The Role of Molecular Hinges in the Structure and Function of Iron Regulatory Protein I

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THESIS
Submitted as partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology and Immunology in the Graduate College of the University of Illinois at Chicago, 2014

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ACKNOWLEDGEMENTS

I would like to thank my family for supporting my education goals. I would also like to thank my research advisors Dr. Karl Volz and Dr. William Walden. I would like to especially thank my dissertation committee members Dr. Chen, Dr. Freitag, Dr. Shukla, Dr. Bouvier and Dr. Yee Kin Ho for their advice throughout the years. Finally, I would like to thank my research colleagues for their helpful comments and advice.
TABLE OF CONTENTS

I. INTRODUCTION .................................................................................................................. 1
   Epidemiology and relevance of iron homeostasis disorders ............................................. 2
   Systemic iron homeostasis ............................................................................................... 4
   Cellular iron homeostasis ............................................................................................... 6
   Regulation of IRP1 by the ubiquitin proteasome system, oxidative stress and
   phosphorylation ............................................................................................................. 10
   Structure and function of Iron Regulatory Protein 1 ...................................................... 11
   Molecular hinges and mechanisms of domain closure .................................................... 16
   Proteolytic degradation of apo-IRP1 by chymotrypsin .................................................. 19
   Statement of Purpose ....................................................................................................... 22

II. METHODS ........................................................................................................................... 23
   Site directed mutagenesis ............................................................................................... 24
   Strains and transformation ............................................................................................. 25
   Cell culture ...................................................................................................................... 25
   Glutamate auxotrophy ..................................................................................................... 25
   Preparation of yeast cell extracts .................................................................................. 26
   Measurement of the enzymatic conversion of isocitrate to cis-aconitate ....................... 26
   SDS-PAGE ....................................................................................................................... 27
   Western blots ................................................................................................................... 27
   Iron sulfur cluster reconstitution .................................................................................... 28
      Iron sulfur cluster reconstitution of IRP1 in cell extracts ............................................ 28
      Iron sulfur cluster reconstitution using purified IRP1 (with NifS) .............................. 28
      Iron sulfur cluster reconstitution using purified IRP1 (without NifS) ......................... 29
   Synthesis of radiolabeled RNA probe .......................................................................... 29
   Quantitative analysis of the interaction between IRP1 and Ferritin-L IRE ................. 30
   Luciferase assay .............................................................................................................. 30
   Limited proteolysis ......................................................................................................... 31
   Protein purification ......................................................................................................... 32
   Small angle x-ray scattering ......................................................................................... 32
   Ab initio modeling .......................................................................................................... 33
   Rigid-body modeling ..................................................................................................... 33
      Comparison of scattering curves .............................................................................. 34
         Generation of hypothetical intermediate structures ............................................. 34
      Ensemble optimization method ............................................................................... 34
      Dynamic light scattering ......................................................................................... 35

III. THE SOLUTION STRUCTURE OF APO-IRON REGULATORY PROTEIN 1 ........................................ 36
   Introduction ..................................................................................................................... 37
   Results ............................................................................................................................. 39
      Purification of apo-IRP1 ............................................................................................. 39
      Dynamic light scattering .......................................................................................... 41
      Small angle x-ray scattering .................................................................................... 43
      Pair distribution function ......................................................................................... 44
      Ab initio structure of apo-IRP1 ................................................................................ 45
      Rigid-body modeling ............................................................................................... 50

iv
TABLE OF CONTENTS (continued)

Comparison of ab initio and rigid body models ........................................... 52
Intermediate conformations of apo-IRP1 .................................................. 52
Ensemble optimization method ............................................................... 53
Discussion .................................................................................................... 56

IV. THE ROLE OF THE β4-α4 MOLECULAR HINGE IN THE STRUCTURE AND
FUNCTION OF IRON REGULATORY PROTEIN 1 ........................................... 60
Introduction ................................................................................................. 61
Results ........................................................................................................ 64
  Purification of apo-IRP1 mutants .............................................................. 64
  The effect of hinge mutations on the SAXS properties of apo-IRP1 ......... 64
  Ensemble optimization method analysis ................................................. 73
  The effects of hinge mutations on aconitase activity ............................. 85
The effects of hinge mutations on RNA binding function ....................... 89
Discussion .................................................................................................... 91
  Analysis of proteolytic susceptibility results ......................................... 93
  The differential cleavage pathway of the D87P and G90A/P92A mutants ... 94
  Analysis of global structural changes by acidic and basic proteases ......... 96
  Specific β4-α4 hinge mutations alter the enzymatic function of IRP1 ....... 96
  The RNA binding function of IRP1 β4-α4 hinge mutants ....................... 97
  Analysis of secondary structure changes in the β4-α4 molecular hinge .... 98

V. GENERAL CONCLUSIONS AND FUTURE DIRECTIONS ............................. 101
  Apo IRP1 adopts an open conformation in solution .................................. 102
  Insight regarding the case of IRP1 structural interconversion .................. 102
  Possible mechanisms of IRP1 domain closure ....................................... 104
  Time resolved SAXS may provide additional details about the mechanism of
  conformation change ............................................................................. 105
  The β4-α4 hinge mutants alter the enzyme activity of IRP1 ...................... 105
  The altered proteolytic susceptibility of IRP1 ......................................... 106
  Analysis of the α28-α29 hinge as a target for future study .................... 107
  Physiological implications of the hinge mutations .................................. 108

VI. CITED LITERATURE .................................................................................. 111

VII. VITA ...................................................................................................... 122
LIST OF FIGURES

1. The Iron Responsive Element ........................................................................... 7
2. Regulation of ferritin mRNA by IRP1 ................................................................. 8
3. The aconitase superfamily ............................................................................... 13
4. Domain organization of different members of the aconitase superfamily ....... 14
5. Crystal structures of Iron Regulatory Protein 1 ................................................. 17
6. The two molecular hinges of IRP1 .................................................................. 19
7. The chymotrypsin degradation pathway of wild type apo-IRP1 ................... 20
8. Diagram showing the location of chymotrypsin sensitive sites of IRP1 .......... 21
9. Purification of his-tagged apo-IRP1 by nickel chelate affinity chromatography . 39
10. Purification of apo-IRP1 by anion exchange chromatography ....................... 40
11. Correlogram of wild type apo-IRP1 ................................................................. 41
12. Dynamic light scattering data of wild type apo-IRP1 ....................................... 47
13. Small angle x-ray scattering of IRP1 ............................................................... 42
14. Pair distribution functions of IRP1 ................................................................. 44
15. Ab initio models of apo-IRP1 ....................................................................... 46
16. Composite and filtered ab initio models of apo-IRP1 ..................................... 48
17. Low resolution molecular envelopes for the ab initio model and crystal structures .... 49
18. Diagram of isolated domains used in rigid body modeling ............................ 50
19. Rigid body models of apo-IRP1 ..................................................................... 51
20. Comparison of ab initio and rigid body models of apo-IRP1 ......................... 52
21. Chi vs. Rg plot of hypothetical apo-IRP1 structures ........................................ 53
22. Ensemble optimization method analysis of apo-IRP1 ...................................... 54
23. Fit of the experimental scattering curve with the scattering curve of the ensemble of structures ........................................................................................................ 55
24. The secondary structure of the β4-α4 region for the two different forms of IRP1 ................................................................. 62
25. Crystal structure of the α4 helix in cytosolic aconitase and the IRP1-RNA complex .... 62
26. Purification of the apo-IRP1 D87P mutant ...................................................... 71
27. Purification of the apo-IRP1 G90A mutant ..................................................... 72
28. Purification of the apo-IRP1 P92A mutant ...................................................... 73
29. Purification of the apo-IRP1 G90A/P92A mutant ........................................... 74
30. Scattering curves for apo-IRP1 apo-IRP1 hinge mutants ............................... 75
31. Guinier plots for apo-IRP1 hinge mutants ................................................... 76
32. Radius of gyration for apo-IRP1 hinge mutants ............................................ 77
33. Pair distribution functions of apo-IRP1 hinge mutants ................................. 78
34. Ensemble optimization method analysis for β4-α4 hinge mutants ................. 80
35. Comparison of scattering curves for ensemble and experimental models .......... 81
36. Protease susceptibility of IRP1 mutants in the presence of GluC, Trypsin, LysC and AspN .... 82
37. Chymotrypsin susceptibility of WT apo-IRP1 .............................................. 84
38. Chymotrypsin susceptibility of D87P apo-IRP1 ............................................ 85
39. Chymotrypsin susceptibility of G90A apo-IRP1 ........................................... 86
40. Chymotrypsin susceptibility of P92A apo-IRP1 ............................................ 87
41. Chymotrypsin susceptibility of G90A/P92A apo-IRP1 ................................. 88
LIST OF FIGURES (continued)

42. Analysis of apo-IRP1 chymotryptic digests ................................................................. 89
43. Mass spectroscopy of apo-IRP1 proteolytic fragments .................................................. 90
44. Glutamate dependent growth of IRP1 hinge mutants ......................................................... 91
45. Growth kinetics of yeast cells expressing IRP1 hinge mutants in media lacking glutamate .... 92
46. Aconitase activity in 0615d extracts containing IRP1 mutants ............................................ 93
47. IRE-Luciferase assay ........................................................................................................... 95
48. Filter binding assays ............................................................................................................ 96
49. Schematic depicting proposed degradation pathways of the D87P and the G90A/P92A hinge
    mutants ............................................................................................................................ 107
**LIST OF TABLES**

I. WORLDWIDE PREVALENCE OF ANEMIA .................................................................3

II. LIST OF KNOWN MRNAS THAT CONTRAIN IRON RESPONSIVE ELEMENTS ...9

III. LIST OF PRIMERS .................................................................................................24

IV. LIST OF STRAINS ................................................................................................25

V. DYNAMIC LIGHT SCATTERING DATA FOR APO IRP1 ......................................42

VI. CHI VALUES FOR GASBOR MODELS ................................................................47

VII. CONNECTIVITY RESTRAINTS AND CHI VALUES OF RIGID BODY MODELS ...51

VIII. DOUBLING TIMES OF YEAST CELLS EXPRESSING IRP1 HINGE MUTANTS ....86
LIST OF ABBREVIATIONS

AUC – Analytical ultracentrifugation
EOM – Ensemble optimization method
DMT1 – Divalent metal transporter 1
GDF15 – Growth differentiation factor 15
IRE – Iron responsive element
IRP – Iron regulatory protein
mRNA – Messenger ribonucleic acid
OD – Optical density
PCR – Polymerase chain reaction
PVDF – Polyvinyl difluoride
RLU – Relative light units
MW – Molecular weight
RPM – Rotations per minute
SAXS – Small angle x-ray scattering
SD-URA – Synthetic defined media minus uracil
SDS-PAGE – Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SMAD – Contraction of Sma (from sma gene in D. melanogaster) and Mad (from MAD mothers against decapentaplegic D. melanogaster)
Tris – Tris (hydroxymethyl) aminomethane
TTBS – Tween tris buffered saline
TWSG1–Twisted gastrulation protein homolog 1
WT – Wild type
YPAD – Yeast peptone adenine dextrose
SUMMARY

Iron Regulatory Protein 1 (IRP1) is a bi-functional protein that can act either as a post-transcriptional regulator of iron homeostasis genes or as an enzyme (cytosolic aconitase). Crystal structures of IRP1 show that two different conformations are required for RNA binding and enzyme function. Additionally, the crystal structures of IRP1 show that portions of the β4-α4 molecular hinge transition between α-helix and β-turn secondary structures. This secondary structure change corresponds to global conformation changes of IRP1. Elucidations of crystal structures of IRP1 in the cytosolic aconitase and RNA binding conformations greatly expanded knowledge regarding the mechanisms associated with IRP1 structural and functional plasticity. However, because the crystal structure of apo-IRP1 is unavailable and little is known about the structural components involved in conformation change, a comprehensive understanding of the structural and functional plasticity of IRP1 has been precluded. Therefore, the aims of this study were to determine the structure of apo-IRP1 and to determine if the β4-α4 molecular hinge is important in the structure and function of IRP1. The initial hypothesis was that stabilization of the helical structure of the β4-α4 molecular hinge would lock the protein in the closed conformation and prevent RNA binding. This hypothesis was tested using a series of biochemical and biophysical experiments.

Small angle x-ray scattering was utilized to characterize the biophysical properties apo-IRP1 because it provides data about size, shape, molecular structure and distribution of multiple conformational states. The SAXS experiments show that apo-IRP1 is in an open conformation and is predominantly distributed about a radius of gyration of approximately 33.6 ± 0.3 Å. SAXS experiments also show that mutant forms of apo-IRP1 containing the helix destabilizing mutations D87P and G90A/P92A in the β4-α4 molecular hinge have a decreased radius of gyration compared to the wild type protein. Furthermore, limited proteolysis experiments show that the D87P and the G90A/P92A mutants undergo an alternate cleavage pathway compared to
the wild type protein. Together, these results suggest that D87P and G90A/P92A mutations alter the solution conformation of apo-IRP1.

The effects of the mutations on cytosolic aconitase enzyme activity were probed using in vivo and in vitro experiments. Aconitase deficient yeast strains transformed with D87P, G90A and G90A/P92A mutants of cytosolic aconitase had decreased growth rates and were deficient in enzyme activity compared to the wild type protein. More specifically, catalysis of isocitrate was decreased for the G90A, P92A, and G90A/P92A mutants. Interestingly, the D87P mutant was devoid of enzyme activity. The results presented in this study implicate a role for helix destabilizing residues of the β4-α4 hinge in the enzymatic function of cytosolic aconitase.

Analysis of the RNA binding function of the IRP1 hinge mutants revealed that mutants retained their ability to bind RNA with picomolar affinity. Together, these series of experiments shows that hinge mutations neither locked the protein in the closed conformation nor prevented the RNA binding. Rather, the results indicate that the hinge mutations have subtle effects on the structure and enzyme function of IRP1.
I. INTRODUCTION
Epidemiology and relevance of iron homeostasis disorders

Iron deficiency and iron overload affects millions of people worldwide. Combined, both diseases transcend age group, socioeconomic status and gender. In non-industrialized nations anemia is prevalent in greater than 40% of school age children (Table 1) [1]. In America, approximately 44 out of every 10,000 individuals of Northern European descent are homozygous for genes that cause hereditary iron overload [2]. Although treatments for anemia and iron overload are effective, numerous medical examples highlight the complexity of managing disorders related to iron metabolism due to an incomplete understanding of the fundamental biological mechanisms that govern iron homeostasis.

Iron deficiency is characterized by severely low systemic iron levels. Worldwide, this condition is estimated to affect 4–5 billion people [3]. Taking this number into consideration, it should come as no surprise that iron deficiency is one of the most prevalent medical disorders in the world. Non-industrialized nations have the highest burden of individuals afflicted with iron deficiency [4]. However, a significant proportion of preschool children and pregnant women that reside in industrialized nations are predicted to be iron deficient [2]. Anemia related to iron deficiency is generally not life threatening. However, fatigue, a major symptom of anemia can have a significant impact on work force productivity and quality of life [4]. More severe liabilities of iron deficiency have been observed in young children and pregnant women. Studies indicate that reduced brain iron content in juveniles is related to low developmental scores [5]. Additionally, iron deficiency is strongly correlated with low birth weight and decreased neonatal health [6].

At the other end of the spectrum is iron overload. This condition occurs when systemic iron levels exceed the capacity of the body’s iron storage repositories. Primary iron overload is caused by several types genetically inherited disorders that are commonly carried by individuals of Northern European descent [7]. In the United States, an estimated one million people live with
hereditary hemochromatosis [8]. Secondary iron overload arises from multiple origins including but not limited to chronic blood transfusion, severe hemolytic anemia and massive ineffective erythropoiesis [7]. Chronic iron overload can cause hepatomegaly, diabetes and congestive heart failure which arise from iron mediated oxidative damage to cells of the liver, pancreas, endocrine glands and heart [9].

