Biochemical analysis of essential components involved in mitochondrial and cytosolic iron-sulfur protein biogenesis in *Saccharomyces cerevisiae*

Dissertation

zur
Erlangung des Doktorgrades
der Naturwissenschaften
(Dr. rer. nat.)

dem Fachbereich Biologie
der Philipps-Universität-Marburg

vorgelegt von

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Marburg/Lahn, April 2007

Vom Fachbereich Biologie der Philipps-Universität Marburg
als Dissertation angenommen am 8.06.2007

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Tag der mündlichen Prüfung am 14.06.2007

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Marburg, 18 April 2007

Contents

Abbreviations

ε	Molar extinction coefficient
(v/v)	Volume per volume
(w/v)	Weight per volume
Amp	Ampicillin
APS	Ammonium persulfate
bp	Base pair
BSA	Bovine serum albumine
CIA	Cytosolic iron-sulfur protein assembly
cpm	Counts per minute
C-Terminus	Carboxyterminus
DCPIP	2,6-dichlorophenol-indophenol
DMPD	*N,N'*-dimethyl-p-phenylene-diamine
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
dNTPs	Desoxyribonucleosidetriphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EPR	Electron-paramagnetic-resonance
Gal	Galactose
GFP	Green fluorescent protein
Glc	Glucose
GSH	Glutathione, reduced form
HA	Hemagglutinine
His	Hexahistidinyl-tag
IPTG	Isopropyl-ß-D-thiogalactoside
ISC	Iron-sulfur cluster assembly
kb	Kilobases
kDa	Kilodalton
LB	Luria-Bertani-medium
NADH	Nicotinamid-adenine-dinucleotide, reduced
N-Terminus	Aminoterminus
OD_{600}	Optical density at a wavelength of λ= 600 nm
PCR	Polymerase chain reaction
PEG	Polyethylenglycol
PMS	Post-mitochondrial supernatant
PMSF	Phenylmethylsulfonyl fluoride
rpm	Rotations per minute
RT	Room temperature
SC	Minimal-medium („synthetic complete medium“)
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrylamide-gel electrophorese
TAP	Tandem affinity purification
TCA	Trichloroacetic acid
TEMED	*N,N,N´,N´,*-Tetramethylethylendiamine
Tris	Tris(hydroxymethyl)-aminomethane
UV-VIS	Ultraviolet-visible (spectroscopy)
WT	Wild-type

Summary

Iron-sulfur (Fe/S) clusters are inorganic cofactors of many proteins found in nearly all prokaryotic and eukaryotic organisms. Fe/S proteins play important roles in different cellular processes, such as electron transport, enzyme catalysis or gene regulation. Eukaryotes contain Fe/S proteins in mitochondria, chloroplasts, cytosol and nucleus. In *S. cerevisiae* 3 different machineries cooperate to synthesise Fe/S proteins. The mitochondrial ISC-assembly machinery is required for maturation of all cellular Fe/S proteins, whereas the mitochondrial ISC-export and the cytosolic CIA-machineries are specifically involved in the formation of cytosolic and nuclear Fe/S proteins. The ISC-assembly machinery consists of more then ten proteins that were identified and characterized over the last eight years.

In the first part of this study Isd11 was identified as a novel component of the mitochondrial ISC-assembly machinery. Isd11 is an essential protein of 11 kDa localized in the mitochondrial matrix and conserved only in eukaryotes. Depletion of Isd11 using gene-regulated yeast strains resulted in impaired activities of mitochondrial (e.g., aconitase, complex II) and cytosolic (Leu1) Fe/S enzymes. Strong defects were also observed in the *de novo* maturation of several mitochondrial, cytosolic and nuclear Fe/S proteins indicating that Isd11 is required for the biogenesis of all cellular Fe/S proteins. Many yeast mutants defective in members of the ISC-assembly and export machineries display misregulated iron homeostasis manifested by accumulation of iron within mitochondria and induction of genes involved in cellular iron uptake via the activation of the transcription factors Aft1/Aft2. Here, it was shown that cells defective in Isd11 have impaired iron homeostasis. These cells show an increased uptake of cellular iron as indicated by the Aft1-dependent induction of FET3, one of the genes involved in cellular iron uptake. Mitochondria isolated from such cells contain three-fold more iron than those from wild-type cells. All these data indicated that Isd11 represents a novel component of the ISC-assembly machinery.

Isd11 forms a stable complex with the cysteine desulfurase Nfs1 and also can interact with the scaffold protein Isu1. Nfs1 catalyses cysteine desulfuration to provide the sulfur needed for the synthesis of Fe/S clusters on Isu1/2 scaffolds. Surprisingly, Isd11 is not required for Nfs1 activity *in vitro*. However, depletion of Isd11 resulted in a strong reduction of Fe/S cluster formation on Isu1 indicating a function in early steps of biogenesis *in vivo*. Although, Isd11 is not needed for Nfs1 desulfurase activity, it is likely that the Isd11-Nfs1 complex is the physiological cysteine desulfurase, as both proteins are required for the Fe/S cluster assembly on Isu1. In summary, these studies have identified and characterized Isd11 as an essential, novel component of the ISC machinery required for sulfur release from cysteine and Fe/S cluster assembly on the Isu1 scaffold protein.

The second part of the present study was focused on the Nar1 protein. Nar1 is a component of the newly identified CIA-machinery. Biogenesis of extra-mitochondrial Fe/S proteins requires the CIA-machinery, which besides Nar1 encompasses at least three other proteins, Cfd1, Nbp35 and Cia1. Yeast Nar1 is a highly conserved protein with relation to Fe-only hydrogenases and contains eight conserved cysteines, four of them at the N-terminus and the other four at the C-terminus that in hydrogenases are the coordinating ligands of the medial [4Fe-4S] cluster and of the H-cluster. At the beginning of this study it was known that Nar1 is an essential cytosolic protein that is required for the maturation of cytosolic and nuclear Fe/S proteins, but not of mitochondrial Fe/S proteins. Nar1 is an Fe/S protein itself, most likely containing two magnetically coupled Fe/S clusters. Thus, Nar1 is both a target and a component of the CIA-machinery. Therefore, it was important to know whether the conserved cysteine residues are involved in coordination of the two Fe/S clusters.

Using site-directed mutagenesis, it was demonstrated that three of the four N-terminal cysteines (C59A, C62A and C65) are essential residues for yeast cell viability. Exchange of the cysteine residues to alanine (or serine) indicated their important role for Nar1 function in the maturation of cytosolic Fe/S proteins, such as Leu1 or Rli1 as analysed by ^{55}Fe incorporation experiments or by measurements of the Leu1 enzyme activity. To see whether these cysteines are directly involved in the coordination of an Fe/S cluster, ^{55}Fe radiolabelling experiments were performed and the incorporation of ^{55}Fe into Nar1 was analysed. Mutation of three of the N-terminal cysteines resulted in a loss of Fe/S cluster association. Mutation of the forth N-terminal cysteine residue (C20A) showed no effect, yet the combined mutation of both C20 and C65 lead to a more severe phenotype. These results indicate that all four N-terminal cysteines are ligands of an Fe/S cluster. Moreover, the data suggest that the N-terminal Fe/S cluster is required for stable insertion of the second Fe/S cluster at the C-terminus.

Surprisingly, single mutations of the C-terminal cysteines had no influence on the incorporation of the Nar1 Fe/S clusters *in vivo*. However, simultaneous exchange of two cysteine residues at the C-terminus resulted in the loss of the Fe/S cluster located at the C-terminus, whereas the N-terminal cluster was still bound. The data presented in this study clearly indicate that the N- and C-terminal cysteine residues coordinate two Fe/S clusters and that these clusters are essential for Nar1 function in the maturation of cytosolic and nuclear Fe/S proteins. Furthermore, the N-terminal Fe/S cluster was found to be more labile than the C-terminal one. An explanation for this observation was suggested by the structural model of Nar1 which was derived from the crystal structure of Fe-only hydrogenases. The calculated model shows that the N-terminal cluster is surface-exposed, whereas the C-terminal Fe/S cluster is buried inside the protein.

