Structural Changes Associated with Switching Activities of Human Iron Regulatory Protein 1*

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Metazoan iron regulatory protein 1 is a dual activity protein, being either an aconitase or a regulatory factor binding to messenger RNA involved in iron homeostasis. Sequence comparisons and site-directed mutagenesis experiments have supported a structural relationship between mitochondrial aconitase and iron regulatory protein 1. The structural properties of human recombinant iron regulatory protein 1 have been probed in the present work. Although iron-free iron regulatory protein 1 displays a significantly larger radius of gyration measured by small-angle neutron scattering than calculated for mitochondrial aconitase, binding of either the [4Fe-4S] cluster needed for aconitase activity or of a RNA substrate turns iron regulatory protein 1 into a more compact molecule. These conformational changes are associated with the gain of secondary structural elements as indicated by circular dichroism studies. They likely involve α-helices covering the substrate binding cleft of cytosolic aconitase, and they suggest an induced fit mechanism of iron-responsive element recognition. These studies refine previously proposed models of the “iron-sulfur switch” driving the biological function of human iron regulatory protein 1, and they provide a structural framework to probe the relevance of the numerous cellular molecules proposed to affect its function.

Iron regulatory proteins (IRP)³ are cytosolic trans-regulators of metazoan cells that bind to iron-responsive elements (IRE) on the untranslated regions of some mRNA to modulate the synthesis of the encoded proteins. Iron availability affects binding of IRP to IRE; iron withdrawal by strong chelators stimulates binding to IRE on the 5′-side of ferritin mRNA and the 3′-side of transferrin receptor with a consequent decrease and increase, respectively, in the synthetic rates of these proteins. This exquisite sensitivity of IRP to iron resources makes them prominent participants of metazoan iron homeostasis, but several other conditions (such as oxidative/nitrosative stress, hypoxia, hormone or cytokine production, or toxic metals) affect IRP activity. Most cellular and cellular properties of the IRP-IRE system have been recently and abundantly reviewed (1–4).

The most easily detected IRP1 is not the target of a very efficient transcriptional regulation (5), and it is accordingly found in most cellular types and tissues. It is a bifunctional protein (4, 6) that either displays aconitase (citrate-isocitrate interconversion) activity or binds to IRE. The switch between these two activities is driven by the assembly/destruction of a [4Fe-4S] cluster that is required for aconitase substrates. These two activities are mutually exclusive as demonstrated by a wealth of independent methods (7–12). Therefore, IRP1 can be a cytosolic aconitase that resembles its mitochondrial homolog as well as other Fe-S isomerases (13–15). Indeed, these extensive sequence alignments convincingly show that the proteins of this family must share structural similarities under their active conformations.

However, the probable similar folding of mitochondrial and cytosolic aconitases has never been experimentally probed. Moreover, IRP1 is an exception among Fe-S isomerases in that its metal-depleted conformation specifically binds to an RNA target. Therefore, the relevance of the mitochondrial aconitase model (16, 17) for the IRE-binding IRP1 conformation is doubtful in light of the diversity of folds exhibited by RNA-binding proteins (18–21).

To improve the relatively crude model previously proposed to describe the structural changes affecting IRP1 when switching between its two activities (1, 12, 22, 23), pure human recombinant IRP1 has been studied in the present work with methods affording valuable structural information in solution. Small-angle neutron scattering experiments have been combined with circular dichroism measurements to show that metal-depleted IRP1 adjusts to its RNA substrate or its [4Fe-4S] active center by locally folding around these elements. Comparison with the mitochondrial aconitase structure suggests some regions of IRP1 that may be involved in these processes.