**TABLE I**

**WORLDWIDE PREVALENCE OF ANEMIA**

<table>
<thead>
<tr>
<th>Estimated percentages of anemia prevalence based on blood hemoglobin concentration</th>
<th>Industrialized countries</th>
<th>Non industrialized countries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children (0-4)</td>
<td>20.1</td>
<td>39.0</td>
</tr>
<tr>
<td>Children (5-14 years)</td>
<td>5.9</td>
<td>48.1</td>
</tr>
<tr>
<td>Pregnant women</td>
<td>22.7</td>
<td>52.0</td>
</tr>
<tr>
<td>All women (15-59 years)</td>
<td>10.3</td>
<td>42.3</td>
</tr>
<tr>
<td>Men (15-59 years)</td>
<td>4.3</td>
<td>30.0</td>
</tr>
<tr>
<td>Elderly (&gt;60 years)</td>
<td>12.0</td>
<td>45.0</td>
</tr>
</tbody>
</table>

Currently, there are numerous effective therapies for treatment of iron deficiency and iron overload. Iron deficiency can be treated by intravenous iron injection or by oral supplementation with ferrous sulfate [10]. Iron overload can be treated by iron chelation therapy and in some cases phlebotomy [11]. In other cases, therapeutic strategies are not straight forward. For example, iron overload is common in patients with sickle cell disease and thalassemia. However, patients with sickle cell disease have lower instances of iron mediated organ failure than patients with
thalassemia. The underlying molecular mechanism that causes the difference is poorly understood and prevents application of the most effective therapy. The aforementioned medical example highlights the importance of basic research aimed to increase the understanding the molecular basis of iron homeostasis.

**Systemic iron homeostasis**

On average, adult males have total body iron content of approximately 35 – 45 mg/kg body weight [12]. In order to sustain total body iron, about 1 - 2 mg of iron is required each day [13]. Surprisingly, humans have no physiological mechanism to rid excess iron from the body, total body iron content is balanced solely by the rate of intestinal iron absorption. Hepcidin is a peptide hormone secreted by the liver that controls iron absorption by modulating the cell surface expression of the iron export molecule ferroportin [14]. At the cellular level, iron is regulated by Iron Regulatory Proteins (IRPs) which post-transcriptionally regulate iron homeostasis genes [9]. Together, IRPs and hepcidin coordinate the expression of iron homeostasis machinery and ensure that iron is maintained at biologically safe levels.

Systemic iron levels are physiologically maintained by the rate of intestinal iron absorption. Enterocytes located in the duodenum and the upper portion of the jejunum absorb iron and subsequently transports it basolaterally into the bloodstream [15, 16]. These enterocytes express divalent metal transporter 1 (also referred to as DMT1, NRAMP2, DCT1), a transmembrane transport protein that carries iron from the lumen of the intestine into the cell. Before iron can be translocated into the cell, iron atoms must be reduced from Fe$^{3+}$ to Fe$^{2+}$ by ferrireductases [17]. Once iron is transported into the cell, it can be incorporated into proteins, stored or exported. Iron export is achieved by basolateral transportation into the bloodstream via the iron export protein ferroportin [18]. Subsequently, iron is bound by the serum iron carrier protein transferrin. This molecule binds Fe$^{3+}$ with an affinity on the order of $10^{-20}$ M [19] so that
under normal physiological conditions virtually no iron circulates freely in bloodstream. Transferrin distributes iron to cells expressing transferrin receptor. Parenchymal cells receive only a small fraction of iron from the blood. Erythroid precursor cells receive a large portion of iron which is subsequently used to synthesize hemoglobin. Mature erythrocytes circulate in the blood stream and eventually become senescent. Senescent erythrocytes are engulfed by special macrophages in the bone marrow, liver and spleen. The iron acquired by macrophages from erythrocytes is either stored or transported to the bloodstream.

Release of iron into the bloodstream from intestinal enterocytes, macrophages, and hepatocytes is strictly regulated by the iron regulatory hormone hepcidin. Hepcidin is expressed by hepatocytes and is secreted into the blood stream where it circulates bound to its chaperone α-2 macroglobulin [20]. Hepcidin influences systemic iron levels by binding to the iron efflux protein ferroportin and inducing its ubiquitination and subsequent degradation [14]. Since ferroportin is essential for dissemination of iron located in enterocytes, macrophages and hepatocytes, its degradation prevents these cells from releasing iron into the blood stream.

Hepcidin expression is modulated by systemic iron status, inflammation, hypoxia and erythropoietic signals [21]. Studies suggest that, interaction between transferrin receptor 2 and transferrin leads to the formation of a cell signaling complex that activates SMAD transcription activators which induce expression of hepcidin [22]. Similarly, infection or inflammation can lead to transcriptional activation of hepcidin. When inflammatory cytokines IL-1 and IL-6 interact with their cognate receptors, STAT transcription factors become activated and induce the expression of hepcidin [23]. Conversely, both erythropoiesis and hypoxia inhibit hepcidin expression. During erythropoiesis, the molecules GDF15 and TWSG1 are produced by erythroid precursor cells [24, 25]. TWSG1 decreases hepcidin expression via inhibition of the signaling pathways that lead to SMAD activation [24]. The mechanism by which GDF15 represses hepcidin transcription is currently unknown. The effects of hypoxia on the hepcidin expression
are two-fold. First, hypoxia induces the expression of erythropoietin which activates erythropoiesis and leads to expression of the hepcidin inhibitory molecules GDF15 and TWSSG1. Second, the classic response to hypoxia results in the production of HIF1 and HIF2 [26]. These factors have been linked to decreased hepcidin production. However, the mechanism by which HIFs modulate hepcidin production is unknown.

**Cellular iron homeostasis**

At the cellular level, iron homeostasis is generally maintained by one process that sequesters excess intracellular iron and another process that transports iron into the cell. In metazoans, Iron Regulatory Protein 1 (IRP1) and Iron Regulatory Protein 2 (IRP2) post-transcriptionally regulate the mRNAs of iron homeostasis genes in accordance with the cellular iron levels. In the case of IRP1, regulation occurs through the ‘iron sulfur switch’ mechanism [27]. When cellular iron levels are high, IRP1 binds an iron sulfur cluster and acquires the ability to interconvert citrate and isocitrate [28]. Conversely, when cellular iron levels are low IRP1 becomes a post transcriptional regulator capable of binding to hairpin loop mRNA structures called Iron Responsive Elements (IRE) [29].

IREs are composed of 25-30 nucleotides that form stem and loop regions (Fig. 1). The stem region consists of a variable sequence of 9-10 helix forming base pairs. Within the stem is a bulge region that divides the stem into upper and lower segments. The bulge region contains a conserved cytosine nucleotide at the eighth position that is essential for the high affinity interaction between IRP1 and the IRE. The loop region consists of a conserved sequence 5’-CAGUGX-3’, where the first and fifth nucleotides in the sequence form hydrogen bonds with each other. Nucleotides A, G, and U in the loop form interactions with IRP1 [30].

Association of IRP1 with IREs can alter the translation of the transcripts by two separate mechanisms. Generally, when IRP1 binds to an IRE located in the 5’ untranslated region (UTR),
it subverts translation by preventing the small ribosomal subunit from binding the 5’ UTR [31] (Figure 2). In the situation where mRNA has its IRE in the 3’UTR, binding of IRP1 stabilizes the transcript. Currently, 11 mRNA transcripts are known to carry IREs (Table 2) [32]. Among these transcripts, seven have IREs in their 5’UTR and four transcripts have 3’UTR IREs. Additionally, the transcripts for α-hemoglobin stabilizing protein and amyloid precursor protein have been shown to carry IRE like structures that are regulated by IRP1 [33, 34]. More recently, 35 mRNA’s were determined to form complexes with IRP1 and IRP2 [35].

Cellular iron status is modulated by cellular iron uptake, efflux and storage. Iron uptake is mediated by the transferrin receptor molecule. Interaction between diferric transferrin and the transferrin receptor induces endocytosis. Acidification of the endocytotic vesicle releases iron from transferrin and iron is subsequently transported into the cytosol via DMT1 [17, 36]. Once iron is in the cytosol it is either utilized in proteins, exported or stored.

When cellular iron concentration is low IRP1 lacks an iron sulfur cluster, adopts an open conformation and can bind to the IREs with picomolar affinity [37]. In this conformation, IRP1

**Figure 1. The Iron Responsive Element.** Diagram of the canonical Iron Responsive Element.
can bind to the 3’ UTR IREs of the transferrin receptor mRNA, upon binding IRP1 stabilizes the transcript and allows expression of transferrin receptor [38]. Thus, when cellular iron concentration is low iron uptake is increased. Conversely, when cellular iron concentration is high, IRP1 has an iron sulfur cluster, is in a closed conformation and lacks the ability to bind IREs with high affinity. In this situation IRP1 cannot bind to the 5’UTR IRE of the ferritin mRNA, allowing its translation [39]. The overall effect is that cellular iron storage is increased.

**Figure 2. Regulation of ferritin mRNA by IRP1.** When cellular iron is low, IRP1 binds the IRE of the ferritin mRNA and represses its translation. Conversely, when cellular iron concentration is high IRP1 acquires an iron sulfur cluster, changes into a compact globular conformation and is no longer able repress translation of the ferritin mRNA.
TABLE II
LIST OF KNOWN MRNAS THAT CONTAIN IRON RESPONSIVE ELEMENTS

A. Transcripts with 3’ UTR IRE

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transferrin Receptor 1</td>
<td>Serum iron chaperone</td>
</tr>
<tr>
<td>Divalent metal transporter 1</td>
<td>Iron transport</td>
</tr>
<tr>
<td>Dual specificity protein phosphatase CDC14A</td>
<td>Cell cycle control</td>
</tr>
<tr>
<td>Myotonic dystrophy kinase-related Cdc42-binding kinase A</td>
<td>Actin formation</td>
</tr>
</tbody>
</table>

B. Transcripts with 5’ UTR IRE

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythroid 5-aminolevulinate synthase</td>
<td>Heme biosynthesis</td>
</tr>
<tr>
<td>Ferritin-L</td>
<td>Iron storage</td>
</tr>
<tr>
<td>Ferritin-H</td>
<td>Iron storage</td>
</tr>
<tr>
<td>Ferroportin</td>
<td>Iron transport</td>
</tr>
<tr>
<td>Hypoxia inducible factor 2-α</td>
<td>Iron transport</td>
</tr>
<tr>
<td>Mitochondrial aconitase</td>
<td>Energy metabolism</td>
</tr>
<tr>
<td>Succinate dehydrogenase *</td>
<td>Energy metabolism</td>
</tr>
</tbody>
</table>

* This IRE is not present in the corresponding gene for mammals but it is found in some invertebrates
Regulation of IRP1 by the ubiquitin proteasome system, oxidative stress and phosphorylation

IRP1 can adopt at least three conformations in the cell. Under normal physiological conditions, between 4 and 18% of IRP1 molecules are in the RNA binding state [40]. Cellular processes such as the ubiquitin proteasome system, oxidative stress and phosphorylation can alter the abundance of IRP1 in the cell and/or modulate its function.

Low cellular iron concentration leads to the degradation of IRP1 by the ubiquitin proteasome system under conditions where iron sulfur cluster biogenesis is limited [41]. FBXL5 is a component of the ubiquitin ligase complex that has been shown to interact with IRP2 [42]. A similar interaction between FBXL5 and IRP1 is likely to occur. FBXL5 senses cellular iron levels through a hemerythin-like domain that is capable of binding both iron and oxygen. When iron levels are high FBXL5 accumulates. Conversely, when iron or oxygen is limiting FBXL5 is destabilized [42]. Thus, proteasome degradation of IRP1 is linked to cellular iron status and oxygen availability. The physiological significance of proteasome mediated degradation of IRP1 is not well understood.

Redox regulation of IRP1 by either reactive oxygen species or nitric oxide provides a link between oxidative stress and iron metabolism. It has been established that exposure of IRP1 to oxidative reagents damages the iron sulfur cluster and inactivates aconitase activity [43]. In vivo, exposure of macrophages to agents that induce production of nitric oxide activates IRP1 to bind IRE’s and impairs aconitase function [44].

The function of IRP1 can be modulated through protein kinase C dependent phosphorylation [45, 46]. Two phosphorylation sites are present in IRP1, one at S138 the other at S711. Phosphorylation of IRP1 has different effects depending on the site of modification. Experiments with phosphomimetic mutants of IRP1 at S711 revealed that citrate to isocitrate...
conversion was hindered but conversion of isocitrate to citrate was not affected. Additionally, S711 phosphomimetic mutants retain high affinity IRE binding [47]. Other experiments show that phosphomimetic mutants at S138 have decreased stability of the [4Fe-4S] and have increased sensitivity to oxygen and H₂O₂ [48]. As observed with the S711 phosphomimetic mutants, the IRE binding of the S138 mutant is not affected [49].

Knockout of both IRP1 and IRP2 genes leads to embryonic lethality at the blastocyst stage, highlighting the importance of these proteins during early development. Knockout of either IRP1 or IRP2 but not both is non-lethal in mice, a direct example of the functional redundancy between the two proteins [40]. However, mice lacking IRP2 develop microcytic hypochromic anemia, iron overload and have aberrant neurological function, suggesting that IRP1 can not compensate for some of the physiological activities of IRP2. Mice lacking IRP1 show no pathological phenotype suggesting that IREs are predominantly regulated by IRP2 [40].

**Structure and function of Iron Regulatory Protein 1**

The dual functionality of IRP1 is closely related to its structure. IRP1 belongs to the aconitase superfamily, a group of proteins characterized by a shared 4 domain structural organization. The aconitase protein superfamily consists of five discernible subgroups (Figure 3). Among these subgroups, protein function and structural organization is variable. Crystal structures of three different types of aconitases provide significant insight as to how IRP1 can bind RNA and function as an enzyme.

The IRPs provide an example of functional diversity within the aconitase superfamily. IRP1 has the ability to interconvert citrate/isocitrate and bind RNA. In contrast, IRP2 lacks enzymatic function and retains the ability to bind RNA. The mitochondrial and bacterial aconitase A proteins are both [4Fe-4S] cluster enzymes that catalyze the interconversion of isocitrate and citrate. Interestingly, there is mounting evidence suggesting that members of the aconitase A
family bind RNA [50]. The aconitase X proteins bind a [4Fe-4S] cluster and catalyze the interconversion of citrate and isocitrate [51, 52]. Proteins within the aconitase B family have typical aconitase function. Homoaconitases and isopropyl malate isomerase proteins catalyze reactions involving substrates other than citrate and isocitrate. Homoaconitases catalyze the conversion of cis-homoaconitate and homoisocitric acid in the lysine biosynthesis pathway. The Isopropylmalate isomerases catalyze the isomerization between 2-isopropylmalate and 3-isopropylmalate, an essential step in the leucine biosynthesis pathway [53].

In addition to the diverse range in functionality displayed within the aconitase superfamily, there is a diverse array of domain organization (Figure 2) [54]. Variable domain organization is attributed to the manner that the fourth domain is connected to the remaining three domains. In the AcnA-IRP1 family, the first three domains are expressed sequentially and the fourth domain is connected to domain 3 by a linker. The prokaryotic and archean isopropyl malate isomerases have a fourth domain expressed by a separate polypeptide that forms a heterodimer with the remaining three domains [54]. The aconitase B family deviates from all other aconitases because it has five domains. This additional domain, the HEAT domain is located at the N-terminus adjacent to the fourth domain. A linker connects the fourth domain to the first three domains.

The crystal structure of IRP1 reveals its tertiary structure and domain organization. As previously observed in other crystal structures of aconitases, domain four is not physically oriented next to domain three [55, 56]. Rather, the linker region provides an extension that allows domain 4 to orient itself on the opposite side of the molecule next to domain 1. An α-helix from domain 1 forms hydrophobic contacts with domain 4 that stabilize inter-domain connectivity [56].

The crystal structure of IRP1 in complex with the ferritin IRE shows that binding is conferred by two spatially separate sites. Each site establishes multiple interactions with the RNA
Figure 3. The aconitase protein superfamily. The aconitase protein superfamily is separated into five different protein subgroups. Organization is based on phylogenetic analysis of aconitases.
Figure 4. Domain organization of different members of the aconitase superfamily. A. Isopropylmalate Isomerase and the aconitase X family have a 4-1-2-3 organization. B. Aconitase A family members: Iron Regulatory Protein, Bacterial Aconitase and Mitochondrial Aconitase have a 1-2-3-4 domain organization and a linker that connects domain 4 to domain 3. C. Prokaryotic and Archean Isopropylmalate Isomerase express domain 4 from a different reading frame. D. Aconitase B has five domains, the HEAT domain is at the N terminus and the remaining domains have a 4-1-2-3 organization. Both domain 4 and the HEAT domain connected to a linker to domain 1 (Figure adapted from Markova and Koonin, 2003).
(Figure 5). This includes hydrogen bonding interactions between protein side-chain/main chain atoms with the RNA nucleosides/phosphate backbone. Domains 2 and 3 of IRP1 provide a binding surface that forms 11 interactions with the terminal loop of the RNA. At a separate site, a small cavity in the surface of domain 4 accommodates binding of the C8 nucleotide from the lower stem of the ferritin RNA [56]. Together, these interactions confer picomolar affinity between IRPs and IREs.