To define the chemical nature of the two Fe/S clusters present in Nar1, wild-type Nar1 protein was purified from *E. coli* and shown to contain up to four iron and four sulfur atoms. Since EPR studies did not provide evidence for the presence of [2Fe-2S] clusters, it is apparently not possible to isolate Nar1 from *E. coli* with a full complement of two [4Fe-4S] clusters, i.e. 8 Fe and 8 S atoms. EPR analysis of the recombinant wild-type Nar1 protein gave a rhombic signal that may indicate the presence of two magnetically interacting Fe/S clusters as previously suggested. Analysis of the C59S, C177S, C412S and C416A mutants indicated the disappearance of this rhombic EPR signal. These mutant proteins gave an EPR signal typical for $[4Fe\text{-}4S]^{1+}$ clusters. In contrast, the results obtained from *in vivo* experiments in yeast cells demonstrated that C59 is an essential residue for Fe/S cluster association and for Nar1 function. Further, single mutations of C-terminal cysteines C177A(S), C412A(S) and C416A did not show any effects on Fe/S cluster binding *in vivo*. These fundamentally different findings in yeast and in *E. coli* made it likely that *E. coli* does not produce a physiologically relevant Nar1 protein. This conclusion could previously not be obtained by analysing the wild-type Nar1, but only on the basis of the comprehensive mutational data revealed in this study. Since *in vitro* chemical reconstitution of recombinant Nar1 did not improve the iron and sulfide content of Nar1, purification from eukaryotic organisms will be needed for isolation of a physiologically relevant Nar1 protein.

Previous studies demonstrated a specific interaction of Nar1 with Cia1. To find out whether Nar1 interacts with other components of the CIA-machinery, coimmunoprecipitation experiments were performed. Nar1 specifically interacts with Nbp35. The interaction depended on the presence of functional Nar1 protein, as Nar1 mutant proteins did not show any Nbp35 association. Earlier it has been shown that two other components of the CIA machinery, Nbp35 and Cfd1 are essential for Fe/S cluster incorporation into Nar1, indicating that Nar1 must perform its function in the cytosol downstream of Cfd1-Nbp35 complex. Since, on the other hand, Cia1 is not involved in the maturation of Nar1, the latter must function upstream of Cia1. The new interaction between Nar1 and Nbp35 identified here may thus serve to functionally connect early components of the CIA-machinery (Cfd1 and Nbp35) with the late parts of this system (Cia1).

In summary, the *in vivo* and *in vitro* work on Nar1 has characterized the eight cysteines at the N- and C-termini as important residues for insertion of the two Fe/S clusters. The functional communication between the two clusters is indicated by the requirement of the N-terminal cluster for stable assembly of the C-terminal one. On the basis of these findings the molecular function of Nar1 between the CIA components Cfd1/Nbp35 and Cia1 can now be determined.

Zusammenfassung

Eisen-Schwefel (Fe/S) Cluster sind anorganische Kofaktoren zahlreicher prokaryotischer und eukaryotischer Proteine. Diese Fe/S Proteine übernehmen wichtige Aufgaben bei verschiedenen zellulären Prozessen, wie dem Elektronentransport, bei Enzymkatalysen oder bei der Genregulation. In Eukaryoten sind Fe/S Proteine in den Mitochondrien, den Chloroplasten, im Cytosol und im Zellkern lokalisiert. In der Hefe *Saccharomyces cerevisiae* wird die Reifung der Fe/S Proteine von mindestens drei komplexen Maschinerien übernommen. Eine davon ist die in der mitochondrialen Matrix lokalisierte „iron-sulfur cluster“ (ISC) Assemblierungsmaschinerie, die an der Reifung aller zellulären Fe/S Proteine beteiligt ist. Sie besteht aus mehr als zehn Proteinen, die erst in den letzten acht Jahren identifiziert wurden. Dagegen werden die mitochondriale ISC-Export- und die „cytosolic iron-sulfur protein assembly“ (CIA) Maschinerien spezifisch nur für die Reifung cytosolischer und nukleärer Fe/S Proteine benötigt.

Im ersten Teil dieser Arbeit wurde Isd11 als eine neue Komponente der mitochondrialen ISC-Assemblierungsmaschinerie in Hefe identifiziert. Isd11 ist ein essentielles Protein mit einer molekularen Masse von 11 kDa, das in der mitochondrialen Matrix lokalisiert ist. Es ist in allen Eukaryoten, nicht aber in Prokaryoten konserviert. Die Depletion von Isd11 durch regulierte Genexpression in einer Hefemutante führte zur Beeinträchtigung der Aktivität von mitochondrialen (z.B. Aconitase, Komplex II) und cytosolischen (Leu1) Fe/S Enzymen. Es wurden auch starke Defekte in der *de novo* Synthese von mitochondrialen, cytosolischen und nukleären Fe/S Proteinen beobachtet. Diese Ergebnisse deuten darauf hin, dass Isd11 an der Reifung aller zellulären Fe/S Proteine beteiligt ist.

Ein Funktionsverlust der mitochondrialen ISC-Assemblierungsmaschinerie führt zu einer Fehlregulation der zellulären Eisenhomöostase. Die Zellen akkumulieren verstärkt Eisen in den Mitochondrien, und es werden Gene induziert, die in Abhängigkeit von den Transkriptionsfaktoren Aft1/Aft2 an der zellulären Eisenaufnahme beteiligt sind. In dieser Arbeit wurde gezeigt, dass Isd11-defiziente Zellen eine fehl regulierte zelluläre Eisenhomöostase aufweisen. Die Mutanten zeigten eine Aktivierung der zellulären Eisenaufnahme, worauf die Induktion des von Aft1 abhängigen FET3 Gens hindeutete. FET3 ist eines der zentralen Gene des zellulären Eisenaufnahmesystems in *S. cerevisiae*. Mitochondrien aus Isd11-depletierten Mutanten wiesen einen dreifach höheren Eisengehalt auf als Wildtyp-Mitochondrien. Diese Ergebnisse weisen darauf hin, dass Isd11 eine neue Komponente der ISC-Assemblierungsmaschinerie ist.

Isd11 bildet einen stabilen Komplex mit der Cystein-Desulfurase Nfs1 und interagiert, vermutlich indirekt über Nfs1, mit dem Gerüstprotein Isu1. Nfs1 katalysiert die Desulfurierung von Cystein zu Alanin und stellt damit den Schwefel für die Assemblierung eines transienten Fe/S Clusters auf den Gerüstproteinen Isu1/2 zur Verfügung. Zwar wird Isd11 nicht für eine *in vitro* Aktivität von Nfs1 benötigt, doch führte die Depletion von Isd11 zu einer stark verminderten Synthese von Fe/S Clustern auf Isu1. Damit kommt Isd11 eine *in vivo* Funktion in der frühen Phase der Fe/S Proteinbiogenese zu. Obwohl Isd11 nicht für die Aktivität von Nfs1 erforderlich ist, stellt ihr Komplex die physiologische Cystein-Desulfurase dar, die zur Assemblierung eines Fe/S Clusters auf Isu1 benötigt wird. Im Ergebnis hat diese Studie Isd11 als eine neue essentielle Komponente der ISC-Assemblierungsmaschinerie identifiziert und charakterisiert. Isd11 ist für die Freisetzung von Schwefel aus Cystein und zur Assemblierung eines Fe/S Clusters auf Isu1 nötig.