MATERIALS AND METHODS

Recombinant human IRP1 was produced and purified as previously described (24). Aconitase activity of IRP1 was measured by following the decrease of cis-aconitate absorbance at 240 nm with a Hewlett-Packard 8452 spectrophotometer in air-tight cuvettes evacuated and filled with argon (24). The measurement of IRE binding activities was carried out (24) with an α-[32P]UTP-labeled RNA probe corresponding to the human H-ferritin stem-loop IRP1 with the following sequence: GAGACCCGAAUCCAUGCUUCUAACAGUGCUUGGACGGAAUCC in which the underlined bases are involved in the stem structure and the italicized ones build the loop. The unlabeled IRP used in structural studies had the same RNA sequence.

Recombinant human IRP1 was depleted of its Fe-S cluster by treatment of the protein under anaerobic conditions with 10 mM EDTA, pH 8.0, and 0.2 M H2O2 for 1 h at room temperature. The reactants were then separated by filtration through a Sephadex G-25 column (Amer sham Biosciences, Inc.) equilibrated in 20 mM Tris-Cl, pH 7.4, with 1 mM 1,4-dithio-DL-threitol. This procedure afforded a fully active IRP-binding protein with no obvious signs of amino acid modifications (not
shown). It has also been found more efficient than alternative methods involving oxidation with ferricyanide described for mitochondrial aconitase (25), high concentrations of thiols, or low and high pH (6).

For neutron scattering experiments, recombinant human IRP1 samples were dialyzed overnight in a Microdialyzer System 100 (Pierce) against a required buffer prepared with diethyl pyrocarbonate-treated H$_2$O inside an anaerobic chamber ensuring a deoxygenated atmosphere containing less than 2 ppm O$_2$. These samples were centrifuged for 15 min at 125,000 × g (Airfuge, Beckman) before use.

Neutron scattering experiments were carried out at Institut Laue-Langevin on beam line D11. Quartz cuvettes of 1-mm (H$_2$O) or 2-mm (samples with D$_2$O) optical paths containing the samples were kept at 20 °C. Two sets of conditions were implemented for recording data at small scattering angles or over a wider range. Low Q data ($Q = (4 \pi / \lambda \sin \theta$, $2 \theta = $ scattering angle, $\lambda =$ neutron wavelength) were obtained with a wavelength of 10 Å with the two-dimensional detector located at 4 m from the sample, whereas a wavelength of 5 Å and a sample-to-detector distance of 2 m were used at higher Q. These data were treated as described (26), and the scattering intensities of the protein solutions were corrected for those of the empty quartz cell and the buffer. Radii of gyration were calculated from low Q data by linear regression following the Guinier relationship.

\[
\text{ln}(I) = \text{ln}(I_0) - (\frac{1}{3}R_gQ^2)
\] (Eq. 1)

The distance distribution function $P(R)$ was calculated from the scattering data as implemented in the GNOM program (27) and computed in CRYSOL (28) for mitochondrial aconitase.

CD spectra were recorded with a Jasco 810 spectropolarimeter at a scanning speed of 10 nm/min with a bandwidth of 4 nm and a time response of 2 s. Five to 10 scans were summed up to provide convenient signal/noise ratios. Protein solutions were prepared in 10 mM potassium phosphate buffer at pH 7.5. They were contained in argon-filled quartz cuvettes of 0.1-cm optical path in a nitrogen-flushed sample holder.

Protein concentrations and other conditions are indicated in the legends to figures.

RESULTS

The homology between IRP1 (cytosolic aconitase) and mitochondrial aconitase was recognized long ago (13–15), and the structure of the latter protein from pig heart has been solved by X-ray crystallography at high resolution (16, 17). Secondary structure predictions using the Jpred server (29) indicate that the proportions of α-helix and β-sheet in pig aconitase (29 and 16%, respectively) agree with those deduced from the tridimensional structures of the [3Fe-4S] (Protein Data Bank code 7ACN) or [4Fe-4S] (Protein Data Bank code 8ACN) forms of the enzyme (32 and 18%, respectively). Such values are close to those obtained for the human IRP1 sequence, 29% for α-helix and 15% for β-sheet. Moreover, secondary structure elements overlap significantly between the sequences of pig mitochondrial aconitase and human IRP1 with most of the discrepancies at the N terminus. These data validate mitochondrial aconitase as a structural model of human IRP1 that has been used in the present work as a structural framework when appropriate.