The mitochondrial aconitase crystal structure provides insight toward the mechanism of the catalytic conversion of isocitrate and citrate [57-59]. Multiple interactions between the active site residues and substrate confer substrate specificity. The iron atoms of the [4Fe-4S] cluster are ligated by 3 cysteine residues and the fourth iron interacts with both a water molecule and the carboxyl group of the substrate [58]. Catalytic conversion of citrate to isocitrate begins with the abstraction of a proton from the beta carbon of citrate by residue serine 772. Subsequently, the alpha-hydroxyl of citrate is transferred to the fourth iron atom of the cluster. The product of this reaction, cis-aconitate, must leave the active site, rotate by 180° and rebind to the active site. Once cis-aconitate rebinds in the proper orientation, isocitrate is formed by addition of a hydroxyl group to the alpha-carbon and addition of a proton to the beta-carbon. Close analysis of mitochondrial aconitase crystal structures containing and lacking substrate (one structure interestingly contains a sulfate molecule in the active site) suggests that rotation of domain 4 may be required to create a channel for the substrate to enter or exit the active site [57].

The aconitase and RNA binding function of IRP1 occur independently of one another because of the drastic differences in conformational requirements. In the cytosolic aconitase conformation portions of the protein required for IRP1-IRE complex formation are not solvent accessible, precluding any possibility of high affinity protein-RNA interaction. For example, residues within the 534-544 and 436-442 regions are important sites for protein-nucleic acid interactions but are not available to interact with solvent [56]. Additionally, some residues
important for RNA binding and aconitase function are within close proximity to one another. Modification of the cluster ligating residue C437 with N-ethylmaleimide (NEM) severely hinders the ability IRP1 to interact with RNA [60]. Indeed, the close proximity of RNA binding residues T438 and A439 to this reactive cysteine residue may render them vulnerable to subtle changes that disrupt their interaction with RNA.

**Molecular hinges and mechanisms of domain closure**

In order for enzymes to carry out biological reactions they must be able to recognize substrates and provide an optimal environment for catalysis. These separate events require the protein to adopt different conformations. Upon binding a ligand or substrate, most proteins undergo relatively small conformational changes. However, a small fraction of proteins undergo dramatic conformational changes, whereby, individual domains rotate up to 60º [61]. A classic example of this is the conformation change exhibited by hexokinase. Here, glucose induces the small domain to rotate by 17 º, this motion encloses glucose in the active site [62, 63]. Rigid body domain motion exhibited by hexokinase and other proteins can be classified as either hinge or shear type motions.

Shear motions occur when segments of closely packed opposing domains traverse each other’s surface [64]. These motions occur as a result of changes in multiple noncovalent interactions at the interface of two adjacent domains. This includes movement of interdigitated side chains and movement of other overlapping segments by no more than a few angstroms [64]. Shear motions cause small domain rotations that rarely involve deformation of the polypeptide backbone.

Molecular hinges contribute to the conformational flexibility of multi-domain proteins. At a basic level, hinges are composed of β-sheet and/or α-helix secondary structure elements.
These secondary structure elements interconnect opposing domains and contain a screw axis which the mobile domain can rotate about [65]. Domain rotation arises from changes in the torsion angles of the polypeptide backbone in a region close to the rotational axis of the mobile domain. Hinges comprised of β-strands have a high degree of conformational mobility. Conversely, hinges composed of α-helices have restrained movement due to strict hydrogen bonding requirements [64]. In some situations, the termini between secondary structure elements of molecular hinges serve as the region of torsional mobility [64]. Small changes in the torsion angle of a molecular hinge can cause large domain motions. Proteins with the ability to change conformation using both hinge and shear type motions can adopt significantly more conformations compared to proteins that utilize only one type of domain motion.

**Figure 5. Crystal structures of Iron Regulatory Protein 1.** A. The crystal structure of cytosolic aconitase [55] and B. the crystal structure of IRP1 bound to Ferritin H IRE [56].
Crystal structures of IRP1 in two different conformations highlight the large degree of conformation change resulting from transition from either the RNA binding or the cytosolic aconitase state. In one crystal structure, IRP1 has an iron sulfur cluster and is in a closed conformation. In another crystal structure, IRP1 is bound to the ferritin-H IRE and has a significantly elongated conformation compared to the cytosolic aconitase form (Figure 5). The exact mechanism by which domain rotation occurs is unknown. However, the crystal structures show that IRP1 contains two molecular hinges that change conformation upon rigid body domain rotation of domains 3 and 4 (Figure 6). Like other proteins that undergo domain movements; molecular hinges are believed to play an important role in conformation change.

The domain motion that governs conformational change of IRP1 involves rotation of two spatially separate domains around unique molecular hinges. Domain 4 rotates around the β4-α4 molecular hinge by 32º and translates by approximately 14 Å [56]. Domain 3 rotates around the α28-α29 molecular hinge by 52º and translates by 13 Å [56]. The combined motion of these two domains results in either the construction of the enzyme active site or exposure of the RNA binding surface. Both hinges undergo secondary structure changes that correspond with rigid body motions of domain 4 and domain 3 (Figure 6).

The β4-α4 molecular hinge contains a screw axis that passes within a few angstroms of the termini that connect beta strand and alpha helix. In the cytosolic aconitase conformation a portion of the β4-α4 molecular hinge contains an α-helix comprised of helix destabilizing residues glycine and proline. These residues may contribute to the destabilization of the alpha helix and facilitate conformation change in this region.

In the RNA binding conformation, the α28-α29 hinge is composed of an extended α-helix that traverses the surface of both the core domain and domain 3. Similar to the case of the β4-α4 molecular hinge, the linker molecular hinge would have to change conformation upon the transition from the RNA binding to the aconitase conformation.
Figure 6. The two molecular hinges of IRP1. Both figures highlight the two molecular hinges of IRP1 and altered secondary structures depending on the conformation. A. Cytosolic aconitase. B. IRP1 in the RNA binding conformation (RNA omitted).

**Proteolytic degradation of apo-IRP1 by chymotrypsin**

Upon binding of the [4Fe-4S] cluster, IRP1 loses its ability to interact with IREs with high affinity. Additionally, protease susceptibility experiments show that after acquiring a [4Fe-4S] cluster, cytosolic aconitase is resistant to degradation by chymotrypsin [66]. Conversely, when apo-IRP1 is treated with chymotrypsin under the same conditions, it is readily cleaved into four fragments (Figure 7). The predominant cleavage sites have been mapped by N-terminal sequencing and occur at W623, F133 and Y501. The crystal structure of cytosolic aconitase reveals that the predominant chymotryptic residues are oriented such that it may be difficult for chymotrypsin to recognize them (Figure 8). Both residues F133 and Y501 are exposed to the solvent but are in locations that are predicted to be not easily accessible by the protease. Based on analysis of the cytosolic aconitase crystal structure, W623 is predicted to be inaccessible to the
Figure 7. The chymotrypsin degradation pathway of wild type apo-IRP1. WT apo-IRP1 is degraded by chymotrypsin by an initial cleavage at W623 that yields a 71 kDa fragment and a 29 kDa fragment. The large fragment is subsequently cleaved at F133 to yield a 50 kDa fragment. Subsequent cleavage of the 50 kDa fragment at Y501 yields a 42 kDa band. Red stars indicate cleavage products that are visible by SDS-PAGE.
Figure 8. Diagram showing the location of chymotrypsin sensitive sites of IRP1. A. Hypothetical model of apo-IRP1 with FR1, FR2 and FR3 built into the crystal structure of RNA bound IRP1. B. The location of chymotrypsin sensitive sites of apo-IRP1 as they appear in the cytosolic aconitase conformation. Plausible locations of chymotrypsin sensitive residues are colored magenta and both crystal structures are rotated by 180° to the right relative to crystal structures in figure 5.
protease because this residue is not solvent exposed. Analysis of the crystal structure in the RNA binding conformation suggests that regions containing the cleavage site have high B-factors, suggesting that these regions are intrinsically disordered.

Analysis of the protein sequence of IRP1 reveals that there are 78 high specificity chymotrypsin cleavage sites. As discussed, only three sites are readily accessible by chymotrypsin in the apo conformation (Figure 8) [66, 67]. This observation is entirely consistent with limited proteolysis experiments with other proteins which show that cleavage of the peptide bond typically occurs within flexible regions of the protein [68, 69].

**Statement of Purpose**

The ability of IRP1 to change conformation is important in the maintenance of cellular iron homeostasis. Currently, a major gap in understanding the bi-functional nature of IRP1 exists because the structure of apo-IRP1 is unavailable and the molecular details of conformation change are not fully characterized. Therefore, the purpose of my research is to advance the understanding of the dual functionality of IRP1 by determining the structure of apo-IRP1 and by characterizing the role of the β4-α4 molecular hinge in the structure and function of IRP1. Elucidation of the structure of apo-IRP1 by small angle x-ray scattering will provide novel insight toward the molecular mechanism of conformation change. Site directed mutagenesis experiments will be used to generate molecular hinge mutants in order to determine if helix destabilizing residues are involved in rigid body domain rotation of IRP1. Additional biochemical characterization of the IRP1 hinge mutants will provide insight toward the role of the molecular hinges in the physiological function of IRP1. The results from this research will be useful not only to scientists studying iron homeostasis but also to researchers interested in understanding the role of molecular hinges in multi-domain proteins.
II. METHODS
Site directed mutagenesis. Site directed mutagenesis was performed using the Qiagen Quick Change kit (Qiagen). Reverse complementary primers containing the mutation of interest were used to incorporate mutations into a plasmid containing IRP1. The reaction was prepared using 50 ng DNA, 125 ng forward and reverse primer, 2 mM dNTP, and 2.5U Pfu Turbo DNA polymerase. The polymerase chain reaction (PCR) consisted of the following steps; denaturing at 95 °C for 30 seconds, followed by annealing at 55 °C for 3 minutes and elongation at 68 °C for 10 minutes. A total of 15 cycles were used. The PCR reaction was digested with Dpn I at 37 °C for 15 minutes to eliminate methylated parental plasmids that did not contain the mutation(s) of interest [70]. DH5-α cells were transformed with the PCR reaction. Transformants containing the mutated IRP1 construct were selected on ampicillin media. DNA was isolated from transformants and DNA sequence analysis was used to determine if the mutation of interest was present.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
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<tr>
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<td>5’-GCCCGGTCATCTCTGCAGCCCTTCACGCGGTGTACCACATCTGTGGTG-3’</td>
<td>5’-CRAACCACAGATGGTACACCGCGGCTCGAGGATGACACGGGC-3’</td>
</tr>
<tr>
<td>G90A</td>
<td>5’-GCCCGGTCTCATCTGGCAGGACTCTCACGGCTGTACCCATCTGTGGTG-3’</td>
<td>5’-CRAACCACAGATGGTACACCGCGGCTCGAGGATGACACGGGC-3’</td>
</tr>
<tr>
<td>P92A</td>
<td>5’-GCCCGGTCATCTCTGCAGGACTCTCACGGCTGTAGCATCTGTGGTG-3’</td>
<td>5’-CRAACCACAGATGGTACACCGCGGCTCGAGGATGACACGGGC-3’</td>
</tr>
<tr>
<td>G90A/P92A</td>
<td>5’-GCCCGGTCATCTCTGAACCCCTTCACGCGSGCTGTAGCATCTGTGGTG-3’</td>
<td>5’-CRAACCACAGATGGTACACCGCGGCTCGAGGATGACACGGGC-3’</td>
</tr>
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Strains and transformation

Aconitase deficient yeast strain 0615d was used to analyze aconitase activity in vivo. The protease deficient strain BJ5465 was used for expression and purification of recombinant IRP1. Strain AS4742 was used for in-vivo IRE-mRNA repression experiments. AS4742 contained a chromosomal insertion of the IRE-luciferase gene under control of a minimal ADH promoter.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>0615d</td>
<td>Ace1, ura3-52, his3-Δ200, trp1-Δ63, ade2-101, IDP2&lt;sup&gt;up&lt;/sup&gt;</td>
<td>Narahari J.</td>
</tr>
<tr>
<td>BJ5465</td>
<td>Mata, ura3-52, trp1, lue2Δ1, his3Δ200 pep4::HIS3 prbΔ1.6 Rcan1</td>
<td>Jones E.</td>
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<tr>
<td>AS4742</td>
<td>MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0</td>
<td>Selezneva</td>
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<tr>
<td></td>
<td></td>
<td>A.(unpublished)</td>
</tr>
</tbody>
</table>

Cell culture

Yeast strains were streaked on YPAD plates from frozen stocks and incubated for 3 to 5 days at 30 ºC. Individual colonies were selected, inoculated into SD-URA media and grown overnight at 30 ºC with shaking at 300 rpm.

Glutamate auxotrophy

*S. cerevisiae* strain 0615d was grown to logarithmic phase, cells were pelleted by centrifuging at 2,500 rpm and washed twice with sterile water. Optical density was measured using a Beckmann Coulter DU-600 spectrophotometer and samples were resuspended to an
OD$_{600}$ of 0.1. Ten microliters containing between $1 \times 10^2$ and $1 \times 10^5$ cells were spotted on media that contained or lacked glutamate. Cells were incubated at 30 °C for five days before assessing growth.

**Preparation of yeast cell extracts**

*S. Cerevisiae* strain 0615d was grown to logarithmic phase and harvested by centrifugation at 2,500 rpm for five minutes. The supernatant was decanted and cells were washed twice with water. Cells were resuspended in 300 µL of a buffer consisting of 50mM Tris, 50mM NaCl, 10% glycerol and 3mM PMSF at pH 8.0. Cells were lysed by vortexing in the presence of 0.04 - 0.05 mm glass beads. Lysate was separated from the glass beads by pipetting and centrifuged at 14,000 rpm for 10 minutes at 4 °C. The supernatant was removed and the total protein concentration was measured by determining the absorbance at 233 nm and 224 nm. Equation 1 was used to quantify protein concentration [71].

**Equation 1**

$$\text{protein concentration} = \frac{A_{224} - A_{233} \text{(sample dilution factor)} \left(0.1 \text{mg/mL}\right)}{\Delta A_{224-233} (0.564)}$$

**Measurement of the enzymatic conversion of isocitrate to cis-aconitate**

Conversion of isocitrate to cis-aconitate was quantified spectrophotometrically by measuring the change in absorbance at 240nm. The reaction was initiated by mixing cell extracts with a reaction buffer consisting of 100 mM Tris and 20 mM isocitrate at pH 8.0. Units of activity per mg protein extract were determined using the following equation [72].
Cell extracts were prepared as previously described. Equivalent amounts of protein were loaded into 12% Tris-glycine gels and electrophoresis was run at 125 V for 1 hour. Gels were washed in deionized water for ten minutes and stained for 1 hour with Simply Blue Safestain (Invitrogen). Excess dye was removed from the gel by soaking in water with concomitant shaking.

Western blots

Samples were run by SDS-PAGE as described in the previous section. After SDS-PAGE gels were soaked in a transfer buffer containing Tris, glycine, SDS and 20% methanol. PVDF membranes were pre-soaked in 100% methanol then soaked in transfer buffer. Protein was transferred to a PVDF membrane using a tank blot system. Electrophoresis was run at 75 V for 1 hour. Transfer efficiency was checked by staining with Ponceau S dye. Excess dye was removed by rinsing with water. Membranes were blocked using 5% milk TTBS solution. After one hour, membranes were incubated in a 1:1000 dilution of the α-myc antibody for at least one hour in order to detect myc tagged IRP1. Next, membranes were rinsed three times in TTBS and incubated with a 1:10,000 dilution of the α-mouse antibody for at least 1 hour. Traces of the secondary antibody were removed by rinsing three times with TTBS. Bands were visualized using a solution of alkaline phosphatase buffer containing 0.15 mg/mL NBT and 0.30 mg/mL BCIP.

\[
\text{enzyme activity} = \frac{\Delta \text{mAbs/min}}{3.6 \text{mM}^{-1} \text{cm}^{-1}}
\]
Iron sulfur cluster reconstitution

The iron sulfur cluster of IRP1 can be reconstituted from the apo-protein using several methods. Each method has its own advantages and disadvantages. Reconstitution using iron and sulfur salts is technically straightforward but complications arise from the formation of iron sulfide precipitants. To circumvent the issue of including high concentrations of iron and sulfide in the reaction, reconstitution can be performed using cysteine as the sulfur source and a cysteine desulfurase. The sulfide is produced catalytically and remains at a low concentration during the initial phase of the reconstitution reaction, precluding any formation of large quantities of iron sulfide precipitants. Another option is to reconstitute IRP1 in cell extract. This can be performed by addition of iron and a reducing agent. No addition of sulfide is necessary, presumably due to the preexistence of sulfide in the cell extract. The details for three different iron sulfur cluster reconstitution procedures are given below.