Im zweiten Teil der Arbeit lag der Schwerpunkt auf der molekularen und funktionellen Charakterisierung von Nar1, einem Protein der erst vor kurzem identifizierten CIA Maschinerie. Diese Maschinerie wird für die Biogenese von extra-mitochondrialen Fe/S Proteinen benötigt und umfasst neben Nar1 noch mindestens die drei Proteine Cfd1, Nbp35 und Cia1. Nar1 der Hefe ist ein hoch konserviertes Protein, das Homologien zu bakteriellen Fe-Hydrogenasen aufweist und acht konservierte Cysteinreste enthält. Je vier davon sind am N- und C-Terminus lokalisiert. In Fe-Hydrogenasen koordinieren diese Cysteinreste zum einen das mediale [4Fe-4S] Zentrum, zum anderen den so genannten H-Cluster. Auch Nar1 ist ein Fe/S Protein und enthält vermutlich zwei gekoppelte Fe/S Cluster. Folglich ist Nar1 gleichzeitig Ziel und Bestandteil der CIA-Maschinerie. Für eine Charakterisierung von Nar1 war es wichtig zu untersuchen, ob die konservierten Cysteinreste für die Koordination der beiden Fe/S Cluster benötigt werden.

Mit Hilfe einer gerichteten Mutagenese wurde gezeigt, dass drei der vier N-terminalen Cysteinreste (C59, C62 und C65) für das Überleben der Hefe essentiell waren. Ein Austausch der Cysteinreste zu Alanin- oder Serinresten führte zum reduzierten Einbau von radioaktivem Eisen-55 (^{55}Fe) in die Fe/S-Proteine Leu1 und Rli1, sowie zum Verlust der Enzymaktivität von Leu1. Beides wies auf eine essentielle Rolle der entsprechenden Cysteinreste für die Funktion von Nar1 bei der Reifung extra-mitochondrialer Fe/S Proteine hin. Um herauszufinden, ob die genannten Cysteinreste an der Koordination eines Fe/S Cluster beteiligt sind, wurde der Einbau von ^{55}Fe in Nar1 analysiert. Die Mutation von jeweils einem der drei N-terminalen Cysteinreste führte zu einem fast kompletten Verlust an gebundenem Fe/S Cluster. Eine Mutation des vierten N-terminalen Cysteins (C20A) alleine zeigte keinen Effekt, jedoch führte die kombinierte Mutation von C20 und C65 zu einem stärkeren Phänotyp der C65 Mutante. Diese Daten legen nahe, dass alle vier N-terminalen Cysteinreste

koordinierende Liganden sind. Überdies weisen die Ergebnisse darauf hin, dass der N-terminale Fe/S Cluster für die Assemblierung des zweiten Fe/S-Zentrums benötigt wird.
Interessanterweise hatten Mutationen einzelner Cysteinreste am C-Terminus *in vivo* keinen Einfluss auf den Einbau von Fe/S Clustern in Nar1. Wurden jedoch zwei C-terminale Cysteinreste gleichzeitig ausgetauscht, so führte dies zum Verlust des C-terminalen, nicht jedoch des N-terminalen Fe/S Clusters. Damit konnte diese Studie zum einen belegen, dass die N- und C-terminalen Cysteinreste jeweils einen Fe/S Cluster koordinieren, und zum anderen, dass beide Fe/S Cluster essentiell für die Funktion von Nar1 bei der Reifung extra-mitochondrialer Fe/S Proteine sind. Darüber hinaus war der N-terminale Fe/S Cluster labiler als derjenige am C-Terminus. Eine Erklärung für diese Beobachtung ergibt sich aus der modellierten Struktur von Nar1, die aus der Kristallstruktur von Fe-Hydrogenasen berechnet wurde. Im Strukturmodel von Nar1 ist der N-terminale Fe/S Cluster zur Proteinoberfläche hin exponiert, während der C-terminale Fe/S Cluster im Inneren des Proteins verborgen liegt.

Um die chemische Natur der beiden Fe/S Cluster zu klären, wurde Nar1 in *E. coli* exprimiert. Das gereinigte Protein enthielt bis zu vier Eisen- und vier (säurelabile) Schwefelatome. Da EPR-Analysen keinerlei Hinweise auf die Anwesenheit von [2Fe-2S] Zentren ergaben, scheint Nar1 in *E. coli* unvollständig mit [4Fe-4S] Zentren produziert zu werden. Die EPR-Analyse des gereinigten Nar1 ergab ein rhombenartiges Signal, das auf die Anwesenheit von zwei interagierenden Fe/S Zentren hindeutete. Mutationen von einigen der konservierten Cysteinreste (C59S, C177S, C412S und C416A) führten zum Verlust dieses Signals und zur Bildung eines EPR Signals, das für [4Fe-4S]+1 Zentren typisch ist. Im Gegensatz dazu wiesen die oben genannten *in vivo* Experimente mit Hefezellen darauf hin, dass C59 ein essentieller Cysteinrest sowohl für die Bindung eines Fe/S Zentrums als auch für die Funktion von Nar1 ist. Weiter zeigten Mutationen einzelner C-terminaler Cysteinreste (C177A(S), C412A(S) und C416A) keinen Einfluss auf die Bindung von Fe/S Clustern in der Hefe. Diese grundlegend unterschiedlichen Beobachtungen in der Hefe und in *E. coli* legen nahe, dass Bakterien kein physiologisch relevantes Nar1 Protein produzieren. Diese Schlussfolgerung war erst durch die ausführlichen Mutationsuntersuchungen in dieser Studie möglich und konnten bisher durch alleinige Analyse der Wildtyp-Form von Nar1 nicht erhalten werden. Eine chemische Rekonstitution des rekombinanten Nar1 *in vitro* erhöhte den Eisen- und Sulfidgehalt des Proteins nicht. Daher ist für die Isolierung eines physiologisch relevanten Nar1 Proteins die Reinigung aus eukaryotischen Organismen erforderlich.

Frühere Studien zeigten eine spezifische Interaktion von Nar1 mit Cia1. Um zu klären, ob Nar1 mit weiteren Komponenten der CIA-Maschinerie interagieren kann, wurden Co-Immunpräzipitationsexperimente durchgeführt. Dabei offenbarte sich, dass Nar1 spezifisch mit Nbp35 interagiert. Diese Interaktion hing von der Funktionsfähigkeit des Nar1 Proteins

ab, da Nar1 Mutanten nicht an Nbp35 banden. Bereits zuvor war gezeigt worden, dass zwei andere Komponenten der CIA-Maschinerie, Cfd1 und Nbp35, für die Insertion der Fe/S Zentren in Nar1 benötigt werden. Zusammengenommen weisen diese Ergebnisse darauf hin, dass Nar1 seine Funktion im Cytosol erst im Anschluss an die Aktivität des Cfd1-Nbp35 Komplexes ausübt. Da andererseits Cia1 nicht an der Reifung von Nar1 beteiligt ist, muss letzteres seine Aktivität noch vor der Funktion von Cia1 entfalten. Die Interaktion zwischen Nar1 und Nbp35, die hier nachgewiesen wurde, dient daher möglicherweise dazu, eine funktionelle Verbindung zwischen frühen (Cfd1 und Nbp35) und späten (Cia1) Komponenten der CIA-Maschinerie herzustellen.

Zusammenfassend haben die *in vivo* und *in vitro* Arbeiten zu Nar1 gezeigt, dass die acht Cysteine am N- und C-Terminus wichtige Reste für die Bindung der zwei Fe/S Zentren darstellen. Die funktionelle Interaktion zwischen den beiden Fe/S Clustern wird durch die Bedeutung des N-terminalen Fe/S Zentrums für die Insertion des C-terminalen Clusters hervorgehoben. Aufgrund dieser Ergebnisse kann die molekulare Funktion von Nar1 nun zwischen den CIA-Komponenten Cfd1/Nbp35 und Cia1 eingeordnet werden.