CD Spectra of Recombinant Human IRP1—The CD spectrum of human recombinant IRP1 purified as the aconitase enzyme (24) is shown in Fig. 1. As expected from secondary structure predictions, the CD spectrum of [4Fe-4S]-IRP1 displays the general features of α/β proteins (30). However, the protein contains a [4Fe-4S] cluster, the CD contribution of which is expected to be weak (31). The CD spectrum of the inorganic moiety has been estimated under the present experimental conditions by recording CD spectra of Clostridium pasteurianum [2[4Fe-4S]] ferredoxin extending to 185 nm (not shown). C. pasteurianum ferredoxin is devoid of significant secondary structure (32), and its CD spectrum, recorded at a concentration half of that of IRP1 to equalize the cluster concentrations (the ferredoxin has two such clusters), is within the base line above 230 nm. Below 230 nm, a weak positive band around 215 nm and a negative one around 195 nm are far less intense (not shown) than the features displayed by IRP1 in this region (Fig. 1), and they are reminiscent of spectra of unordered proteins (30). These weak bands may arise from the ferredoxin peptide or from its clusters, and they are of inverted differential absorbance compared with the [4Fe-4S]-IRP1 spectrum of Fig. 1. These observations emphasize the very weak contribution of the cluster to the CD spectrum of [4Fe-4S]-IRP1 that has not been corrected for the potential contribution of the cluster in this study.

The [4Fe-4S] cluster of human recombinant IRP1 can be removed in vitro as described above (see “Materials and Methods”) to give the form of the protein that is competent to bind the IRE motif. The CD spectrum of this form of IRP1 is displayed in Fig. 1. Compared with the aconitase form of IRP1, the changes are not large, but they are significant as is more easily seen on the difference spectrum (Fig. 1). The general shape with a negative band peaking at 222 nm with a shoulder around 210 nm and a positive band maximal below 200 nm indicates the secondary structure elements have been lost upon removal of the cluster, α-helices giving the largest CD contribution among them. Therefore, even if at the presence of substrates, the switch between the two activities of IRP1 is paralleled by a change of the structure that we probed by a complementary method.

Neutron Scattering Data of Recombinant Human IRP1—Fig. 2 compares the Guinier plots (low Q) obtained with the aconitase and IRE-binding forms of IRP1. The calculated radii of
gyration for data recorded without added D2O are 32 and 40 Å, respectively. The latter value was deduced in the 1 < QR < 2.2 range (Table I) that somewhat extends from the Guinier approximation because signs of aggregation can be seen at very low Q (Fig. 2). In fact, the likeliness of IRP1 devoid of metals to form highly scattering particles increases with the percentage of D2O added to the sample (Fig. 2), and this effect has impeded the measurement of the radii of gyration for this form. This problem is less important with [4Fe-4S]-IRP1 for which values have been calculated for three D2O concentrations (Table I). The slight decrease of \( R_g \) from 32 to 28.7 Å as a function of D2O in the 0–100% range reflects the non-uniform scattering density of IRP1, as observed with other proteins (33).

The validity of the assumption that [4Fe-4S]-IRP1 is a globular protein, by similarity with the known structure of mitochondrial aconitase, can be checked with neutron scattering data. The pair distribution function \( P(R) \) has been plotted for [4Fe-4S]-IRP1 using the data recorded at 65% D2O over a wider Q range (Fig. 3). A similar plot can be calculated from the x-ray crystal structure of pig mitochondrial aconitase (Fig. 3). This plot displays the frequency of vector lengths connecting scattering elements within the particle. For [4Fe-4S]-IRP1, the pair distribution function is relatively symmetric around a maximum below 40 Å, a feature clearly revealing a single compact particle as expected for a globular protein. The slightly non-monotonous decrease to 0 at high R values may be assigned to the minor aggregation already observed on the Guinier plots (Fig. 2) and introduce some uncertainty in the maximal dimension of [4Fe-4S]-IRP1. However, comparison of the main parts of the curves agrees with both proteins being globular and with the larger (by about 15%) size of [4Fe-4S]-IRP1 compared with mitochondrial aconitase. A radius of gyration of 25.6 Å has been calculated for the latter, as compared with 32 Å for [4Fe-4S]-IRP1 (Table I). The difference between these two values indicates that most of the insertions found in IRP1 as compared with mitochondrial aconitase lie at the surface of the protein and strongly influence its hydrodynamic properties.