Iron sulfur cluster reconstitution of IRP1 in cell extracts

Cells were grown to logarithmic phase and extracts were prepared. Iron sulfur clusters were reconstituted by incubating cell extracts with 0.5 mM ferrous ammonium sulfate and 1 mM DTT for 30 minutes at room temperature. Addition of sulfide was not necessary to reconstitute enzyme activity.

Iron sulfur cluster reconstitution using purified IRP1 (with NifS)

Purified IRP1 was incubated with 0.5 mM DTT, 2 mM cysteine, 0.5 mM ferrous ammonium sulfate and 0.76 mg/mL NifS for at least 30 minutes anaerobically at room temperature [73, 74].
Iron sulfur cluster reconstitution using purified IRP1 (without NifS)

Purified IRP1 was incubated with 0.5 mM DTT, 0.5 mM ferrous ammonium sulfate and 0.5 mM sodium sulfide for at least 30 minutes anaerobically at room temperature [75].

Synthesis of radiolabeled RNA probe

Ferritin light chain IRE was cloned into vector PTZM18R in a region downstream of a T7 phage promoter [76]. This vector was cut with the restriction enzyme Xho I and T7 RNA polymerase was used to generate a runoff transcript of the ferritin light chain IRE. The probe synthesis reaction contained the following components: transcription buffer, 10mM DTT, 3mM each of rATP, rCTP, rGTP, 4mM cold rUTP, 10µg linearized template DNA and 120 µCi alpha α-32P UTP, and T7 RNA polymerase. The reaction mixture was incubated for 1 hour at 37 ºC. After incubation, DNAase was added and the reaction was incubated for 15 minutes at 37 ºC [77]. Subsequently, an equal volume of phenol:chloroform:isoamyl alcohol was added to the solution and vortexed for one minute. The aqueous phase was collected and passed through a G-25 column. RNA was precipitated from this solution by adding three volumes 100% ethanol, 1/10 the volume of 3.0 M sodium acetate and incubating at -20 ºC for one hour. The precipitated RNA was pelleted by centrifugation at 10,000 g for 10 minutes [78]. The supernatant was removed and the sample was air dried, resuspended in RNA loading buffer and heated at 85 ºC for five minutes. Samples were loaded into a denaturing 8M urea, 38:2 acrylamide:bis-acrylamide gel and subjected to electrophoresis for 1 hour at 18 mAmps. Bands were visualized using x-ray film and the portion of the gel containing radiolabeled IRE-RNA was excised. The excised acrylamide gel fragment was placed in a centrifuge tube containing a hole bored by a 16 gauge needle. The gel fragment was crushed by centrifuging at 1,000g for 3 minutes. IRE-RNA was extracted from the fragmented gel by soaking in a buffer containing tRNA and 0.1% SDS overnight at ambient temperature. The eluate was separated from the gel fragments and passed through a G-25 column.
IRE-RNA was precipitated by incubating the solution in 3 volumes ethanol and 1/10 sodium acetate at -20 °C for 1 hour and centrifuging at 16,000 rpm at 4 °C. Supernatant was removed, the pellet containing the IRE-RNA was air dried and resuspended in water. The concentration of the probe was determined by scintillation counting [78].

**Quantitative analysis of the interaction between IRP1 and Ferritin-L IRE**

The dissociation constant between Ferritin-L IRE and IRP1 was measured by nitrocellulose filter binding assays. A constant amount of IRP1 was titrated with a radiolabeled RNA probe ranging from concentrations between 2nM and 600nM. The IRP1-IRE mixture was incubated at 37 °C for 15 minutes and then passed through nitrocellulose filters using a vacuum manifold. Filters were washed twice with an equivalent volume of RNA binding buffer. Filters were dried and radioactive IRP1-IRE complexes bound to the filters were quantified by scintillation counting. Dissociation constants were determined using Prism (GraphPad) [79]. The amount of active IRP1 was determined by titrating IRP1 with radiolabeled IRE probe to saturation. The molar quantity of IRE probe that completely saturated IRP1 was determined using the equation for single site binding kinetics. Taking into consideration the IRP1 stock protein dilution factor and the 1:1 binding stoichiometry of IRP1, the total concentration of active IRP1 was determined.

**Luciferase assay**

The ability of IRP1 mutants to repress translation was tracked *in vivo* by using yeast cells containing a luciferase reporter gene with an IRE in its 5’ UTR. Cells were grown to logarithmic phase and extracts were prepared as previously described. A standard curve was generated using extracts from cells transformed with the empty vector to yield linear measurements between 30,000 and 500,000 RLU. Subsequent measurements were made in the linear region of the standard curve [80]. All samples were measured 3-7 times for each independent experiment.
Protein concentration in cell extracts was quantified and luciferase activity was expressed as ratio between RLU and the total protein concentration.

**Limited proteolysis**

Protease sensitivity was measured by incubating apo-IRP1 with Asp-N, Chymotrypsin, Glu-C, Lys-C or Trypsin in an ice water bath for 5 minutes to 15 hours. Proteolysis was terminated by combining aliquots from the digestion reaction with an equal volume of Laemmli buffer containing 2mM PMSF. Samples were heated at 75 °C for three minutes and proteolytic fragments were separated by SDS-PAGE at 125V for 1 hour. Bands were visualized using Simply Blue Safe Stain (Invitrogen) or Silver Stain (BioRad).

Purified apo-IRP1 was mixed with chymotrypsin at an apo-IRP1:chymotrypsin ratio of 0.78:1, 121:1, or 1210:1. Aliquots of the 0.78:1 reaction were removed after 5, 10, 15, 20 and 30 minutes. Aliquots were removed every 30 minutes for 180 minutes in the reaction containing apo-IRP1 and chymotrypsin at ratio of 121:1. A single aliquot was removed after 15 hours for the sample at the apo-IRP1:chymotrypsin ratio of 1210:1.

Limited proteolysis was also performed by combining IRP1 and Lys-C at a protein to protease ratio of 111:1. The reaction was stopped after 30 minutes. Limited proteolysis was also conducted by combining apo-IRP1 and Asp-N at ratio of 43:1, after 1 hour the reaction was stopped and bands were visualized by silver staining. Apo-IRP1 and Glu-C were combined at a ratio of 2:1 and the reaction was stopped after one hour. Apo-IRP1 and trypsin were combined at a apo-IRP1:trypsin ratio of 0.375:1 and the reaction was stopped after 1 hour. In all proteolysis experiments fragments were separated using SDS-PAGE and gels were washed two times with water. Protein fragments were visualized with Simply Blue Safe Stain (Invitrogen).
Protein purification

Hexa-his tagged rabbit IRP1 was over-expressed in the low protease yeast strain BJ5465 [81]. Cells were grown overnight in YPAD media and then inoculated in SD-URA media to an OD$_{600}$ of 0.05. Once cells reached logarithmic growth phase they were inoculated to an OD$_{600}$ of 0.1 in SD media containing 2% raffinose and grown overnight. Protein expression was induced by adding galactose to a final composition of 2% and cells were grown for 6–9 hours before harvesting. Cells were harvested by centrifugation and washed once with sterile water. All purification steps were performed aerobically. Lysis was performed by manual shaking with 0.04–0.06 mm diameter glass beads and the lysate was centrifuged at 14,000 rpm for 1 hour at 4°C. The supernatant was filtered, passed through a column containing Ni-NTA resin (Qiagen) and washed with 500 mM NaCl in 20 mM sodium phosphate pH 6.3. Protein was eluted from the column with 500 mM NaCl and 300 mM imidazole in 20 mM sodium phosphate buffered to pH 6.3. The eluate was dialyzed overnight against 5 mM NaCl and 100 mM Tris, pH 8.0. Subsequently, the dialysate was loaded on a WPQUAT anion exchange column and eluted with a linear 5–500 mM gradient of NaCl. Fractions containing ≥ 90% pure IRP1 were pooled and dialyzed overnight against 100 mM NaCl, 1 mM DTT, 100 mM Tris, pH 7.4 and 10% glycerol [82]. The extinction coefficient of IRP1 was determined from the IRP1 protein sequence using the program ProtParm [83]. Protein concentration was determined by measuring absorbance at 280 nm and using the extinction coefficient IRP1 of 89,500 M$^{-1}$ cm$^{-1}$. Samples were checked for monodispersity using the Malvern Zetasizer Nano-S dynamic light scattering apparatus. Samples along with their corresponding dialysis buffers were stored at -80 °C until needed.

Small angle x-ray scattering

All scattering experiments were conducted at the BioCAT beamline 18-ID of the Advanced Photon Source, Argonne National Laboratory [84]. Protein concentrations of 0.5, 1.0
and 2.0 mg/mL IRP1 were subjected to SAXS analysis. At least three samples for each protein concentration were analyzed. Scattering data sets were collected at a $q$ range between 0.006 and 0.356 Å$^{-1}$. Samples were loaded in a quartz capillary flow cell and exposed to 12 keV x-rays for 1 second in 5 second intervals, twenty times. The temperature of the flow cell was maintained at 23 °C. Scattering data were measured using a CCD detector positioned 2422 mm from the sample. Radiation damage was monitored by analysis of the scattering data and by analysis of protein samples SDS-PAGE. Data reduction was performed using Igor-Pro (WaveMetrics Inc., Lake Oswego, OR, USA) equipped with custom SAXS analysis tools designed for the BIO-CAT beamline.

**Ab initio modeling**

The program GASBOR [85] was used to construct 30 dummy-residue models from the scattering curves of apo-IRP1. The DAMAVER [86] suite of programs was used to select a set of closely corresponding models, superimpose them and generate a final composite model. An envelope representation of the final bead model was prepared with the program Situs [87].

**Rigid-body modeling**

The IRP1 molecule (PDB ID: 3SNP, molecule A) was divided into 3 parts: a static core (domains 1 and 2), and the two flexing domains 3 and 4. Rigid-body modeling was performed by two separate methods using the programs RigiMOL [88] and SASREF [89]. A total of 11 connectivity restraints were applied to the rigid body system. These restraints were designed to retain the continuity of the polypeptide backbone and establish connections between interdomain atom pairs whose relative distances were predicted to not change upon domain rotation.
Comparison of scattering curves

Scattering curves of computer generated models were compared to scattering curves of the experimental data using equation 3 [90]. \( N_{\text{obs}} \) represents the number of data points, \( I_{\text{obs}} \) is the experimental scattering intensity, \( \sigma_{\text{obs}} \) is the observed error and \( I_{\text{cal}} \) is the theoretically determined scattering intensity.

\[
\chi = \sqrt{\frac{1}{N_{\text{obs}}} \sum \left( \frac{I_{\text{obs}} - I_{\text{cal}}}{\sigma_{\text{obs}}} \right)^2}
\]

Generation of hypothetical intermediate structures

The program RigiMOL was employed to generate 50 hypothetical structures of apo-IRP1. These structures were in conformations ranging from the cytosolic aconitase state to a hyper-extended state. Hypothetical models were generated such that domains 3 and 4 were flexed away from the molecules center of mass in 2 to 3 degree increments to yield progressively extended forms apo-IRP1. The scattering curves of the intermediate structures were compared to the experimental scattering data using the program CRY SOL [90].

Ensemble optimization method

The ensemble optimization method (EOM) utilizes the fact that the scattering curve of flexible multi-domain proteins arise from multiple protein conformations in solution. EOM analysis generates a scattering curve from a pool of predetermined structures using the following equations. In equation 4, the scattering intensity of each model in the ensemble is calculated. \( I_n(s) \) is the scattering intensity of the nth molecule in the ensemble and \( s=4\pi \sin \theta/\lambda \). In equation 5, the scattering curve of the ensemble model is compared to the experimental data. \( K \) represents the
number of experimental points $\sigma(s_j)$ is the standard deviation and $\mu$ is the scaling factor. A chi value around or below 2 signifies that the ensemble model matches the experimental data.

\textbf{Equation 4} \\
\[ I(s) = \frac{1}{N} \sum_{n=1}^{N} I_n(s) \]

\textbf{Equation 5} \\
\[ \chi^2 = \frac{1}{K-1} \sum_{j=1}^{K} \left[ \frac{\mu(s_j) - I_{\text{exp}}(s_j)}{\sigma(s_j)} \right]^2 \]

A pool of fifty hypothetical structures of IRP1 was generated such that molecules ranging from the compact globular state to a hyperextended state were represented. Theoretical scattering curves of the models were generated using the program CRYSOL. The EOM was applied using an ensemble size of 50 and a 1000 generations were run [91].

\textbf{Dynamic light scattering} \\
Dynamic light scattering experiments were conducted on solutions of apo-IRP1 using the Malvern Zeta Sizer Nano S. Purified apo-IRP1 was measured at concentration of 1.09 mg/mL in a buffer containing 100 mM NaCl, 1 mM DTT, 100 mM Tris, pH 7.4 and 10 % glycerol. Samples were centrifuged at 10,000 g for 10 minutes and subsequently filtered through a 0.1 µM filter. Samples were allowed to equilibrate to 25 °C prior to data collection. At least three measurements were made for each sample.
III. THE SOLUTION STRUCTURE OF APO-IRON REGULATORY PROTEIN 1
**Introduction**

Crystal structures of IRP1 in the cytosolic aconitase conformation and in complex with RNA provide a foundation to understand the mechanism by which IRP1 can act as both an enzyme and a RNA binding protein [55, 56]. In this study, small angle x-ray scattering experiments were conducted in order to determine the solution structure of apo-IRP1 and to expand knowledge regarding the molecular mechanisms of IRP1 conformation change. Prior to this study, the structural and biochemical properties of apo-IRP1 were characterized by limited proteolysis, analytical ultracentrifugation (AUC) and small angle neutron scattering experiments. However, these studies only provided a loose characterization of apo-IRP1 structure.

Limited proteolysis studies provided insight to understand the structural properties of apo-IRP1, cytosolic aconitase and the IRP1-IRE complex. In the presence of chymotrypsin apo-IRP1 is degraded into four major fragments (Figure 7). However, under the same experimental conditions cytosolic aconitase is resistant to degradation by chymotrypsin [66]. Studies examining degradation of the IRP1/IRE complex by proteases revealed that the RNA partially shields IRP1 from proteolysis [92].

Analytical ultracentrifugation and small angle neutron scattering experiments revealed the biophysical solution properties of apo-IRP1. The AUC experiments provided evidence that IRP1 is capable of forming dimers in solution. Additionally, hydrodynamic frictional ratios reported in the AUC studies indicated that apo-IRP1 is a globular and slightly elongated protein [93]. Neutron scattering experiments corroborate the AUC experiments by showing that the radius of gyration for apo-IRP1 decreases upon the acquisition of either the iron sulfur cluster or RNA [94].

By using a sophisticated series of experimental approaches to analyze the SAXS data for apo-IRP1, high quality biophysical data was obtained. Subsequent analysis of the SAXS data by *ab initio* modeling revealed the low resolution solution structure of apo-IRP1. Analysis of the
SAXS data using the ensemble optimization method (EOM) revealed the distribution of apo-IRP1 conformers in solution.
Results

Purification of apo-IRP1

Hexa his-tagged rabbit IRP1 was over expressed in *S. cerevisiae* strain BJ5465. A two step process was used to purify IRP1 [82]. First, nickel chelate affinity chromatography was used as the first purification step (Figure 9). Next, anion exchange chromatography was used to fractionate IRP1 (Figure 10). Approximately 1L of cell culture yielded 1 mg of apo-IRP1.