1. Introduction

1.1. Chemistry and toxicity of iron

Iron belongs to the sub-family of transition elements and, with rare exceptions, is an essential element for nearly all studied organisms from *Archaea* to man because many cellular processes rely on proteins that contain iron or iron-containing prosthetic groups. Iron, as a transitional element can reversibly modify its oxidation state and according to this it can be found in a variety of cofactors, e.g., iron-sulfur clusters or heme. The iron-containing proteins play an important role in many metabolic processes including electron transport, regulation of gene expression, synthesis of deoxyribonucleotides, the metabolism of amino acids or oxygen transport (hemoglobin).

Despite its relative abundance in nature, the amount of bioavailable iron is very limited. At physiological oxygen concentrations the stable state of iron in most of its biological complexes is Fe^{3+} (ferric iron). Under aerobic conditions iron is rapidly oxidized to ferric oxyhydroxides with low solubility. Reduction reactions therefore have a critical role in iron metabolism because most reactions within a cell make use of ferrous iron. On the other hand, excess of free Fe^{2+} (ferrous iron) in the cell is detrimental because its ability to catalyse the Fenton reaction, which leads to the generation of toxic oxygen radicals under aerobiosis. Therefore, the organisms have developed mechanisms for iron solubilization and uptake. Thus, cellular systems involved in the uptake and utilization of iron are regulated according to the availability of iron and the cellular requirements for iron.

1.2. Iron uptake in *S. cerevisiae* and its cellular distribution

S. cerevisiae cells can take up iron using two uptake systems: high- and low affinity iron uptake systems. In *S. cerevisiae* the high-affinity system can be divided in two iron uptake mechanisms: a reductive and a non-reductive mechanism (Kosman, 2003). The reductive iron uptake mechanism depends on the reduction of ferric to ferrous iron. The reductive system of baker's yeast consists of several plasma membrane reductases, such as Fre1 and Fre2 which are involved in the reduction of Fe^{3+} to Fe^{2+}. The ferrous iron is then used as substrate for Fet3/Ftr1 complex. Fet3 is a plasma membrane Cu^{2+}-dependent ferroxidase (Hassett *et al.*, 1998) that oxidizes the Fe^{2+} to Fe^{3+} which is then transported into the cell by the Ftr1 permease (Figure 1.1) (Kosman, 2003; Philpott, 2006).

The cellular iron uptake must be regulated to avoid the accumulation of Fe^{2+} within the cell, as the ferrous iron can form highly toxic free radicals via the Fenton reaction. In *S. cerevisiae* the uptake of iron is regulated by two transcription factors, Aft1 and Aft2. Under iron-deplete conditions, Aft1 translocates from cytosol into the nucleus and activates the transcription of genes involved in Fe uptake.

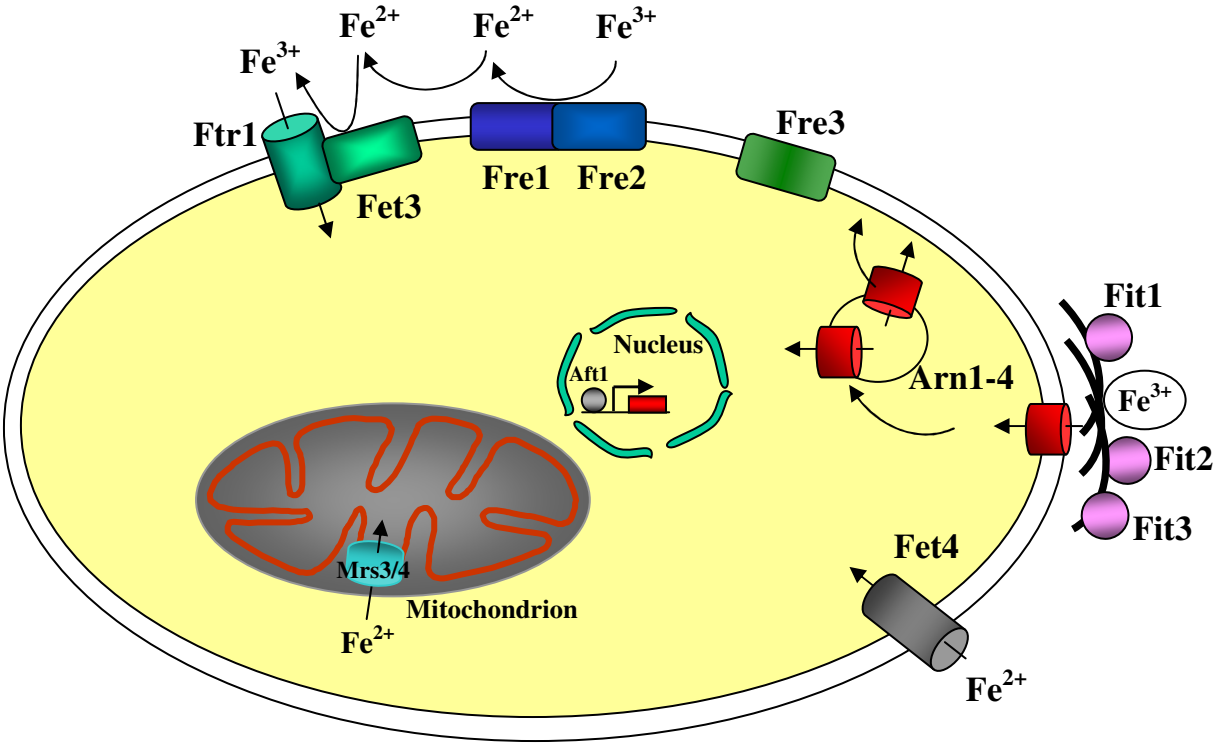


Figure 1.1 Iron uptake and its cellular distribution in *S. cerevisiae*. Iron is taking up by the yeast cell via the plasma membrane-localized high-affinity (Fet3/Ftr1) and low affinity (Fet4) iron uptake systems. Two metalreductases, Fre1-Fre2 reduce the Fe^{3+} to Fe^{2+} and supply the ferrous iron to the Fet3/Ftr1 complex. Fet3/Ftr1 complex oxidizes ferric to ferrous iron which is then imported into the cell by the Ftr1 permease activity. The Fit manoproteins in the cell wall facilitate retention of siderophore-bound iron in the cell wall. Siderophore-bound iron can be released from the siderophore and reduced by the Fre reductases. Intact siderophore-iron chelates can be taken up via members of the Arn transporter family. Inside the cell, ferrous iron is imported from the cytosol into the mitochondrial matrix by Mrs3/4 and used for the synthesis of Fe/S clusters and heme. Aft1 is the transcriptional regulator of iron uptake that under conditions of iron starvation induces the expression of genes involved in iron uptake. Adapted from (Rutherford *et al.*, 2004).

Cellular iron is mainly used by mitochondria for the synthesis of Fe/S clusters and heme. The import of ferrous iron (Fe^{2+}) into mitochondria is facilitated by the carrier proteins Mrs3/Mrs4. The Fet4-mediated iron uptake plays a more indirect role due to kinetic disadvantages, as the K_m values of 35 µM (Dix *et al.*, 1997; Dix *et al.*, 1994) are higher than those for the Fet3/Ftr1 complex (K_m- 0.2 µM) (Dancis *et al.*, 1992).