**Protein-RNA Interactions with Recombinant Human IRP1**

The [4Fe-4S] cluster-depleted recombinant human IRP1 is fully activated to bind its RNA substrate, and the structural changes induced by IRE binding on IRP1 have thus also been probed with the same methods.

**CD Changes upon IRE Binding to IRP1**

The CD spectrum of the RNA fragment containing the human H-ferritin IRE motif used in this work is displayed in Figs. 4 and 5. The positive band around 270 nm and the negative one around 210 nm are typical of “single strand” nucleic acids (34). At equimolar concentrations, the CD signal intensity of the IRE probe is far smaller than that of the IRE protein.

Upon addition of IRP1 at a 1:1 molar ratio, the CD spectrum displays the general shape of the sum of the individual CD contributions of IRE and IRP1 (Fig. 4). However, when the experimental spectrum of IRE is added to that of IRP1 and the result is compared with the spectrum of the mixture, subtle differences are observed. These differences are likely due to conformational changes induced by the binding of IRP1 to the IRE.

**Table I**

Radii of gyration calculated from small angle neutron scattering data

| D2O | [4Fe-4S]-IRP1 | apo-IRP1 | IRP1:IRE (1:1) |
|-----|---------------|----------|----------------|
| %   | \( R_g \) (Å)  | \( R_g \) (Å) | \( R_g \) (Å) |
| 0   | 32.0 (0.5)    | 39.7 (0.7) | **a**          |
| 62  | 30.6 (0.4)    | **b**    | 35.7 (1.2)     |
| 100 | 28.7 (0.2)    | **b**    | **b**          |

**a** Calculated value cannot be compared because of the large IRE contribution.

**b** Aggregated sample.
differences are evidenced, such as the presence of a more intense negative contribution centered at 222 nm for the IRE/H18528 IRP1 complex as compared with the sum of the individual components (Fig. 4). The dissociation constant for the IRP1/H18528 IRE complex has been estimated at a few tens of picomoles in different laboratories (e.g. Ref. 9), which implies that the concentration of this complex is several orders of magnitude above that of the free protein in the present experimental conditions.

Fig. 5 shows the CD spectrum of a mixture of IRP1 with a slight (3-fold) excess of IRE. When this sample was kept at 21 °C, we observed some evolution of the spectra over the time needed to record them; the (dotted) spectrum in Fig. 5 is an average taken during the first hour after mixing. The sample was then heated at 37 °C for 15 min and returned to 22 °C. The resulting spectrum was stable over several hours, and it is also shown in Fig. 5. The difference spectrum between those recorded after and before heating at 37 °C is drawn in the bottom panel.

The difference spectrum of Fig. 5 also displays a negative band at 270 nm at the positive maximum of IRE. Therefore, the intensity of this band decreases when IRE binds to IRP1; this most probably reflects some change of the RNA structure upon interaction with the protein (34). The variations of the difference CD spectrum below 210 nm are more difficult to assign as they arise from a combination of changes affecting both IRP1 and IRE. Nevertheless, the positive difference between 190 and 200 nm may be mainly associated with the negative one at 222 nm due to the protein that has a larger spectral contribution than the RNA fragment in this region.