![Figure 9. Purification of his-tagged apo-IRP1 by nickel chelate affinity chromatography. SDS-PAGE analysis of all purification steps are shown. Lane 1: Molecular weight marker. Lane 2: crude cell extract, lane 3: column flow through, lane 4 and 5: wash, lane 6: eluate.](image-url)
Figure 10. *Purification of apo-IRP1 by anion exchange chromatography*. Apo-IRP1 was loaded on WP-QUAT column and eluted with a linear gradient of NaCl from 20 mM to 500 mM. A. Molecular weight marker and fractions. B. Continued fractionation.
Dynamic light scattering

Dynamic light scattering experiments were conducted on solutions of purified apo-IRP1 (Figures 11 and 12). Analysis of the dynamic light scattering data revealed three individual peaks (Figure 12). The first peak has a mode at 0.2916 nm and contributes to approximately 7.5% of the total scattering intensity. The second peak has a mode centered on 4.253 nm and contributes to approximately 78.2% of the total scattering intensity. The third peak at 156.3 nm is associated with a particle of an extraordinarily high molecular weight and contributes 13.8% of the overall scattering intensity. The molecular weight of peak two corresponds to the molecular weight of IRP1. Additionally, the overall polydispersity index of this peak is 14.7%, a strong indication that the scattering molecule is monodisperse (Table 5).

Figure 11. Correlogram of wild type apo-IRP1. A solution of apo-IRP1 was analyzed by dynamic light scattering. The time for the signal to decay is plotted.
Figure 12. Dynamic light scattering of wild type apo-IRP1. A solution containing apo-IRP1 was analyzed using dynamic light scattering. This histogram depicts the intensity of scattering particles of various sizes.

<table>
<thead>
<tr>
<th></th>
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</table>

Table 5. Analysis of dynamic light scattering of apo-IRP1. Mode of \( r_H \) values for all three peaks. The percent polydispersity (%Pd) defines distribution of scattering particles within a given peak. Molecular weight (MW) provides and estimation of the mass of the scattering particle of the corresponding peak. The intensity (% intensity) describes the total intensity contributed by each peak. The % Mass describes the total mass that the scattering particle contributes to in the solution.
Small angle x-ray scattering

SAXS scattering profiles of apo-IRP1 were measured at protein concentrations of 0.54, 1.09, and 2.18 mg/ml at scattering ranges ($q$) of 0.065–0.356 Å$^{-1}$ (Figure 13 A). The radius of gyration ($R_g$) for apo-IRP1 is 33.6 ± 0.3 Å as determined from solution scattering profiles at a $q R_g$ limit of 1.3 using the Guinier approximation (Figure 13 B).

Figure 13. Small angle x-ray scattering of IRP1. A. Scattering intensity of apo-IRP1 at 0.54 mg/mL (brown), 1.09 mg/ml (orange) and 2.18 mg/ml (green). B. The Guinier plots of apo-IRP1 at three different concentrations 0.54 mg/mL, 1.09 mg/m and 2.18 mg/ml.
The scattering intensities agreed with the Guinier approximation at the two lowest concentrations but the 2.18 mg/ml samples had poor fits at the lowest scattering angles and were not used in further analysis.

**Pair distribution function**

Pair distribution functions were calculated for apo-IRP1 using the experimental scattering data, the crystal structures of IRP1-IRE complex (PDB ID: 3SNP) and cytosolic aconitase (PDB ID: 2B3X) (Figure 14). The shape of the pair distribution function for apo-IRP1 is very similar to that for the protein component in the IRP1-IRE complex and clearly distinct from that for cytosolic aconitase. Apo-IRP1 has the largest $D_{\text{max}}$ of 118 Å, the IRE-bound form of IRP1 has a smaller $D_{\text{max}}$ of 108 Å and cytosolic aconitase has the smallest $D_{\text{max}}$ of 92 Å.

![Image of pair distribution functions](image)

**Figure 14.** Pair distribution functions IRP1 in different conformations. Black, cytosolic aconitase (PDB ID: 2B3X). Green, IRP1 (PDB ID: 3SNP). Red, apo-IRP1.
**Ab initio structure of apo-IRP1**

Thirty *ab initio* models of apo-IRP1 were generated from the small angle x-ray scattering data using the program GASBOR (Figure 15) [85]. The normalized spatial discrepancy provides a quantitative measurement of the similarity among models. Out of a pool of thirty models, two models had normalized spatial discrepancy values greater than the acceptable $2\sigma$ threshold and were not used in further analysis. The average normalized spatial discrepancy among all structures was 1.2, indicating an acceptable degree of similarity among the models. As shown in figure 15, multiple *ab initio* reconstructions using the apo-IRP1 scattering data yield similar dummy residue models. Additionally, comparison of the *ab initio* scattering curves with the experimental data revealed a strong correlation. Chi values provide a quantitative assessment of the similarity between the scattering curve of the experimental data and the dummy atom models (Table 6). The average chi value among all scattering curves is 1.2 indicating an acceptable degree of similarity. A superimposition of 28 *ab initio* dummy residue models is presented in figure 16A. This model contains a large amount of low occupancy dummy atoms that are not consistently found in all structures. Removal of these low occupancy dummy atoms produces an unambiguous low resolution model of apo-IRP1. The molecular envelope for the final model apo-IRP1 is displayed in figure 17 along with envelopes and crystal structures for cytosolic aconitase and the protein component of the IRP1-IRE complex.
Figure 15. *Ab initio* models of apo-IRP1. Scattering data was used to generate dummy residue models of apo-IRP1 using the program GASBOR. Eight out the 28 models are shown above.
Table VI

CHI VALUES FOR GASBOR MODELS

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<th>Ab initio model</th>
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<tr>
<td>30</td>
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</tr>
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</table>

Corresponding models are shown in figure 15
Figure 16. Composite and filtered *ab initio* models of apo-IRP1 Dummy atoms (2.4 Å green spheres) were used to approximate the low resolution molecular structure of apo-IRP1 A. Composite of 28 dummy atom models. B. Non overlapping dummy atoms were removed from the composite model and a molecular envelope that closely fits the dummy atom model was added.
Figure 17. Low resolution molecular envelopes for the \textit{ab initio} model and crystal structures. The molecular envelopes for the three different forms of IRP1 are shown in this figure. A. The molecular envelope of apo-IRP1 was calculated from the experimental scattering data. B. The theoretical molecular envelope of the protein component from the IRP1-IRE complex (PDB ID: 3SNP). C. The theoretical molecular envelope for cytosolic aconitase was calculated from the crystal structure (PDB ID: 2B3X).
Rigid-body modeling

Rigid-body modeling was performed using the program SASREF [89]. Three rigid bodies were used to approximate the structure of apo-IRP1 against the experimental scattering data. The first rigid body consisted of the core domain and included residues: 2-87, 119-146, 171-367, 605-630. The second rigid body consisted of domain 4 and consisted of residues: 88-118, 147-170, 631-889. The last rigid body consisted of domain 3 and included 368-604 (Figure 18). The orientation of rigid bodies relative to each other was established by applying connectivity restraints based upon continuity of the polypeptide backbone and distances between domains (Table 7A). The scattering curves of rigid body models were compared to experimental scattering data, yielding an average χ value of approximately 1.2 (Table 7 B), indicating good agreement with the experimental data. Superimposition of the α-carbon trace of all seven models revealed an RMSD of 3.4 Å (Figure 19).

Figure 18. Diagram of isolated domains used in rigid body modeling. Components from the crystal structure of IRP1 were divided into three rigid body domains, the core, domain 3 and domain 4.
TABLE VII
CONECTIVITY RESTRAINTS AND CHI VALUES OF RIGID BODY MODELS

A.

<table>
<thead>
<tr>
<th>Rigid bodies connected</th>
<th>Linked residues</th>
<th>Distance (Å)</th>
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<td>1 and 3</td>
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<td>10</td>
</tr>
<tr>
<td>1 and 3</td>
<td>604, 605</td>
<td>10</td>
</tr>
<tr>
<td>2 and 3</td>
<td>88, 87</td>
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<tr>
<td>2 and 3</td>
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<tr>
<td>2 and 3</td>
<td>146, 147</td>
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</tr>
<tr>
<td>2 and 3</td>
<td>170, 171</td>
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</tr>
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<td>2 and 3</td>
<td>631, 630</td>
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<td>2 and 3</td>
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<td>2 and 3</td>
<td>820, 47</td>
<td>50</td>
</tr>
<tr>
<td>3 and 1</td>
<td>328, 600</td>
<td>50</td>
</tr>
<tr>
<td>3 and 1</td>
<td>365, 581</td>
<td>50</td>
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</table>

B.

<table>
<thead>
<tr>
<th>Model</th>
<th>Chi value</th>
<th>Total number of restraints</th>
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<tbody>
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</table>

Table 7. Connectivity restraints used for rigid body modeling. Rigid body domains were spatially oriented relative to each other using connectivity restraints. Restraints were based on continuity of the polypeptide backbone and relative positions of residues located on different domains. A. Table of residues and maximum distance that the pair can be located from one another during rigid body modeling. B. Chi fit for seven individual rigid body models and corresponding number of restraints for the fit.

Figure 19. Rigid body model of apo-IRP1. Least squares superimposition of the c-alpha backbone of seven apo-IRP1 rigid body models. The average RMSD among all models was 3.4 Å.
Comparison of ab initio and rigid body models

In order to cross validate the modeling results, the apo-IRP1 rigid body model was superimposed against an envelope representation of the dummy-atom model. All domains of the rigid body model aligned within the molecular envelope (Figure 20). The NSD value from this superimposition was 0.94, indicating that the ab initio and rigid body models were similar.

Figure 20. Comparison of ab initio and rigid body models. The ab initio model and rigid body model were superimposed and NSD values were calculated. The NSD of the superimposition was 0.96 indicating a good fit between the models.

Intermediate conformations of apo-IRP1

Theoretical models of full-length IRP1 in varying degrees of extension were generated from the crystal structures of cytosolic aconitase (PDB ID: 2B3X) and RNA-bound IRP1 (PDB ID: 3SNP) by rotating each domain stepwise by 2 – 3 degrees [88]. The chi values between each model and the experimental data were calculated and plotted (Figure 21). The results show that structures with a radii of gyration less than 32 Å or greater than 33 Å have poor fits to the experimental scattering data. Consistent with the ab initio and rigid body modeling results, structures with a radius of gyration between 32.6 and 33.1 Å had the lowest chi values, indicating a good fit between these models and the experimental data (Figure 21).
Figure 21. Chi vs. Rg plot of hypothetical apo-IRP1 structures. Plot of chi values of fifty hypothetical structures of apo-IRP1 compared to experimental scattering data. Short dashed lines represent the radius of gyration of cytosolic aconitase and long dashed lines represent the radius of gyration the protein component of the IRP1-IRE complex.

**Ensemble optimization method**

The ensemble optimization method was employed to determine the contribution of different conformers of IRP1 to the experimental scattering data. Fifty structures ranging from the closed conformation of IRP1 to a hyper-extended conformation comprised the pool of structures to which the scattering curves of IRP1 were compared. The results show that molecules of IRP1 are narrowly distributed around a radius of gyration of about 33.5 Å (Figure 22). A minor fraction of IRP1 molecules are distributed around a radius of gyration of 31.6 Å. The final scattering curve representing the ensemble of structures was compared to the scattering curves of apo-IRP1.
The chi value between the two curves is 1.35, suggesting both scattering curves are similar (Figure 23).

**Figure 22. Ensemble optimization method analysis of apo-IRP1.** EOM analysis was performed using the experimental SAXS scattering data. The pool of structures included 50 evenly distributed structures with Rg values ranging from approximately 28-36 Å. The final model shows that the scattering data consist of structures predominantly distributed between a Rg values of 33-34 Å. A smaller population is distributed between 31-32 Å. The dashed lines represent the Rg values for c-aconitase (28.7 Å) and apo-IRP1 (33.6 Å).
Figure 23. Fit of the experimental scattering curve with the scattering curve of the ensemble of structures. The curve of the experimental scattering data (black) was compared to the scattering data of the ensemble of structures (red).
Discussion

In this study the low resolution solution structure of apo-IRP1 was determined using SAXS. Both *ab initio* models and rigid body models show that apo-IRP1 adopts an open conformation that is similar to IRP1 bound to IRE. Additionally, the results provide new insight regarding the distribution of molecules of apo-IRP1 in solution. Consistent with previous neutron scattering experiments, these results show that apo-IRP1 is elongated compared to both cytosolic aconitase and the IRP1-IRE complex. Taken together, the findings from this study provide a framework to postulate the mechanism of IRP1 conformation change and develop a global picture of how the structure of apo-IRP1 facilitates its function.

The *ab initio* method generates molecular models using “dummy atoms” from the experimental scattering data. It provides robust and accurate molecular structures at resolutions around 20 Å. Analysis of the experimental SAXS data in this study by *ab initio* and rigid body modeling reveals the structure of apo-IRP1. Superimposition of the *ab initio* model with the crystal structure of the protein component of the IRP1-IRE complex and subsequent quantitative analysis of the fit between the structures shows that the NSD value is 1, a clear indication that the structures are similar. Rigid body modeling using SAXS data and the domains obtained from IRP1 crystal structures are consistent with structures obtained from *ab initio* modeling. Since two different experimental approaches yielded the same result, it can be concluded that apo-IRP1 adopts an open conformation in solution.

The open conformation of apo-IRP1 depicted by both *ab initio* and rigid body models appears particularly suitable for binding IRE-RNA stem loops. Only local rearrangements of the protein and nucleic acid may be required to achieve complex formation as observed in the IRP1-IRE crystal structure [56, 95]. Complex formation *in vivo* may require a more complicated model than complex formation *in vitro*. First, molecular crowding theory argues that flexible
macromolecules favor their closed conformations [96, 97] in cells. Therefore, if IRP1 is in the open conformation under crowded conditions, then different mechanisms may be at play in the IRP1-IRE association. This can be investigated through SAXS experiments on IRP1 and IREs with the appropriate crowding agents [98].

Understanding the in vivo conversion of apo-IRP1 to cytosolic aconitase is also challenging because iron sulfur cluster biogenesis is a complex process involving multiple steps [99]. The cluster assembly proteins would conceivably need access to a large surface area of IRP1 in order to access cluster ligating cysteine residues. Thus, the open form of apo-IRP1 seems amenable for the initial steps of iron sulfur cluster assembly and insertion. The understanding of the structural biology of iron sulfur cluster biogenesis is in its infancy and the few successful investigations rely on experimentally designed mutations for complex characterization and stabilization [100, 101]. The sequence of events immediately following cluster insertion in IRP1 can only be speculated: whether there is an open intermediate of assembled cytosolic aconitase or how it closes is not known. It is generally accepted that the holo forms of iron-sulfur cluster proteins are more stable than their apo counterparts, so one would expect spontaneous closure for the sake of solvent exclusion and stability.

The results from this study provide new insight toward the solution distribution of molecules of apo-IRP1. EOM analysis is designed to determine the contribution of molecules with different conformations to the experimental scattering data. Thus, this method was applied to determine if apo-IRP1 exists in multiple conformational states [91, 102]. Analysis of SAXS data using the ensemble optimization method shows that the majority of apo-IRP1 molecules are narrowly distributed between Rg values around 33 –34 Å (Figure 20). A small peak accounting for 4% of the structures was detected around 31 Å. It is conceivable that this peak is a less stable form of apo-IRP1 because it is 24 times less abundant than the main peak. Whether or not this
subset of structures can be converted to cytosolic aconitase or if it is capable of binding RNA remains to be determined. No structures with fully-closed conformations were present in the final ensemble, suggesting that the formation of the closed apo-protein is an energetically less favorable process than the formation of the open apo-protein. This observation is consistent with the research showing that other multidomain proteins and enzymes typically adopt an open conformation in the absence of their ligands [61, 103].

Analysis of the scattering data using the Guinier approximation reveals that the radius of gyration of apo-IRP1 is 33.6 ± 0.3 Å, whereas, the radii of gyration for cytosolic aconitase and the IRP1-IRE complex are 28.7 Å and 33.4 Å respectively. Based on rigid body analysis, apo-IRP1 can theoretically have a maximal $R_g$ of approximately 36Å. However, $R_g$s greater than 36 Å could only be achieved through an unlikely physical separation of the domains.