The non-reductive iron uptake involves the siderophore-mediated uptake of iron. Although, *S. cerevisiae* does not synthesize or secrete siderophores, the baker's yeast produces at least four distinct facilitators for their uptake, the Arn1-4 proteins (De Luca *et al.*, 2000; Haas, 2003; Yun *et al.*, 2001; Yun *et al.*, 2000). *S. cerevisiae* cells also express three cell wall mannoproteins (Fit1-3) which contribute to the retention of siderophore-bound iron in the cell wall and enhance the siderophore-mediated iron uptake.

1.3. Structure and cellular localization of Fe/S proteins

Fe/S clusters are bioinorganic cofactors which consist of iron cations (Fe^{2+}; Fe^{3+}) and sulfide anions (S^{2-}). The Fe/S clusters are bound to the polypeptide chain via coordination of iron ions usually by the cysteine residues, but also histidine, nitrogen or serine have been shown to represent coordination sites. The simplest and most common forms of Fe/S clusters are the rhombic [2Fe-2S] cluster and the cubane [4Fe-4S] cluster. The [2Fe-2S] clusters are present in, e.g., ferredoxins, biotin synthase or ferrochelatase. The Rieske Fe/S proteins contain a [2Fe-2S] cluster that is coordinated by two cysteine and two histidine residues. The [4Fe-4S] clusters are found in many proteins such as aconitase and aconitase-like proteins, bacterial ferredoxins and complex I and II of the respiratory chain. The complexes of the respiratory chain possess many subunits that contain Fe/S clusters. The complex I of respiratory chain contains 8 (eukaryotes, not *S. cerevisiae*) and 9 (bacteria) Fe/S clusters (Hinchliffe *et al.*, 2005). Likewise, complex II contains a [2Fe-2S], a [3Fe-4S] and a [4Fe-4S] cluster (Sun *et al.*, 2005). Many proteins contain more complex forms of Fe/S clusters. Nitrogenases possess as cofactors the so-called P-cluster, where two [4Fe-3S] clusters are linked together via cysteines. The sulfite reductase of *E. coli* contains a [4Fe-4S] cluster linked via a cysteine to the iron in a siroheme. Several proteins contain Fe/S clusters with different metals coordinated, e.g., molybdenum or nickel. An example is the FeMo cofactor in bacterial nitrogenases (Beinert, 2000; Rees, 2002; Rees *et al.*, 2003).

In yeast and other eukaryotes, Fe/S proteins are found in different cell compartments. Many Fe/S proteins are localized in mitochondria such as the well-known aconitase involved in TCA cycle, biotin synthase (Bio2) involved in the biotin biosynthesis in the mitochondrial matrix. The latter enzyme contains a [2Fe-2S] and a [4Fe-4S] cluster. Other matrix Fe/S proteins are Ilv3, Lip5 and Yah1 that play a role in the biosynthesis of amino acids, lipoic acid and Fe/S cluster synthesis, respectively. In the yeast cytosol (Figure 1.2) several Fe/S proteins are found such as the [4Fe-4S]-containing isopropylmalate isomerase (Leu1) and

sulfite-reductase (Ecm17), enzymes involved in the biosynthesis of leucine, glutamate and methionine. Rli1 is a cytosolic Fe/S protein which plays an essential role in ribosome biogenesis (Kispal *et al.*, 2005). Cfd1, Nbp35 and Nar1 are Fe/S-containing proteins involved in the maturation of extra-mitochondrial Fe/S proteins. At least one Fe/S protein shows a nuclear localization, the DNA-glycosylase Ntg2, an enzyme playing a role in DNA repair (Figure 1.2).

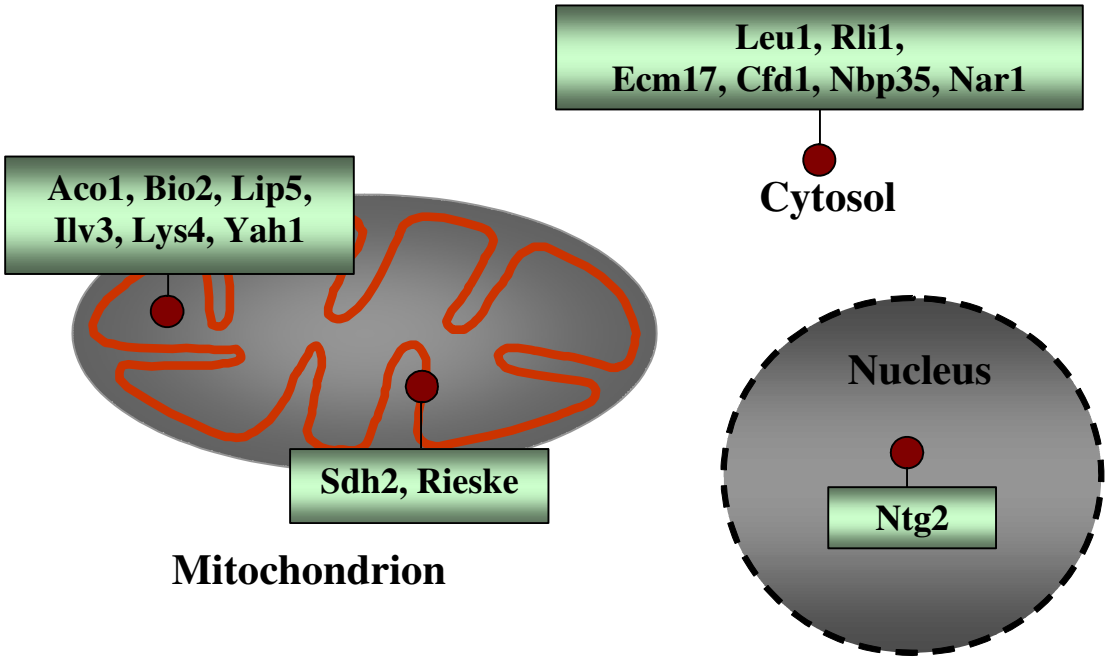


Figure 1.2 Subcellular localization of Fe/S proteins in *S. cerevisiae*. Fe/S proteins are found in mitochondria, cytosol and nucleus of yeast cells.

1.4. Function of Fe/S proteins

1.4.1. Electron transfer

One of the main functions of Fe/S proteins is in electron transfer through the ferrous iron (Fe^{2+}) and ferric iron (Fe^{3+}) oxidations states. The protein environment of Fe/S clusters enables a wide range of redox potentials from -600 to +400 mV (Beinert, 2000). Typical Fe/S proteins involved in redox reactions are found in the respiratory complexes of mitochondria, ferredoxins and plant photosystem I. An example of electron transfer is indicated by the respiratory chain complexes I and II, where electrons are transferred from the substrates, NADH and succinate, respectively, to ubiquinone and then via the Rieske Fe/S cluster and the heme centers of complex III to cytochrome c which thereby becomes reduced.

1.4.2. Catalysis

Fe/S clusters can serve also as the active sites of catalytic enzymes. A classical example for a role of Fe/S clusters in enzyme catalysis is aconitase. Aconitase, which is an essential enzyme of the citric acid cycle isomerizes citrate to isocitrate. One iron atom of the [4Fe-4S] cluster is non-coordinated and serves as a Lewis acid to bind the substrate catalysing the re-arrangement of the hydroxyl group of the substrate (Beinert, 2000). A similar mechanism to the aconitase can be found in the [4Fe-4S] cluster-containing aconitase-like proteins, such as homoaconitase (Lys4) involved in lysine biosynthesis and isopropylmalate-isomerase (Leu1) with a role in leucine metabolism.