Small Angle Neutron Scattering Measurements on the IRE-IRP1 Complex—The contribution of IRE binding to the hydrodynamic properties of IRP1 has been assessed by small angle neutron scattering. The Guinier plot from data recorded without D2O is of little value for direct comparison with that obtained for the protein alone (Fig. 2) because the RNA piece significantly contributes to the scattering intensity. More readily valuable data have been obtained at 62% D2O, close to the isopycnic value of RNA, where the contrast between solvent and IRE is near 0 (33), hence canceling the contribution of the latter. Under these conditions, free IRP1 shows signs of aggregation (see above), so that the IRE-IRP1 complex has been compared with [4Fe-4S]-IRP1 only (Fig. 6). As shown above for
other D$_2$O concentrations (Fig. 2), data obtained for [4Fe-4S]-IRP1 (Fig. 6) provide a linear Guinier plot over a wide $Q^2$ range (0.6 < $RQ$ < 1.8). In the case of the IRE-IRP1 complex the intensities at very low $Q$ values do not properly align indicating that there is residual aggregation, but the linear approximation is as good as that for [4Fe-4S]-IRP1 for larger $Q$ values. Therefore, the data for [4Fe-4S]-IRP1 and IRE-IRP1 can be compared over a narrower range (0.9 < $RQ$ < 1.8). The resulting slope of the Guinier plot for the IRP1-IRE complex is consistently larger than that of [4Fe-4S]-IRP1 (Fig. 6). Still, this difference is smaller than that measured between metal-free IRP1 and [4Fe-4S]-IRP1 in the absence of D$_2$O (Table I). Because the variations of $R_g$ measured for [4Fe-4S]-IRP1 are relatively minor (Table I), the large increase of this value between [4Fe-4S]-IRP1 and metal-free IRP1 (+25%) is reversed in part by the binding of IRE to the protein.

**DISCUSSION**

The [4Fe-4S] Cluster of Cytosolic Aconitase Contributes to the Folding of Secondary Structure Elements—CD spectroscopy and neutron scattering experiments converge in showing that the globular compact structure of human cytosolic aconitase unfolds partly upon [4Fe-4S] cluster complete removal. Both a significant increase of the radius of gyration and the loss of secondary structure elements occur. These observations agree with the structural role the cluster may play in cytosolic aconitase besides its catalytic function. By analogy with mitochondrial aconitase, cytosolic aconitase is expected to be organized in four globular domains (I–IV), each lining the active site with a 24-amino acid linker between domains III and IV. Three of the ions are bound to cysteine residues, and there is a hydrogen bond between the side chain nitrogen of an asparagine residue of domain III and one of the sulfur atoms of the cluster (16). In addition, several other residues of domain I interact with molecules, such as substrates or inhibitors, bound to the cluster (17). The loss of these stabilizing contributions through destruction of the cluster led to the proposal that the cytosolic aconitase structure opens, possibly via the hinge region linking domain IV to the rest of the molecule, to accommodate the large IRE substrate (1, 12, 22, 23).

The increased radius of gyration estimated for IRE-binding competent IRP1 (Fig. 2, Table I) agrees with this model. However, the conformational change is not limited to a movement of domain IV around the hinge because the linker does not display large secondary structure elements in mitochondrial aconitase, except for a few turns. The decrease of CD intensity between 190 and 222 nm observed on the spectra upon converting cytosolic aconitase into IRE-binding IRP1 (Fig. 1) must involve rearrangements of at least one of the main domains of the protein with loss of α-helices or β-sheets. Likely candidates include domains I and II with helices 147–152, 166–174, and 250–263 and strand 67–73 (the amino acid numbering is that used for the mitochondrial aconitase structure) that line the substrate binding cleft and lie close to the [4Fe-4S] cluster.

