substrates. In this regard, the iron-replete form of the protein is identical to both the [4Fe-4S] and the [3Fe-4S] cluster states of the purified protein. This finding is not unexpected, in that the [3Fe-4S] and the [4Fe-4S] forms tightly bind substrate (23). The apoprotein, which does not tightly bind substrate, is unaffected in its RNA-binding properties by aconitase substrates. It is this sensitivity to substrate that characterizes the RNA-binding form of the protein isolated from iron-deprived cells. The current data suggest that the cell is capable of more profoundly altering the Fe-S cluster of the IRE-BP and that the physiologically relevant form of the IRE-BP in iron-deprived cells is the apoprotein.

The types of manipulations required to disassemble the Fe-S cluster in cell lysates or in preparations of the purified protein are harsh, requiring either high amounts of oxidant (1 mM ferricyanide) or high concentrations of reductant (50–100 mM dithiothreitol) at elevated pH (pH 8.9) (22, 29). While more gentle treatments (10 nmol ferricyanide) are sufficient to remove the labile fourth iron, only the harsh treatments described above produce a protein that resembles the IRE-BP derived from iron-deprived cells. These observations suggest that cells are likely to utilize a specific, perhaps enzymatic process to disassemble this Fe-S cluster. One possibility is that the IRE-BP Fe-S cluster in cells may be undergoing constant disassembly and reassembly. The ability of the protein to "sense" the level of available iron may depend on the capacity of the cell to reassemble the cluster. While the precise status of the iron or the sulfur of the cluster in the RNA-binding form of the protein has yet to be ascertained, the intracellular lability of this cluster provides both a novel regulatory role for these clusters and an opportunity to study the cellular process of cluster assembly and disassembly.

The effects of the cluster and substrate in determining whether or not this protein binds to its cognate RNA point to the active-site cleft as a critical region for RNA binding. A clue as to how both cluster and substrate might contribute to a protein conformational change is suggested by the crystal structure of the mitochondrial aconitase (28), if we assume that the structure of the IRE-BP is similar. The latter protein consists of three tightly folded domains (termed 1–3) connected via an apparently flexible hinge/linker to a fourth domain. The active site is composed of residues from all four domains in the region of the Fe-S cluster. While multiple potential interactions exist between domain 4 and the rest of the protein across the cleft, the residues lining the cleft are primarily hydrophilic and water is found in the cleft. These

properties suggest that exposure of the cleft to solvent through a conformational change might be energetically favored. In the crystal structure, the substrate forms multiple contacts with domain 4 as well as the cluster, which is ligated to domain 3. In addition, the substrate contacts amino acid residues within the first three domains. Thus, one way in which both cluster and substrate could influence the structure of the protein would be if the "closed" state that does not bind RNA involved the apposition of domain 4 to domains 1–3 to produce the active-site cleft. The "open" state of the protein (able to bind to its target RNA) would be produced by motion about the hinge/linker in which domain 4 would no longer be apposed to the rest of the protein.

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