Several investigations have indicated that aconitases are capable of forming dimers in solution [104, 105]. Under the experimental conditions used in this study, dimerization was neither observed in dynamic light scattering nor in SAXS experiments. Only minor higher-order aggregation was observed at protein concentrations of ~20 μM. Aggregation was also observed in previous neutron scattering experiments [94] which may have been caused by either the high protein concentration or differences in the buffer composition. A likely explanation behind previous dimerization observations is that the phenomena is a result of intermolecular disulfide bond formation between the solvent exposed cysteine residues of apo-IRP1.

The flexible and hinged architecture of IRP1 enables it to bind IRE and to act as an enzyme [56]. Proteins evolve for functional purpose. IRP1 is not an exception, having adapted to the unusual role of binding two completely unrelated ligands. Based on molecular taxonomy of other aconitases, it is likely that IRP1 was an enzyme long before it acquired IRE binding functionality [51, 54]. The open conformation may have been initially useful for cluster insertion rather than IRE binding because the closest homologues of IRP1 are the aconitases from plants.
and the aconitases-A from bacteria, organisms which do not have functioning IRP-IRE systems [106].

Most members of the aconitase superfamily [54] retain the basic four domain organization, only a subset exhibit odd variations in placement such as permuted sequence order. The similar domain architecture raises the question of whether all proteins in the IRP-aconitase A family are capable of adopting an open conformation. Interestingly, it has been observed that bacterial aconitases interact with polynucleotides [6, 107]. By analogy to IRP1, high-affinity nucleic acid binding would require domains three and four to rotate away from one another. Although it is an exciting possibility that other aconitases offer clamshell-like access for purposes of cluster assembly and IRE binding, there is as yet no definitive evidence to support this speculation. SAXS experiments with other apo-aconitases may provide answers regarding conformational variability in the aconitase superfamily.

A clear picture of the mechanism of conformation change emerges when the findings from this research are considered with the crystal structures of the IRP1-IRE complexes and cytosolic aconitase (Figure 16) [55, 56, 95]. Taken together these results indicate that both cluster-ligating and IRE-binding surfaces are exposed to solvent. Exposure of these functionally important surfaces enables IRP1 to rapidly respond to the physiological state of the cell. In cells with sufficient iron, cysteine residues would be able to bind an iron sulfur cluster and subsequently undergo a large conformation change whereby domains 3 and 4 rotate toward each other. Conversely, in cells containing low levels of iron, apo-IRP1 is in an optimal conformation that allows for immediate interaction with the IREs. In contrast to the large transformation from apo-IRP1 to cytosolic aconitase, shifting from the apo to the IRE-binding form would require minimal rotations of domains 3 and 4 in order to stabilize the high affinity protein-IRE interactions.
IV. THE ROLE OF THE β4-α4 MOLECULAR HINGE IN THE STRUCTURE AND FUNCTION OF IRON REGULATORY PROTEIN 1
Introduction

Cytosolic aconitase and IRP1 crystal structures reveal the presence of two molecular hinges which are hypothesized to allow domains 3 and 4 to undergo rigid body domain rotation. The β4-α4 molecular hinge contains an axis about which domain 4 rotates. The β4-α4 molecular hinge is composed of an α-helix and a β-strand that establishes a connection between domains 1 and 4. When IRP1 is bound to an IRE, the hinge contains a type II’ β-turn conformation [108]. Conversely, in the cytosolic aconitase conformation, residues 86-89 that were previously in the type II’ β-turn conformation adopt an α-helix conformation.

Currently, the mechanistic details of IRP1 conformation change are not well characterized and the specific components of the protein which are important for conformation change are unknown. Based on crystal structures of cytosolic aconitase and IRP1 it was predicted that the β4-α4 molecular hinge is involved in the conformation change of IRP1 and that the transition between the α-helix and β-turn secondary structures is a crucial component of the structural transformation. The hypothesis is that mutations designed to stabilize the α-helical structure of the β4-α4 molecular hinge would drive IRP1 to adopt a closed conformation, prevent RNA binding and stabilize aconitase activity. In order to test this hypothesis, two mutations were designed to stabilize the α-helix by replacing helix destabilizing residues at glycine 90 and proline 92 with helix stabilizing alanine residues. Another mutation was introduced to convert aspartate residue 87 to proline. When IRP1 is bound to IRE, aspartate 87 is located in
Figure 24. The secondary structure of the β4-α4 region for the two different forms of IRP1. The above diagram shows the sequence of the β4-α4 molecular hinge and the corresponding secondary structure when IRP1 is in the c-aco or in a complex with RNA.

Figure 25. Crystal structure of the α4 helix in cytosolic aconitase and the IRP1-RNA complex. A. α4-helix in c-aconitase B. α4-helix and β-turn in the IRP-RNA complex C. The β-turn in IRP1-RNA complex. Dashed lines represent α-helix hydrogen bonds.
position two of the β–turn (Figure 25 C). This position requires that the amino acid have a positive ϕ angle. Since proline can’t accommodate the required positive ϕ angle at this position, it is anticipated that this mutation will prevent the formation of the β-turn. Additionally, the aspartate to proline mutation at position 87 is in an ideal location to stabilize the amino terminus of the α-helix [109].

Four variants of the β4-α4 hinge mutants were generated. Three variants contained the single mutations G90A, P92A and D87P. One variant contained the two helix stabilizing mutations G90A/P92A. This set of proposed mutations will be used to determine if the presence of multiple helix stabilizing mutations has a synergistic effect on both structure and function of IRP1.

The effects of the β4-α4 mutations on the structural properties of apo-IRP1 were probed by small angle x-ray scattering and limited proteolysis experiments. Additionally, a series of biochemical assays were used to characterize aconitase and RNA binding function of the mutant proteins.
Results

Purification of apo-IRP1 mutants

The his-tagged IRP1 hinge mutants were purified by nickel chelate affinity chromatography and anion exchange chromatography (Figures 26-29). IRP1 purified by nickel affinity chromatography produced IRP1 of 80% - 90% purity. Anion exchange chromatography was used to fractionate the protein sample using a linear salt gradient. This method successfully separated most of contaminating proteins from IRP1 and allowed for the selection of highly pure fractions. All of the β4-α4 hinge mutants behaved similarly to the wild-type protein in both steps of purification. Small quantities of a 40 kDa protein often co-eluted with IRP1. Subsequent analysis of this product by mass spectroscopy revealed its identity to be glyceraldehyde 3-phosphate dehydrogenase (G3PDH). The low levels of contaminating G3PDH did not interfere with subsequent biochemical or biophysical measurements.

The effect of hinge mutations on the SAXS properties of apo-IRP1

The biophysical properties of apo-IRP1 hinge mutants were determined by small angle x-ray scattering. The scattering curves of all mutants were flat in the low q region and scattering intensities decreased exponentially at mid-range q values (Figure 30). Guinier analysis shows that scattering intensities are linear up to a q•Rg limit of 1.3 for all mutants (Figure 31). Only negligible deviations from linearity are evident at Q^2 values below approximately 0.2.

Radii of gyration were determined from the Guinier plots. The D87P, G90A, P92A, and G90A/P92A mutants had radii of gyration of 32.56 ± 0.62 Å, 33.68 ± 0.73 Å, 33.37 ± 0.59 Å, and 32.80 ± 0.33 Å respectively (Figure 32). Pair distribution functions were calculated for all hinge
mutants and each mutant had a skewed asymmetrical curve (Figure 33). The Dmax for each mutant was within the range of 112 – 118 Å.

**Figure 26. Purification of the apo-IRP1 D87P mutant.** A. Isolation of apo-IRP1 D87P by nickel chelate affinity chromatography. B. Protein was loaded on WP-QUAT column and eluted with a linear gradient of NaCl ranging from 20 mM to 500 mM. C. Continued fractionation from panel B. The arrow indicates bands that coincide with apo-IRP1.
Figure 27. Purification of the apo-IRP1 G90A mutant. A. Isolation of apo-IRP1 G90A by nickel chelate affinity chromatography. B. Apo-IRP1 G90A was loaded on a WP-QUAT column and eluted with a linear gradient of NaCl ranging from 20 mM to 500 mM. The arrow indicates bands that coincide with apo-IRP1.
Figure 28. Purification of the apo-IRP1 P92A mutant. A. Isolation of apo-IRP1 P92A by nickel chelate affinity chromatography. B. Apo-IRP1 P92A was loaded on a WP-QUAT column and eluted with a linear gradient of NaCl ranging from 20 mM to 500 mM. C. Continuation of elution from panel B. The arrow indicates bands that coincide with apo-IRP1.
Figure 29. Purification of the apo-IRP1 G90A/P92A mutant. A. Isolation of apo-IRP1 G90A/P92A by nickel chelate affinity chromatography. B. Apo-IRP1 was loaded on a WP-QUAT column and eluted with a linear gradient of NaCl ranging from 20 mM to 500 mM. The arrow indicates bands that coincide with apo-IRP1.
Figure 30. Scattering curves of the apo-IRP1 hinge mutants. Small angle scattering curves for apo-IRP1 hinge mutants A. D87P B. G90A C. P92A D. G90A/P92A.
Figure 31. Guinier plots of the apo-IRP1 hinge mutants. Plots of apo-IRP1 hinge mutants. Guinier plots are displayed apo-IRP1 hinge mutants A. D87P B. G90A C. P92A D. G90A/P92A.
Figure 32. Radius of gyration for apo-IRP1 hinge mutants. Radius of gyration for WT and hinge mutants were determined from Guinier plots.
Figure 33. Pair distribution functions of apo-IRP1 hinge mutants. Pair distribution functions are displayed for apo-IRP1 hinge mutants A. D87P B. G90A C. P92A D. G90A/P92A.
**Ensemble optimization method analysis**

Fifty theoretical structures of IRP1 were generated ranging from the closed conformation to a hyper-extended conformation. For each of the hinge mutants, the ensemble optimization method was applied to determine the fraction of molecules within the pool that contributed to the experimental scattering intensity (Figure 34). The D87P sample had a bimodal distribution with one peak centered between 31 and 33 Å. A second minor peak was detected between 35 Å and 36 Å. Similarly, the G90A/P92A mutant had a bimodal peak. The minor peak was around 31.5 Å and the major peak was around 33.5 Å. The G90A sample had a single major peak between approximately 33 and 34 Å. Similarly, the P92A mutant had a single major peak between 33 and 34 Å. A small tail extended from the major peak and ended at 35 Å. The agreement between the scattering curves of each ensemble was quantified using chi values. The curves corresponding to the D87P mutant had a chi value of 1.56. The curves for the G90A mutant had a chi value of 1.63. The curves for the P92A mutant had a chi value of 2.12 and the G90A/P92A curve had a value of 2.17 (Figure 35). These chi values indicate that the ensemble model calculations are robust and have a good fit to the experimental scattering data.

**The effect of β4-α4 hinge mutations on apo-IRP1 protease susceptibility**

The effect of mutating the β4-α4 hinge region was analyzed by limited proteolysis experiments. The proteases Glu-C, Asp-N, Lys-C, Trypsin and Chymotrypsin (Figures 36 - 41) were used to probe for structural differences between WT apo-IRP1 and hinge mutants. Cleavage of apo-IRP1 with LysC yielded two fragments of 85 kDa and 15 kDa for both the wild type and all the hinge mutants (Figure 36 D). Cleavage with
**Figure 3.4. Ensemble optimization method for β4-α4 hinge mutants.** Frequency of selection for different conformers of apo-IRP1 from a pool of structures with Rg ranging from approximately 28 – 35 Å. A. D87P B. G90A C. P92A D. G90A/P92A. The dashed lines represent the R_g values for c-aconitase (28.7 Å) and apo-IRP1 (33.6 Å).
Figure 35. Comparison of scattering curves for ensemble and experimental models. The scattering intensity of the ensemble models was compared to the scattering intensity of the experimental model.
Glu-C yielded a complex digestion pattern consisting of 5 predominant fragments with molecular weights corresponding to 80 kDa, 64 kDa, 41 kDa and 35 kDa (Figure 36 B). No difference in protease susceptibility was observed between the wild type and hinge mutants upon treatment with Glu-C. Cleavage of apo-IRP1 with trypsin yields a complex digestion pattern consisting of multiple bands with molecular weights corresponding to 85 kDa, 64 kDa, 40 kDa, 45 kDa, 30 kDa and 15 kDa (Figure 36 C).
Cleavage of wild type and hinge mutants of apo-IRP1 with chymotrypsin yields 4 main bands with molecular weights corresponding to 71 kDa, 29 kDa, 50 kDa, and 42kDa. Limited proteolysis of G90A and P92A mutants yielded proteolytic susceptibility patterns similar to wild type. Interestingly, limited proteolysis experiments with the D87P and G90A/P92A mutants reveal conspicuous differences when compared to the wild type protein. Notably, the 71 kDa and 29 kDa bands of the D87P and G90A/P92A mutants have a decreased intensity compared to the wild type. Analysis of the kinetics of the appearance of the p29 band relative to the full length IRP1 shows that the p29 band of both the D87P mutant and the G90A/P92A protein appear at much slower rate than wild type protein (Figure 42 A). Another difference in the digestion patterns is that the 42 kDa band is more prominent in the D87P and G90A/P92A mutants than in the wild type protein. Analysis of the ratio between the 50 kDa and the 42kDa fragments shows that the ratio is lower for the both the D87P and the G90A/P92A mutants (Figure 42 B).

Mass spectroscopy was utilized to further characterize the protease susceptibility of the D87P and the G90A/P92A mutants (Figure 43). The peak ratios for the major cleavage products were consistent with the SDS-PAGE results. Interestingly, a 58 kDa peak was visible in samples from both the D87P and the G90A/P92A double mutant but not in samples of the wild type protein (Figure 42).
Figure 37. Chymotrypsin susceptibility of WT apo-IRP1. WT apo-IRP1 was digested with chymotrypsin at a 121:1 protein:protease ratio. Aliquots were removed in 30 min intervals for 180 minutes and subsequently visualized by SDS-PAGE.
Figure 38. Protease susceptibility of D87P apo-IRP1. D87P apo-IRP1 was digested with chymotrypsin at a 121:1 protein:protease ratio. Aliquots were removed in 30 min intervals for 180 minutes and subsequently visualized by SDS-PAGE.
Figure 39. **Protease susceptibility of G90A apo-IRP1.** WT apo-IRP1 was digested with chymotrypsin at a 121:1 protein:protease ratio. Aliquots were removed in 30 min intervals for 180 minutes and subsequently visualized by SDS-PAGE.
Figure 40. Protease susceptibility of P92A apo-IRP1. WT apo-IRP1 was digested with chymotrypsin at a 121:1 protein:protease ratio. Aliquots were removed in 30 min intervals for 180 minutes and subsequently visualized by SDS-PAGE.
Figure 41. **Protease susceptibility of G90A/P92A apo-IRP1.** G90A/P92A apo-IRP1 was digested with chymotrypsin at a 121:1 protein:protease ratio. Aliquots were removed in 30 min intervals for 180 minutes and subsequently visualized by SDS-PAGE.
Figure 42. Analysis of apo-IRP1 chymotryptic digests. A. Time course of the Full length IRP1:p29 ratio. B. Graph of p50:p42 band ratios after 180 minutes digestion with chymotrypsin.
Figure 43. Mass spectroscopy of apo-IRP1 proteolytic fragments. IRP1 was incubated with chymotrypsin at a ratio protein to protease ratio of 121:1 at 4°C. After 150 minutes the samples were analyzed using MALDI-TOF. A. The spectra for WT apo-IRP1 with predominant peaks indicated by arrows. B. The spectra for the D87P digestion with 4 predominant peaks indicated.
by arrows C. The spectra for the G90A/P92A mutant with predominant peaks indicated by arrows.

**The effects of hinge mutations on aconitase activity**

The effects of hinge mutations on the aconitase function of IRP1 were assessed using an aconitase deficient yeast strain. Aconitase deficient yeast fail to grow on media lacking glutamate due to their inability to generate a precursor in glutamate biosynthesis, α-ketoglutarate. Transformation of aconitase deficient yeast cells with IRP1 enables cells to grow in the absence of glutamate because IRP1 catalyzes the conversion of citrate to isocitrate [110]. A second enzyme, isocitrate dehydrogenase catalyzed the conversion of isocitrate to α-ketoglutarate that is necessary for restoration of glutamate prototrophy.