Several enzymes of the S-adenosylmethionine (AdoMet) group utilise Fe/S clusters to initiate radical catalysis and formation of stable protein-centered radicals. These enzymes use a [4Fe-4S] to donate an electron initiating a radical-dependent reaction with the formation of an adenosylradical and methionine. Such enzymes are the biotin synthase, lysine 2, 3-aminomutase, pyruvate formate-lyase, ribonucleotide reductases (Cheek *et al.*, 2001). A similar mechanism has been established for 2-hydroxyacyl-CoA-dehydratases (Buckel *et al.*, 2004).

1.4.3. Regulatory role of Fe/S clusters

Fe/S clusters also play a regulatory role, in which they sense molecular iron, oxygen, superoxide ion, and nitric oxide concentrations. The regulatory role of several Fe/S-containing proteins is discussed bellow.

SoxR

The *E. coli* SoxRS system enhances the production of a set of proteins in response to superoxide exposure, including those involved in detoxification (manganese superoxide dismutase). This is achieved by SoxR activating expression of the transcription factor SoxS. SoxR from *E. coli* is a homodimer, each subunit containing one [2Fe-2S] cluster and is involved in sensing both, superoxide and nitric oxide stress (Green *et al.*, 2004).

Oxygen-sensor FNR

FNR is a global regulator of *E.coli* that controls expression of many genes in response to oxygen limitations. *E. coli* FNR activates the expression of genes that encode components of alternative electron transport chains and it also represses the expression of some aerobic functions under anaerobic growth conditions. In its active DNA-binding form,

FNR is a homodimer containing one [4Fe-4S] $^{2+}$ per subunit. The ability of FNR to function as a regulator depends on the integrity of the [4Fe-4S] cluster (Crack *et al.*, 2006).

IscR

IscR is a regulatory Fe/S protein that is involved in the biosynthesis and the repair of Fe/S clusters. In *E. coli,* both the *sufABCDSE* and *iscRSUA* operons are induced under oxidative stress conditions and iron starvation (Lee *et al.*, 2004; Outten *et al.*, 2004; Zheng *et al.*, 2001). IscR contains a [2Fe-2S] cluster and serves as a repressor of the *iscRSUA* gene expression under anaerobic condition as a [2Fe-2S]-bound form. Oxidative stress could cause inactivation of IscR as a repressor, leading to derepression of the operon (Schwartz *et al.*, 2001). When IscR is present in its apo-form the transcription factor retains another regulator activity, in this case becomes activator for the *sufABCDSE* operon, another Fe/S assembly system in *E. coli* (Yeo *et al.*, 2006).

IRP1

In mammalian cells, two proteins IRP1 and IRP2 play an important role as cytosolic iron sensors. Even though IRP2 contains the conserved cysteines, it cannot assemble a [4Fe–4S] cluster. IRP2 protein levels are diminished during iron-sufficient condition, but protein synthesis is unaffected by cellular iron status (Hentze *et al.*, 2004; Wallander *et al.*, 2006).

IRP1 can bind an Fe/S cluster and in this situation it functions as a cytosolic aconitase. Under iron starvation conditions that lead to the loss of the Fe/S cluster of IRP1, its apoform binds to RNA stem-loop structures (iron regulatory elements-IRE) at the 5´- or 3´-untranslated regions of specific mRNAs. Formation of an IRP1-IRE complex at the 5´-untranslated region results in the inhibition of translation and leads to low protein synthesis activity, whereas binding of IRP1 to IREs at the 3´-untranslated end in mRNAs leads to the stabilisation of mRNAs thus preventing their degradation and resulting in enhanced protein synthesis. IREs are found in the mRNAs of proteins involved in iron uptake (transferrin-receptor, DMT1), storage (H- and L-ferritin), heme synthesis (5-aminolevulinate synthase) or iron export (ferroportin).

Ferritin and transferrin-receptor are regulated in opposite ways. When cells are iron-deficient, IRP1 bind IREs with high affinity inhibiting the translation of ferritin leading to a decrease in ferritin expression and thus more iron will be available. IRP1-dependent stabilization of mRNAs containing 3′ IREs, such as transferrin-receptor mRNA leads to an increase in its abundance. When cells are iron-replete, IRP1 looses its high affinity mRNA binding capacity and, due to association with an Fe/S cluster, fails to bind to IREs. Consequently, ferritin synthesis is activated, while transferring-receptor mRNA is degraded.

1.4.4. DNA-binding proteins

Another role proposed for Fe/S clusters is the stabilisation of protein structure. The [4Fe-4S] centers in endonuclease III and Mut Y, members of the base excision repair enzyme superfamily, have been proposed to play purely structural roles (Cunningham *et al.*, 1989; Guan *et al.*, 1998; Kuo *et al.*, 1992). Recent studies have shown that the [4Fe-4S] clusters in these enzymes are invloved in recognizing, positioning and distorting duplex DNA for the base removal activity (Lukianova *et al.*, 2005). In *Saccharomyces cerevisiae*, *NTG1* and *NTG2*, encode proteins with similarity to endonuclease III. Both contain the highly conserved helix-hairpin-helix motif, whereas only Ntg2 harbors the characteristic Fe/S cluster of the endonuclease III family (Alseth *et al.*, 1999).

1.5. Biogenesis of Fe/S proteins in Bacteria

Fe/S clusters can be assembled *in vitro* on apoproteins from ferrous iron (Fe^{2+}) and sulfide (S^{2-}) in the presence of a thiol compound (such as dithiothreitol) (Malkin *et al.*, 1966). Free iron and sulfide may exist in cells, but they are toxic to the cells and therefore the intracellular free concentrations have to be rather low, as the *in vitro* chemical reconstitution requires high concentrations of iron and sulfide. Therefore, Fe/S cluster assembly in cells cannot occur spontaneously. Bacteria, as well as eukaryotes have developed systems to assemble and insert Fe/S clusters into apoproteins (Frazzon *et al.*, 2002; Johnson *et al.*, 2005; Lill *et al.*, 2005; Lill *et al.*, 2006b; Zheng *et al.*, 1998).

Fe/S cluster biosynthesis in eubacteria requires at least three separate systems containing several genes organized in operons. The first system identified was the *nif* (nitrogen fixation) system from the nitrogen-fixing bacterium, *Azotobacter vinelandii* (Frazzon *et al.*, 2003). The genes in the *nif* operon code for proteins that are involved in the assembly of Fe/S clusters of nitrogenase, a complex metalloenzyme responsible for the reduction of atmospheric N_2 to the metabolically usable NH_3. Nitrogenase consists of two components, the Fe-protein and the molybdenum-iron (MoFe) protein. The Fe-protein is a dimer that coordinates a single [4Fe-4S] cluster and serves as a specific source of electrons required for substrate reduction. The MoFe-protein is an $\alpha_2\beta_2$ heterotetramer and contains two metalloclusters, the FeMo-cofactor and the P-cluster ([8Fe-7S]). Inactivation of many *nif* genes results in defects in maturation of either the Fe-protein or the MoFe-protein, but deletion of either of two linked genes, designated *nifU* and *nifS*, uniquely results in substantial loss in activity of both the Fe-protein and MoFe-protein (Jacobson *et al.*, 1989). This was the

first indication that the *nifS* and *nifU* are involved in the formation of Fe/S clusters, because their presence is the only common feature of MoFe-protein and Fe-protein. NifS is a homodimeric pyridoxal-phosphate (PLP)-dependent enzyme that catalyses the conversion of cysteine to alanine and liberation of sulfur *in vitro*. Further studies indicated that NifS-catalysed desulfurization of L-cysteine involves formation of an enzyme-bound persulfide intermediate (Zheng *et al.*, 1994a; Zheng *et al.*, 1994b; Zheng *et al.*, 1993). NifU serves as a scaffold for Fe/S cluster assembly. NifU is a homodimer that contains one permanent [2Fe-2S] per monomer. The N- and C-terminal regions can transiently bind an Fe/S cluster *in vitro* (Agar *et al.*, 2000b; Smith *et al.*, 2005; Yuvaniyama *et al.*, 2000).