**The Structural Role of IRE Binding to IRP1 and the Mode of RNA-Protein Interaction**—When the IRE substrate binds to IRP1 the radius of gyration of the protein decreases (Fig. 6, Table I) and the CD intensity in the 190–225-nm region increases (Figs. 4 and 5). However, this form of IRP1 is less compact and it displays a slightly smaller proportion of secondary structure elements than cytosolic aconitase (Fig. 1). Because IRE is a larger molecule than the aconitase substrates, IRP1 is expected to adopt a more voluminous conformation to adjust to the RNA molecule, whereas cluster-depleted IRP1 lacks some of the interactions that help stabilize a compact structure. Nevertheless, such conformational changes do not necessarily imply that secondary structure should develop upon IRE binding as detected on CD spectra (Figs. 4 and 5).

IRP1 is functionally and structurally quite remote from other RNA-binding proteins. Yet, upon binding IRE, IRP1 displays some features that are reminiscent of proteins or peptides interacting with RNA, such as the domain of the HIV-1 protein Rev that increases its α-helical content when bound to its RNA substrate (36). Recent surveys of RNA-protein structural interactions (20, 21) recognized the occurrence of two main classes of RNA-binding proteins, one that binds single strand RNA on β-sheet surfaces and another that inserts a secondary structural element (helix, loop . . . ) into an RNA helix (groove binding). Because most of the IRE sequence involved in IRP1 recognition folds as a stem-loop RNA with a bulge (37), both types of interaction modes may be relevant for the IRE-IRP1 complex. However, from the mitochondrial aconitase structure, hardly any strand covers the active site cleft that is expected to be strongly involved in IRE recognition as indicated by site-directed mutagenesis studies, protection from proteases upon substrate binding, and cross-linking experiments (7–12). It is then likely that IRP1 binding to IRE involves groove binding to the stem structures of RNA with formation of secondary structure elements. On the basis of the residues identified as important for IRE binding (9, 11, 12) and by comparison with the mitochondrial aconitase structure (16), these elements may organize around residues of domain I, such as those belonging to the 80–187 fragment (human IRP1 numbering). Because the loss of secondary structure in domains I and II was associated above with loss of aconitase activity, it is proposed that IRP1 unfolds locally in domains I or II by removal of the [4Fe-4S] cluster and recovers some secondary elements in domain I upon IRE binding. An alternative or addition to the latter partial recovery of secondary structure may involve fragments of domain IV (those surrounding arginines 721, 728, or 732 for IRP1, the structure of IRE appears to be affected by its interaction with the protein, as witnessed by the negative band at 270 nm in Fig. 5. Such behavior occurs upon formation of many RNA-protein complexes (18, 19). In this induced fit mode of recognition where both partners experience structural changes,
Structural and Functional Implications—The exact location of the structural elements involved in protein-RNA recognition will certainly be clarified when high resolution structural data become available, but the present work provides an unprecedented view of how IRP1 responds to substrate binding and switch of activities. The previously proposed increase of the aconitase volume to accommodate the IRE substrate (1, 12, 22, 23) is now experimentally confirmed, but our data indicate that it may not merely involve the flexible hinge predicted to link the fourth globular domain of the protein to the other three. Some structural changes including α-helices or β-sheets affect the active site, and it seems likely that a subset of these secondary structural elements is important in substrate binding. For this reason, further work aiming at probing the mechanistic details of IRP1 conversion between its different active forms will certainly benefit from the spectroscopic signatures delineated herein.

The structural data obtained in this work suggest that a relatively modest perturbation, such as that afforded by phosphorylation (23, 41), should be enough to switch activities because folding/unfolding of a small number of secondary structure elements (Figs. 1, 4, and 5) translates into sizable changes in the IRP1 volume (Figs. 2 and 6, Table I). Indeed, IRP1 efficiently responds to a large variety of cellular changes, but the molecular mechanisms underlying these responses seem to involve complex signaling pathways (24, 42, 43), with the possible exceptions of conditions leading to large concentrations of superoxide (23, 44) or reactive nitrogen species (45, 46). The actual cellular components able to trigger such changes by direct reaction with IRP1 remain to be identified, but, once suspected, their involvement may be conveniently assessed in light of the data presented herein.

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