**Figure 44. Glutamate dependent growth of IRP1 hinge mutants.** Yeast cells expressing variants of IRP1 were grown on media containing or lacking glutamate. Cells expressing IRP1 mutants D87P failed to grow on media lacking glutamate. The G90A, P92A and G90A/P92A grew similar to wild-type on media lacking glutamate.
Figure 45. Growth kinetics of yeast cells expressing IRP1 hinge mutants in media lacking glutamate. Yeast cells expressing variants of IRP1 hinge mutations were grown in media lacking glutamate. IRP1 mutants G90A, P92A, and G90A had a decreased growth rate compared to wild type, whereas, IRP1 D87P failed to grow on media lacking glutamate.

TABLE VIII
DOUBLING TIMES OF YEAST CELLS EXPRESSING IRP1 HINGE MUTANTS

<table>
<thead>
<tr>
<th>IRP1 construct</th>
<th>Doubling time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>7.5 ± 1.4</td>
</tr>
<tr>
<td>D87P</td>
<td>No growth</td>
</tr>
<tr>
<td>G90A</td>
<td>8.7 ± 2.3</td>
</tr>
<tr>
<td>P92A</td>
<td>7.9 ± 1.4</td>
</tr>
<tr>
<td>G90A/P92A</td>
<td>9.3 ± 2.3</td>
</tr>
</tbody>
</table>
Figure 46. Aconitase activity in 0615d extracts containing IRP1 mutants. A. Conversion of isocitrate to cis-aconitate was measured in cell extracts. B. Conversion of isocitrate to cis-aconitate after iron sulfur cluster reconstitution. C. Western blots from extracts expressing IRP1 hinge mutants (left to right empty vector, wild type, D87P, G90A, P92A, G90A/P92A).*ND Not detected.
On solid media lacking glutamate, cells expressing IRP1 with the hinge mutations G90A, P92A or G90A/P92A grew similarly to wild type. Conversely, cells transformed with either empty vector or D87P mutant failed to grow (Figure 44). The doubling time of cells growing in liquid media lacking glutamate was also determined. No difference in growth rate was observed between cells transformed with the P92A mutant compared to cells transformed with the wild type protein. Cells containing wild type IRP1 and the P92A mutant had the fastest growth rates (Figure 45). An intermediate growth rate was observed in cells transformed with the G90A or the G90A/P92A mutants (Table 8). Cells expressing IRP1 with the D87P mutation failed to grow during the entire course of the experiment.

The observed decrease in growth rate of yeast cells expressing hinge mutants was hypothesized to be directly correlated with the aconitase function of IRP1. In order to test this hypothesis the aconitase activity in cell extracts was determined using the isocitrate to cis-aconitate assay [111] (Figure 46 A). The cell extracts expressing the wild type IRP1 or the P92A mutant had similar enzyme activities. Conversely, extracts from cells containing the G90A or the G90A/P92A mutants had less than half the enzyme activity compared to wild type. Extracts prepared from cells expressing the D87P mutant had no detectable enzyme activity. To further assess the mechanism behind the observed alteration in enzyme activity, cell extracts were subjected to iron sulfur cluster reconstitution and subsequent measurement of aconitase activity (Figure 46 B). After iron sulfur reconstitution, the enzyme activity of the wild type, G90A, P92A and G90A/P92A mutants increased by at least 25 times compared to non-reconstituted extracts. Interestingly, even after iron sulfur cluster reconstitution the D87P mutant failed to show any evidence of enzyme activity. As a final step, western blots were performed using cell extracts. IRP1 was detected for wild-type and all the mutant proteins (Figure 46 C).
The effects of hinge mutations on RNA binding function

The RNA binding functionality of the IRP1 hinge mutants was investigated using a quantitative filter binding assay and an IRE-Luciferase assay [80] [112]. Yeast cells containing the IRE-luciferase gene were used to determine if the IRP1 hinge mutants were capable of repressing translation in vivo. Cells transformed with an empty vector had higher luciferase activity compared to cells transformed with wild type IRP1 (Figure 47). Luciferase activity of hinge mutants was decreased by approximately 5 fold compared to the cells transformed with empty vector. The filter binding assays show that all mutants retain picomolar RNA binding affinity. All mutants examined had a dissociation constant between 49 and 103 pM (Figure 48).

![Figure 47. IRE-Luciferase assay. IRP1 hinge mutants were transformed into a strain containing a chromosomal insertion of IRE-luciferase. All mutants have decreased lucifersase activity compared to the strain transformed with the empty vector.](image-url)
Figure 48. Filter binding assays. A IRP1 was titrated with radiolabeled Ferritin L IRE RNA. A. WT B. D87P C. G90A D. P92A E. G90A/P92A F. Table containing the dissociation constants derived from binding curves.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Kd pM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>39 ± 4</td>
</tr>
<tr>
<td>D87P</td>
<td>49 ± 15</td>
</tr>
<tr>
<td>G90A</td>
<td>100 ± 9</td>
</tr>
<tr>
<td>P92A</td>
<td>73 ± 11</td>
</tr>
<tr>
<td>G90A/P92A</td>
<td>103 ± 22</td>
</tr>
</tbody>
</table>
Discussion

In this study, the role of the β4-α4 molecular hinge in the structure and function of IRP1 was determined by mutating helix destabilizing residues in the molecular hinge to helix stabilizing residues. The SAXS and limited proteolysis experiments provide evidence that helix stabilizing mutations in the β4-α4 hinge of IRP1 can alter the structure of apo-IRP1. Additionally, both in vivo and in vitro experiments suggest that the enzymatic function of IRP1 is altered. Contrary to the original hypothesis, these mutations did not lock the protein in the closed conformation. Even so, these experiments provide novel insight toward the mechanism of IRP1 conformation change.

Small angle x-ray scattering experiments provide evidence that D87P and G90A/P92A mutations alter the structure of apo-IRP1. To investigate the effects of β4-α4 mutations on the structure of apo-IRP1, the ensemble optimization method was used to determine the contribution of different apo-IRP1 conformers to the experimental scattering data. The results show that unlike the wild type protein which consists of one predominant population (Figure 22), both the D87P and G90A/P92A mutants consist of two distinct populations of apo-IRP1 molecules (Figure 34 A and D). One population is distributed around a radius of gyration of 33.5 Å and the second population is distributed around a smaller radius of gyration of 31 Å. This altered distribution suggests that the mutations shift the equilibrium of apo-IRP1 molecules by increasing the abundance of structures that have a decreased radius of gyration relative to structures that have a radius of gyration similar to the wild type protein. This altered distribution of apo-IRP1 molecules may be accompanied by changes in the dynamic properties of apo-IRP1. Since the enzymatic function requires multiple cycles of binding and release of substrate [113], it is conceivable that any mutation that effects domain rotation may also hinder substrate binding and
release. Interestingly, the D87P mutant has a higher proportion of molecules distributed around 31 Å compared to the G90A/P92A mutant, suggesting that this mutation has a stronger effect on modifying the conformation of apo-IRP1. Furthermore, the G90A/P92A mutant has decreased enzyme activity and D87P mutant is devoid of enzymatic activity, suggesting a possible link between the altered solution distribution of molecules and enzymatic function. Unlike the aforementioned mutants, the G90A and P92A mutants have EOM distributions similar to the wild type protein, suggesting that these two mutations alone have little effect on the structure of apo-IRP1.

Comparison of the scattering curves of the ensemble models and the solution scattering data reveals that chi values all fall within a range of 1.56 – 2.17, indicating a match between the ensemble model and the experimental scattering data (Figure 35). At higher scattering angles the fit between curves deviates from the experimental scattering data. Despite this deviation, the data at high angles are within the range of experimental error. Moreover, since the analysis utilizes the data in the low angle portion of the curve, deviations at higher scattering angles do not change the interpretation of the results.

Analysis of the SAXS data for the D87P and the G90A/P92A mutants reveal a decreased mean radius of gyration compared to wild type protein (Figure 31). However, the radii of gyration for the D87P and G90A/P92A mutants are not statistically different from the wild type IRP1. This can be attributed to the fact that the difference in radii between the wild type and the mutants is only 2 fold above the errors observed in radii of gyration measurements. Nevertheless, the decreased radius of gyration is consistent with the EOM results that show a higher abundance of molecules with a decreased radius gyration compared to the wild type protein. Analysis of the pair distribution function shows that all mutant proteins have an open conformation.
Analysis of proteolytic susceptibility results

Limited protease susceptibility assays provide supporting evidence that helix stabilizing mutations in the β4-α4 hinge alter the solution conformation of apo-IRP1. In particular, limited proteolysis of the D87P and G90A/P92A mutants with chymotrypsin reveal differences in both the ratios of cleavage products and in the kinetics of cleavage compared to the wild type protein (Figure 42). Previous studies indicate that apo-IRP1 is first cleaved at W623 to generate small and large polypeptide fragments (named p29 and p71 in this study) [67]. Plots of the full length IRP1:p29 ratios as a function of time for the D87P and G90A/P92A mutants suggest that the cleavage at W623 occurs at a slower rate in these mutants compared to wildtype. An unlikely alternative explanation is that cleavage at W623 takes place at a normal rate and the p71 and p29 fragments are rapidly degraded. This scenario is unlikely because rapid degradation p71 would leave behind visible bands of the p29 fragment. Furthermore, the scenario for rapid degradation of the p29 fragment is unlikely because it does not contain any of the of three predominant cleavage sites making it extremely stable in the presence of chymotrypsin. In addition to differences in cleavage rate, a decrease in the p50:p42 band ratio is prominent for both the D87P and the G90A/P92A mutants. This decrease in band ratio is likely to arise from an initial cleavage at Y501 rather than W623. Cleavage at Y501 would generate 58 kDa and 42 kDa polypeptides. Subsequent degradation of the 58 kDa polypeptide at F133 would generate a 50 kDa polypeptide (Figure 49). The mass spectroscopy results support this interpretation by showing the presence of a 58kDa peak in the D87P and G90A/P92A chymotryptic digests. Conversely, this 58kDa peak is absent in the wild type protein, suggesting that D87P and G90A/P92A mutations confers a structural change that causes the mutants to be cleaved by chymotrypsin by an alternate pathway.

One inconsistency observed in the analysis of the protease susceptibility assays is that the molecular weights of the cleavage products from the SDS-PAGE and mass spectroscopy for the
p50 (p56 on SDS PAGE) fragments do not match. This inconsistency can be explained by the fact that polypeptides migrate according to multiple factors including shape, charge and ratio of SDS binding to the polypeptide. Incomplete denaturation or abnormal SDS binding to the polypeptide may lead to aberrant migration during electrophoresis [114]. Since the mass spectroscopy results correspond to the expected molecular weights of the peptide fragments according to the protein sequence, they represent a more accurate estimation of the molecular weights of the proteolytic fragments than the SDS-PAGE results.

**The differential cleavage pathway of the D87P and G90A/P92A mutants**

Based on the EOM and limited proteolysis experiments, it is proposed that the D87P and G90A/P92A mutants undergo two simultaneous cleavage pathways. The degradation pathways can be classified into major and minor pathways by careful analysis of the degradation patterns. The minor cleavage pathway yields low levels of the p71 and p29 fragments as a result of decreased initial cleavage at W623. Conversely, the major pathway yields high levels of the p50 and p42 fragments as a result of initial cleavage at Y501. How can we be confident regarding the distinction between the “major” and “minor” cleavage pathways? First, during early stages of the experiments it was established that the differences in cleavage pathways are dependent upon the protein:protease ratio. Inclusion of lower amounts of protease amplified the prominence of the alternate cleavage pathway for the D87P and G90A/P29A mutants while higher concentrations masked the effect. Second, further experiments indicated that differences in the cleavage patterns were independent from the extent of the degradation of the full length protein. In samples where up to 90% of the full length protein was degraded, the characteristic pattern of the alternate cleavage pathway persisted (exhibiting a concomitant increase in the respective proteolytic fragments). It is not conceivable that the D87P and G90A/P92A mutants undergo the same
cleavage pathway as the wildtype protein because the protease susceptibility clearly show
differences in the abundance of cleavage products resulting from the initial proteolysis in W623
and Y501. Taken together, the results suggest the D87P and G90A/P92A mutants confer a
structural change to apo-IRP1 that alters its susceptibility to chymotrypsin.

The relationship between EOM results and protease susceptibility assays

IRP1 mutants that have altered protease susceptibility also have an altered EOM profile.
The consistency between these two different experiments provides supporting evidence that the
D87P and G90A/P92A mutants confer structural changes to apo-IRP1. Unlike the EOM result for
wild type IRP1 which is unimodal, the EOM results are bimodal for both the D87P and
G90A/P92A mutants. The most likely interpretation of the bimodal EOM plots is that the
dynamic properties of apo-IRP1 are altered. The global structural shift depicted in the EOM
results maybe accompanied by a local structural change in the protease susceptible loop
containing W623. A decrease in the the flexibility in the loops that contain target proteolytic
residues may render them unable to adopt the conformation required to enter the active site of
chymotrypsin. Alternatively, the mutations may bury W623 in the surface of the protein,
rendering them impossible to be recognized by chymotrypin. An intersting possibility is that the
population of molecules with a radius of gyration around 31.0 Å are intially cleaved at Y501,
whereas, the molecules with the radius of gyration around 33.5 Å are intially cleaved at W623.
This interpretation is less likely because one would expect a much higher proportion of the p29
and p71 fragments that result from cleavage at W623. Thus, the observed differences in
proteolytic susceptibility are most likely to result from a more complex mechanism that can’t be
deduced by sole consideration of the EOM results. A drawback of the SAXS method is that it
only provides information about overall size and shape of the molecule. Discerning if specific
regions were altered by the hinge mutations is not possible. However, analysis, of several
IRP1:IRE crystal structures show that W623 can adopt different orientations, suggesting that this region is highly flexible (Volz K., unpublished). Moreover, in the aconitase crystal structure W623 is not solvent exposed. It is feasible that the D87P and G90A/P92A hinge mutations indirectly alter the solvent exposure of W623 and hampers the ability of chymotrypsin to target this residue.

**Analysis of global structural changes by acidic and basic proteases**

Analysis of the proteolytic susceptibility of IRP1 with LysC, GluC, Trypsin and AspN reveals no significant alteration in the proteolytic susceptibility of the hinge mutants compared to the wildtype protein. Since no major differences were observed upon digestion of the D87P and G90A/P92A mutants with multiple types of proteases, it can be deduced that changes are restricted to a very specific region of the protein. Based on these results, it is hypothesized that the D87P mutants confer a structural change that results in W623 becoming less accessible to the protease. In absence of this cleavage site Y501 becomes the the predominant target for chymotrypsin. These two events combined provide a likely explanation for the cleavage pattern of the D87P and G90A/P92A mutants.

**Specific β4-α4 hinge mutations alter the enzymatic function of IRP1**

Several independent lines of investigation provide evidence that the enzymatic function of IRP1 is altered by the β4-α4 hinge mutations. Growth of aconitase deficient yeast cells transformed with the G90A and G90A/P92A mutants was decreased compared to wild type. More interestingly, the D87P mutant completely failed to grow. Subsequent aconitase assays conducted using cell extracts revealed a similar trend. After iron-sulfur cluster reconstitution, enzyme activities from extracts expressing the wild type proteins G90A, P92A, G90A/P92A hinge mutants increased 25 fold. Surprisingly, no aconitase activity was detected in the extracts.
expressing the D87P mutant suggesting that this mutant either lacks the ability to convert substrate to product or is unable to bind an [4Fe-4S] cluster. Another possibility is that mutation of the hinge region may perturb the active site. Consistent with the latter interpretation is the observation that enzyme activity is most affected by mutations that are closest to the substrate binding residue glutamine 86. In other proteins, mutations that alter hydrogen bonding within the active site have been shown to affect catalysis [115]. Therefore, a similar mechanism may account for the altered enzyme activity of the β4-α4 hinge mutants. As a whole, in vivo and in vitro aconitase assays indicate that the D87P, G90A, and G90A/P92A molecular hinge mutants alter the enzymatic function of IRP1.