Identification of a second desulfurase activity in *A. vinelandii* led to the discovery of the *iscS* gene, which is part of the *isc* (iron-sulfur cluster) gene cluster (Zheng *et al.*, 1998). Further studies identified that the *isc* gene cluster (*iscRSUAhscBAfdx*) is conserved in almost all bacteria and represents a second system that is required for the generation of the majority of cellular Fe/S proteins and hence may serve a "housekeeping" function for Fe/S cluster assembly in bacteria (Takahashi *et al.*, 1999; Zheng *et al.*, 1998). The central players of the ISC system are the IscS and IscU which have similar functions to the NifS and NifU, respectively. IscS delivers the sulfur for Fe/S cluster synthesis, as this has been demonstrated, both *in vivo* and *in vitro* (Urbina *et al.*, 2001). IscU shows sequence similarities to the N-terminus of NifU and functions also as a scaffold protein for Fe/S clusters synthesis (Agar *et al.*, 2000a; Mansy *et al.*, 2002). The *isc* operon encodes, besides IscS and IscU, other proteins needed for Fe/S clusters synthesis. IscA may serve as an alternative scaffold, IscR is known as transcriptional repressor of the *isc* gene cluster (Ding *et al.*, 2004; Krebs *et al.*, 2001; Schwartz *et al.*, 2001). HscA and HscB are two chaperones of the DnaK/Hsp70 and DnaJ/Hsp40 families, (Seaton *et al.*, 1994) that may be involved in the release of Fe/S clusters during the maturation of target Fe/S proteins. A [2Fe-2S]-containing ferredoxin (Fdx) is also encoded by the bacterial *isc* operon and required for the maturation of cellular Fe/S proteins (Ta *et al.*, 1992a; Ta *et al.*, 1992b; Takahashi and Nakamura, 1999; Tokumoto *et al.*, 2001).

In *E. coli* deletion of the *isc* gene cluster is not lethal. This surprising finding is due to the partial complementation by a biogenesis system encoded by the *suf* operon (Takahashi *et al.*, 2002). The *suf* operon consists of six genes, *sufABCDSE*, homologues of which were found in many bacteria, cyanobacteria and plants, but they are absent in fungi. SufS and SufE form a complex that catalyses the conversion of L-cysteine into L-alanine and sulfane sulfur via the formation of a protein-bound persulfide intermediate. Only SufS displays cysteine desulfurase activity, whereas the SufE has no desulfurase activity, but it greatly enhances the

SufS catalytic activity. (Loiseau *et al.*, 2003; Outten *et al.*, 2003). SufA shows homology to IscA (Ollagnier-de Choudens *et al.*, 2003), and it functions as a scaffold protein for the Fe/S cluster synthesis, as it can transiently bind an Fe/S cluster (Ollagnier-de-Choudens *et al.*, 2004). SufC is an ATPase of the ABC superfamily and it forms a complex with SufBD to assist the assembly or repair of Fe/S clusters (Nachin *et al.*, 2001; Nachin *et al.*, 2003; Rangachari *et al.*, 2002). The SufBCD complex can enhance the SufS cysteine desulfurase activity, acting synergetically with SufE (Outten *et al.*, 2003). The SUF system does not contain any homologs to IscU or to the chaperones HscA and HscB. The SUF system may act as an alternative to the ISC machinery for Fe/S cluster assembly, especially under stress conditions such as oxidative stress and iron starvation (Fontecave *et al.*, 2005; Outten *et al.*, 2004). Under these conditions the expression of the *suf* operon is strongly increased, and may thus backup the oxygen-sensitive ISC system.

1.6. Biogenesis of Fe/S proteins in *S. cerevisiae*

1.6.1. Biogenesis of mitochondrial Fe/S proteins

Homologs of bacterial *nif* and *isc* genes were discovered in eukaryotes. In yeast, the ISC system is localized in mitochondria suggesting that the ISC machinery has been inherited from the bacterial endosymbiont that gave rise to mitochondria (Johnson *et al.*, 2005; Lill *et al.*, 2006a).

In *S. cerevisiae*, at least 13 proteins comprise the mitochondrial ISC-machinery (Figure 1.3). Fe/S cluster assembly in *S. cerevisiae* mitochondria is based on a similar principle as that of bacteria. In the biosynthetic process of Fe/S proteins, a central role is played by the Isu1/2 proteins that serve as scaffolds for Fe/S cluster assembly. The Isu proteins show homology to the N-terminal domain of NifU and to the bacterial IscU (Garland *et al.*, 1999; Mühlenhoff *et al.*, 2003; Schilke *et al.*, 1999). The cysteine desulfurase Nfs1 provides the sulfur for Fe/S cluster assembly. Nfs1 is a homodimeric pyridoxal-phosphate (PLP)-dependent enzyme and catalyses the conversion of cysteine to alanine and sulfane sulfur. The catalytic mechanism of Nfs1 may be similar to that of its related proteins NifS/IscS/SufS (see above) (Smith *et al.*, 2001; Urbina *et al.*, 2001; Zheng *et al.*, 1994b; Zheng *et al.*, 1993). The sulfur transfer from Nfs1 to Isu1 implies a physical interaction between these two proteins (Gerber *et al.*, 2003). Besides its role in Fe/S cluster synthesis, yeast Nfs1 as its bacterial counterparts provide sulfur for the biosynthesis of vitamins and

thionucleosides of mitochondrial tRNA (Kambampati *et al.*, 2000; Lauhon *et al.*, 2000; Mühlenhoff *et al.*, 2004; Nakai *et al.*, 2004).