The RNA binding function of IRP1 β4-α4 hinge mutants

The RNA binding function of the β4-α4 hinge mutants was assessed using nitrocellulose and IRE-luciferase filter binding assays. The nitrocellulose filter binding assays show that all mutants retain picomolar binding affinity. This observation is consistent with the fact that all mutants have radii of gyration that correspond with open structures. EOM results show that a subpopulation of D87P and the G90A/P92A mutants adopt conformations with a radius of gyration around 31 Å. It may be possible that the molecules with the decreased radius of gyration do not bind RNA because they are not in the optimal conformation and most likely have a decreased surface area for RNA binding. The observed RNA binding may arise solely from molecules with the radius of gyration similar to the wild type protein. A contrasting idea is that the two populations may be in a dynamic equilibrium and constantly interconvert between the compact and normal state. Accordingly, all molecules would eventually be able to interact with RNA with high affinity.
Analysis of secondary structure changes in the $\beta_4$-$\alpha_4$ molecular hinge

One assumption in these experiments is that the G90A and P92A mutants exerts its effect through stabilization of the $\alpha$-helix secondary structure. The EOM and biochemical experiments indicate that the double mutants have a greater effect on both the structure and enzymatic function compared to the single mutants. For example, monomodal distributions were observed in EOM analysis of the G90A and P92A apo-IRP1 mutants. However, when these two mutations are combined, the EOM distribution shift to a bimodal distribution, characterized by the appearance of a peak a around 31 Å. The synergistic effect conferred by these mutations may be caused by restoration of multiple hydrogen bonds required for $\alpha$-helix formation. This observation is consistent with the hypothesis that inclusion of helix stabilizing residues would alter the conformation of apo-IRP1. Another hypothesis is that the G90A and P92A single mutants induce formation of an $\alpha$-helix in the molecular hinge but do not alter the global conformation change. Some credence can be given to this hypothesis because a decrease in enzyme activity was observed, which could signify structural aberrations near to the active site. If the $\beta_4$-$\alpha_4$ molecular hinge changes its secondary structure, this may be manifested as a change in the dynamic properties of IRP1 that effect the binding and release of substrate. Elucidation of the crystal structures for the mutant apo-IRP1 would allow us to compare the secondary structures and hydrogen bonding patterns of the single and double mutants.

In this study, the role of the $\beta_4$-$\alpha_4$ hinge in the structure and function of apo-IRP1 was investigated. The original hypothesis was that the $\beta_4$-$\alpha_4$ mutations would drive the protein to adopt a closed conformation, prohibit RNA binding and stabilize aconitase activity. Contrary to original the hypothesis, these experiments show that the helix stabilizing $\beta_4$-$\alpha_4$ hinge mutations do not lock the apo-IRP1 in the closed conformation. Rather, the D87P and G90A/P92A mutations exert subtle effects on the structure of IRP1 that are characterized by a decrease in
average radius of gyration and the appearance of a small subpopulation of apo-IRP1 molecules with a decreased radius of gyration. Finally, these mutations show no significant effect on RNA binding function. This study provides the first preliminary characterization of a molecular hinge of IRP1 that is involved in its conformational change. On a broader spectrum these results expand knowledge regarding mechanisms that multidomain proteins utilize to achieve domain closure.
Figure 49. Schematic depicting proposed degradation pathways of the D87P and the G90A/P92A hinge mutants. Analysis of proteolytic fragments by SDS-PAGE and mass spec of IRP1 samples D87P and G90A/P92A degraded with chymotrypsin suggest an altered cleavage mechanism compared to the wild type protein. Here it is proposed that D87P and G90A/P92A mutants are degraded by two simultaneous pathways. The major pathway cleaves the protein at Y501 to yeild p58 and p42 fragment. Subsequent degradation of the p58 fragment yields the p50 fragment. In the minor pathway the initial cleavage occurs at W623. Subsequent cleavage of p71 yeilds the p50 fragment. Cleavage of p50 at Y501 yeilds a 42 kDa fragment. Red stars represent fragments visible by SDS-PAGE.
V. GENERAL CONCLUSIONS AND FUTURE DIRECTIONS
Apo IRP1 adopts an open conformation in solution

Apo-IRP1 is the first form of the protein that interacts with the cytosol. Therefore, the ability of apo-IRP1 to rapidly sense the cellular environment and change its conformation is important for the regulation of cellular iron homeostasis. The SAXS experiments show that apo-IRP1 adopts an L-shaped conformation in solution. The L-shape conformation is suitable to accommodate stem-loop IRE’s. Only local structural rearrangements and rotation of domains three and four by 2 to 3 degrees would be required for apo-IRP1 to bind RNA. Conversely, in order to achieve domain closure as observed in cytosolic aconitase, domains three and four would need to rotate by 60° and 30°, respectively.

Interestingly, the EOM results show that molecules of apo-IRP1 are narrowly distributed around a radius of gyration of 33.5 Å in solution. This narrow distribution was unexpected because large differences were observed in the orientation of domains three and four in crystal structures of the cytosolic aconitase and the IRP1-IRE complex. Yet, experiment results indicated that only a small subpopulation of molecules accounting for 4% of the total wild type apo-IRP1 conformers had a conformation different from IRE bound IRP1.

Insight regarding the case of IRP1 structural interconversion

The issue concerning structural interconversion of apo-IRP1 in-vivo is complex. Previous experiments show that treatment of cells with iron sulfur cluster perturbants increases total cellular IRE binding without concomitant production of new protein [116]. Moreover, it has been shown that addition of iron to IRE-IRP1 complexes weakens the interaction between the protein and RNA [117]. Together, these results highlight the potential for structural interconversion. Other experiments indicate that cytosolic aconitase can bind IRE with a nanomolar affinity [66]. Although intriguing, it may be possible that RNA binding observed in the aforementioned
experiment is caused by contaminating IRP1, thus more rigorous experimentation is required to identify a IRP1 - [4Fe-4S] - IRE complex.

In this study, no evidence for large scale conversion between structures similar to cytosolic aconitase and apo-IRP1 was observed. Based on the SAXS results, the open conformation appears to be the most stable form. Structural interconversion may involve complex factors such as molecular crowding, accessory proteins or molecules that were not present under the SAXS experiment conditions. It is possible that the iron sulfur cluster, substrate and the molecular hinge all play a role in the domain closure of IRP1. Previous studies indicate that the [4Fe-4S] cluster plays a major role in the domain closure of IRP1. However, the mechanism by which the [4Fe-4S] induces domain closure is perplexing because all iron ligating cysteine residues of IRP1 are located on the same domain within mere angstroms of one another. Thus, it is unlikely that cluster assembly alone establishes cross domain interactions necessary for maintaining domain closure. Rather, the iron sulfur cluster may be one component of several that forms a structural link between residues located on different domains. The substrate could provide a bridge between the cluster binding domain and other domains of the protein. Analysis of opposing domains shows that domain 4 is of particular interest because it provides both a mechanism for domain closure through the molecular hinge and sites for interaction with the substrate. An interesting theoretical mechanism for domain closure is that initial formation of the iron sulfur cluster may only induce partial closure of IRP1. Complete domain closure may be caused when the substrate comes in contact with the substrate binding residue of the β4-α4 molecular hinge. Indeed, several studies have exemplified that association of substrates with molecular hinges can serve as a trigger for conformation change [118].
Possible mechanisms of IRP1 domain closure

To understand the mechanism of IRP1 conformation change, the results from this study must be compared to the current theories that aim to describe domain closure of multidomain proteins. The diffusional collision theory and the sequential model of domain closure both describe possible mechanisms of domain closure. The diffusional collision theory stipulates that the ligand binds to one domain and subsequent diffusion and collision of domains results in a stabilizing interaction between domains. The results from this study show that IRP1 is predominantly in one conformation in solution with less than 4% of the protein adopting a conformation with a decreased radius gyration. Additionally, the EOM results for the wild type apo-IRP1 show that no molecules with closed conformations were detected, suggesting that the diffusion collision model of domain closure is not applicable in the case of IRP1. The sequential model of domain closure stipulates that the ligand drives the protein to the closed conformation [118] and conformation change occurs after the ligand binds to specific amino acids in the binding domain. Special residues in the binding domain interact with the substrate. Since these residues are commonly located near to molecular hinges it is proposed that interaction with the substrate induces domain closure. Glutamine residue 86 of IRP1 meets the criteria to be a closure inducing residue because it is situated in the β4-α4 molecular hinge of IRP1 and forms interactions with the carboxyl group of the substrate. Interaction between glutamine 86 and the substrate may induce the torque that causes domain 4 to rotate. This hypothesis can be tested by examining the SAXS profiles of IRP1 with mutations at glutamine 86 in the presence and absence of substrate.
**Time resolved SAXS may provide additional details about the mechanism of conformation change**

SAXS experiments provide an ideal experimental platform to elucidate the mechanism of conformation change due to its ability to capture multiple conformation states of molecules in solution. Time resolved SAXS experiments [119-121] can be applied to track the conversion of apo-IRP1 to cytosolic aconitase upon iron sulfur cluster reconstitution. One complication that may arise from this type of experiment is the formation of insoluble iron sulfide aggregates. This problem could potentially be solved by using a capillary flow cell connected to a filter designed to remove aggregates immediately before flowing through the measurement compartment. EOM analysis of the time resolved scattering data against a library of structures corresponding to all plausible conformations of IRP1 may reveal the conformational landscape of proteins undergoing domain closure.

**The β4-α4 hinge mutants alter the enzyme activity of IRP1**

The results from this study show that IRP1 containing mutations D87P, G90A, and G90A/P92A have altered enzyme activity compared to wild type protein. Two plausible mechanisms by which enzyme activity becomes altered include perturbation of active site geometry and inability of the enzyme to undergo the conformational changes necessary to bind and release substrate. Determination of crystal structures of the IRP1 hinge mutants should provide information regarding the active site geometry. Further investigations directed toward the determination of Km values and examining the temperature dependance on the enzyme activity should provide information on whether the mutant proteins have an altered ability to bind and release substrate. In the case of the D87P mutant, the loss of enzyme activity may be attributed to
the inability to bind an iron sulfur cluster. Electron paramagnetic resonance (EPR) experiments should provide a clear answer to whether the D87P mutant binds an iron-sulfur cluster.

Several experiments were conducted with the aim of characterizing the specific enzyme activity of IRP1. Enzyme activity was quantifiable relative to the total protein concentration using both isocitrate and cis-aconitase assays. However, quantification of specific activity proved to be a challenge because less than 5% of the protein was able to be converted to cytosolic aconitase by \textit{in vitro} iron sulfur cluster reconstitution. Several methods were applied to quantify iron content. Iron chelation experiments were used to quantify iron content after iron sulfur cluster reconstitution. However, the values were too close to the detection limit of the assay and did not provide useful data. As an alternative, UV-Visible spectroscopy was used to determine if the proteins contained an iron sulfur cluster. The [4Fe-4S] cluster of cytosolic aconitase has a characteristic broad absorbance around 340 nm. However, after reconstitution of the protein, the characteristic peak was not detected. Since iron sulfur proteins are relatively sensitive to oxygen, it was hypothesized that aerobic purification had a negative impact on the amount of active protein. Therefore, attempts were made to increase the percent of active protein by purifying IRP1 anaerobically. However, anaerobic purification of IRP1 did not yield a greater quantity of active protein. A possibility exists that the \textit{S. cerevisiae} expression system is not the best for producing fully active cytosolic aconitase. Overexpression of IRP1 in a bacterial or an insect cell based expression system may yield cytosolic aconitase with a higher composition of enzymatically active protein.

\textbf{The altered proteolytic susceptibility of IRP1}

The protease susceptibility results show that the D87P and G90A/P92A mutants are initially cleaved at Y501 instead of W623. The mechanism that accounts for the altered
susceptibility is unknown. However, it is clear that the alteration in protease susceptibility is not a direct effect of mutagenesis because the β4-α4 hinge is located 40 Å away from W623 and 23 Å away from Y501. A plausible hypothesis is that the D87P and G90A/P92A mutants indirectly alter either the solvent exposure of W623 or the flexibility of the loop containing W623. In the cytosolic aconitase crystal structure W623 is not solvent exposed. In other crystal structures, W623 has a variable position (Volz, unpublished data). A crystal structure of either the D87P or G90A/P92A mutant should provide information regarding the position of W623. Additionally, fluorescence quenching of tryptophan residues can be used to determine the proportion of tryptophan residues exposed to the solvent and to characterize the conformational flexibility of the linker region containing W623[122, 123].

**Analysis of the α28-α29 hinge as a target for future study**

In this study, emphasis was placed on the characterization of the β4-α4 molecular hinge. However, IRP1 contains another molecular hinge that includes the axis of rotation for domain 3. This molecular hinge is comprised of helix destabilizing residues P606 and G607. The helix destabilizing residues in the linker hinge of IRP1 were replaced with the corresponding leucine and serine residues of IRP2. These mutations were predicted to stabilize the open conformation of apo-IRP1. The enzyme activity was measured in cell extracts expressing the linker hinge mutants. The G607S mutant had similar activity to wild type. Conversely, the P606L and the P606L/G607S mutants had decreased enzyme activity compared to the wild type protein. These results suggest that flexibility at the linker hinge may be an important factor in the enzyme functionality of IRP1. Unlike the β4-α4 mutations which may alter enzyme activity because of its close proximity to the active residues, the linker hinge is unlikely to exert a direct effect on active site residues because it is located approximately 27 Å from the active site residues. Thus,
interpreting the enzyme assay results from the α28-α29 mutants would be more straight forward. Further analysis of these mutants could provide answers regarding why mutation of molecular hinges alter the enzyme activity of IRP1.

Currently, it is unclear if domains 3 and 4 rotate independently from one another or if the domain motion is linked. Preliminary SAXS experiments with the α28-α29 mutants indicate that the radius of gyration is increased compared to wild type. SAXS characterization of the structural properties of the α28-α29 mutants alone or in combination with the D87P or G90A/P92A mutant may provide additional insight to the mechanistic aspects of domain rotation.

**Physiological implications of the hinge mutations**

A physiologically important link between the molecular hinge and the cellular environment is established via interaction between the substrate and residues belonging to the molecular hinge. Previous studies have implicated a role for the [4Fe-4S] in IRP1 conformation change. However, it remains unknown how domain closure is established and maintained by an iron sulfur cluster which binds to three cysteine residues all located on the same domain [55, 56]. It is likely that other factors are required to form stabilizing interactions with opposing domains. The results from this study implicate a novel role for the β4-α4 hinge in domain closure. In light of this discovery, it is worthwhile to test a new model that integrates the [4Fe-4S] cluster, molecular hinge and cell physiology into the mechanism for IRP1 domain closure.

A plausible model for IRP1 domain closure is one where the L-shaped apo-protein responds to physiologically high iron concentrations by acquiring an iron sulfur cluster and undergoing partial domain closure. Subsequent binding of the substrate would induce complete domain closure. Although it is commonly believed that cluster acquisition alone induces complete
domain closure, my hypothesis is that the [4Fe-4S] cluster induces partial domain closure because (i) cluster binding alone does not form sufficient contacts to stabilize the interaction with opposing domains and (ii) there is no crystal structure of an aconitase devoid of substrate or molecule within the active site. This raises the question whether a bridging molecule is required to stabilize the closed conformation by providing a connection between opposing domains 3 and 4. It is conceivable that natural substrates of cytosolic aconitase act as bridging molecules because they form interactions with both the [4Fe-4S] cluster and the molecular hinge of domain 4. Association of the substrate with the molecular hinge could induce a conformation change that brings the cluster binding region on domain 3 and the substrate binding region on the molecular hinge of domain 4 in close proximity. In support of this postulation, previous studies have shown that addition of oxalomalate (a competitive inhibitor of aconitase) to cell cultures decreases the total IRE binding capacity of IRP1 [124]. This suggests that oxalomalate may interact with IRP1 and induce a conformation change.

The ability of IRP1 to maintain normal physiological iron levels depends on its ability to change conformation in response to the prevailing cellular conditions. The G90A/P92A and the D87P mutants both retain the ability to bind and regulate IREs. But how would these mutants respond in a cell that has just experienced a rapid change in iron content? Previous studies show that IRP1 responds to changes in cellular iron status in a matter of hours and that this response does not require a de novo protein synthesis [116]. This suggests that the response requires preexisting IRP1 molecules to change conformation. Since the SAXS results for the G90A/P92A and the D87P suggests a change in the dynamic properties of apo-IRP1, it may be plausible that the mutants respond at a different rate compared to the wild type protein.
In conclusion, the results from this study show that apo-IRP1 adopts an open conformation. Contrary the original hypothesis, the mutations that were tested did not lock IRP1 in the closed conformation. Rather, the effects on the global structure of IRP1 were subtle. This indicates that multiple factors in addition to β4-α4 molecular hinge are required to stabilize the closed form of IRP1. Additional factors may include the iron-sulfur cluster and substrate.
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