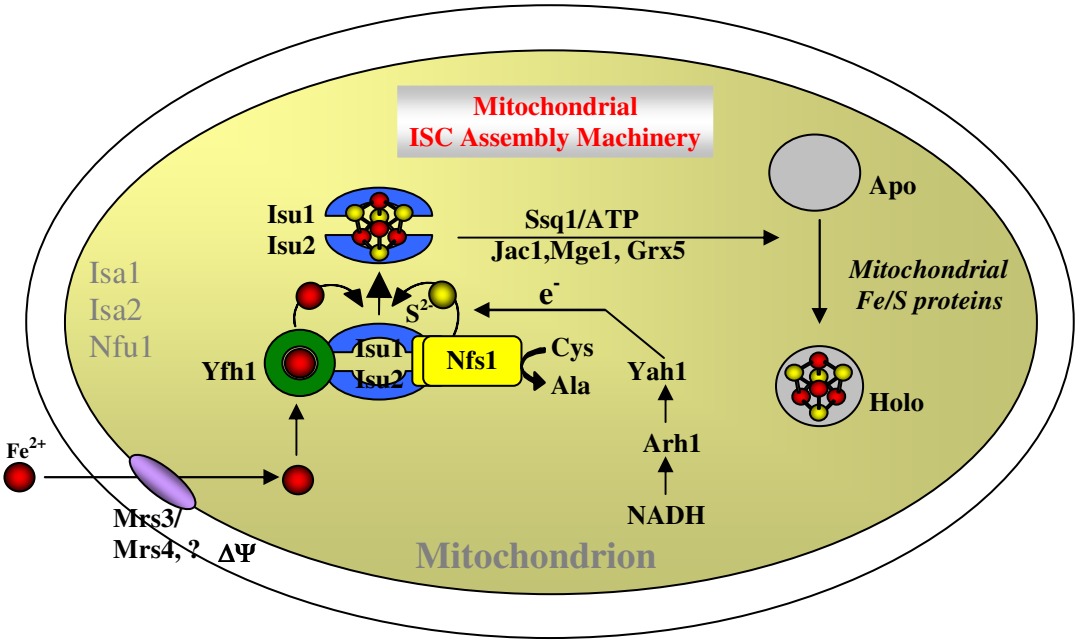


Figure 1.3 Model for the function of the mitochondrial ISC-assembly machinery. Isu1/2 proteins serve as scaffold for Fe/S cluster formation. Nfs1 provides the sulfur, a step that likely needs an electron transport chain formed by NADH, Arh1 and Yah1 to reduce the sulfur to sulfide. Import of ferrous iron (Fe^{2+}) into mitochondria is facilitated by the carrier proteins Mrs3/4 and unknown components (?) and is membrane potential ($\Delta\Psi$)-dependent. Delivery of iron to the Isu proteins may involve the yeast frataxin homolog (Yfh1), which directly binds to Isu1. After Fe/S cluster synthesis on the scaffold Isu proteins several other components are needed for its transfer to the target mitochondrial Fe/S apoproteins. These components include a chaperone system consisting of Hsp70 chaperone Ssq1, its co-chaperone Jac1 and a nucleotide exchange factor Mge1. The glutaredoxin Grx5 is also required in this later step of Fe/S proteins biogenesis. Isa1/2 proteins are required for the maturation of mitochondrial aconitase-like proteins (not depicted here). The function of Nfu1 is still unresolved.

Iron is imported into mitochondria in its reduced ferrous (Fe^{2+}) form. The iron import depends on the mitochondrial membrane potential (Lange *et al.*, 1999) and is facilitated by the mitochondrial inner membrane carriers Mrs3 and Mrs4 (Foury *et al.*, 2002; Muhlenhoff *et al.*, 2003). The simultaneous deletion of these two transporters (Δ*mrs3/4*) showed that Mrs3/4 are not essential in yeast indicating that additional transporters are involved in mitochondrial iron import (Muhlenhoff *et al.*, 2003; Zhang *et al.*, 2005). The form of iron in which it is stored in the mitochondrial matrix before its use in Fe/S cluster formation is unknown.

After import into mitochondrial matrix, iron may be delivered to the Isu scaffold by the yeast frataxin homolog, Yfh1. Several groups investigating Fe/S proteins biogenesis could

show that purified Yfh1 binds and oxidizes iron, even though this binding might be unspecific (Aloria *et al.*, 2004; Isaya *et al.*, 2004; Nair *et al.*, 2004; Yoon *et al.*, 2003). It has been shown that frataxin is required for the assembly of a transient Fe/S cluster on Isu1 (Mühlenhoff *et al.*, 2003) and that Yfh1 binds to Isu1, an interaction which is stimulated by iron (Gerber *et al.*, 2003).

Frataxin is a well studied protein because it is of medical interest. Reduced levels of frataxin in humans are the cause of the Friedreich ataxia, a neurodegenerative disease which is associated with defects in iron homeostasis (Pandolfo, 2002; Wilson, 2003). Several studies indicated that frataxin plays additional roles in heme synthesis or iron storage. However, it seems that most of these effects that were observed in frataxin-deficient cells are secondary consequences of an impaired Fe/S cluster assembly and iron accumulation into mitochondria.

The biogenesis of Fe/S clusters on Isu1 scaffold proteins requires two additional components. The [2Fe-2S]-ferredoxin Yah1 (Barros *et al.*, 1999) and the ferredoxin reductase Arh1 (Lange *et al.*, 2000; Manzella *et al.*, 1998) form an electron transport chain. The electrons are provided by NADH and most likely are used for the reduction of sulfur (S^0) formed in the cysteine desulfurase reaction to sulfide (S^{2-}) present in Fe/S clusters (Muhlenhoff *et al.*, 2003). The experimental evidence for the role of Yah1 and Arh1 in the sulfur reduction is still missing and it might be possible that these proteins are additionally required at a later step in biogenesis.

After Fe/S cluster assembly on Isu scaffold proteins the cluster is transferred to the mitochondrial Fe/S apoproteins. At this step, a chaperone system is needed and consists of the Hsp70 chaperone Ssq1, its J-type co-chaperone Jac1 and the nucleotide exchange factor Mge1 (Dutkiewicz *et al.*, 2003; Kim *et al.*, 2001; Lutz *et al.*, 2001; Schilke *et al.*, 1999; Voisine *et al.*, 2001). The proteins are not required for Fe/S cluster assembly on Isu1/2. Rather, upon their depletion an up to 5-fold accumulation of Fe/S cluster on Isu1 was observed (Dutkiewicz *et al.*, 2006; Mühlenhoff *et al.*, 2003). Ssq1 is specifically interacting with Isu1 via a conserved LPPVK motif present in Isu1 and this binding is facilitated by the co-chaperone Jac1 (Dutkiewicz *et al.*, 2004; Dutkiewicz *et al.*, 2003). This interaction was observed first in bacteria were the scaffold IscU binds to the Ssq1/Jac1 homologous chaperone system Hsc66/Hsc20 (Hoff *et al.*, 2000; Hoff *et al.*, 2002). At present, it can only be speculated that these proteins may facilitate Fe/S cluster transfer to the mitochondrial apoproteins, that they may stabilize the conformation of the Isu scaffold or regenerate the scaffold proteins for the next round of Fe/S cluster synthesis.

Another component of the mitochondrial ISC-machinery acting late in the biogenesis is the monothiol glutaredoxin Grx5. Depletion of Grx5 in yeast results in a general Fe/S defect with the accumulation of transiently bound Fe/S cluster on Isu scaffold proteins (Mühlenhoff *et al.*, 2003). The exact role of Grx5 remains to be elucidated.

Yeast mitochondria contain two proteins, Isa1 and Isa2 that are related to the bacterial IscA. They form a hetero-dimer and deletion of *ISA1* and *ISA2* genes in *S. cerevisiae* leads to respiration deficiency, loss of mitochondrial DNA and no cell growth on non-fermentable carbon sources (Jensen *et al.*, 2000; Kaut *et al.*, 2000; Pelzer *et al.*, 2000). Initial characterization of these two Isa proteins indicated a general defect in Fe/S proteins. A more detailed study has observed that the Isa proteins are required for the maturation of mitochondrial aconitase-like Fe/S proteins (Aco1, Lys4), whereas the maturation of other mitochondrial Fe/S proteins , such as ferredoxin Yah1 or Rieske Fe/S protein was unaffected (U. Mühlenhoff, personal communication). Moreover, a recent study has shown that the Isa proteins are essential for the catalytic activity of biotin synthase Bio2 *in vivo*, but not for the *de novo* synthesis of any of the two Fe/S centers of Bio2 (Muhlenhoff *et al.*, 2007). This suggested that the Isa proteins play a role in the regeneration of the Fe/S clusters of Bio2.

The mitochondrial protein Nfu1 exhibits sequence similarity to the C-terminal domain of bacterial NifU and it is conserved in eukaryotes (Johnson *et al.*, 2005; Schilke *et al.*, 1999). Deletion of yeast *NFU1* has no phenotype, but simultaneous deletion with the *ISU1* is lethal, indicating an auxiliary function of Nfu1 in Fe/S cluster formation (Schilke *et al.*, 1999). In contrast, NifU-like proteins play an essential role in the maturation of Fe/S proteins in cyanobacteria or plant chloroplasts, as their deletion results in growth defects and impaired Fe/S cluster assembly (Balk *et al.*, 2005b; Leon *et al.*, 2003; Touraine *et al.*, 2004; Yabe *et al.*, 2004).

1.6.2. Biogenesis of extra-mitochondrial Fe/S proteins

Biogenesis of extra-mitochondrial Fe/S proteins requires a functional ISC-assembly machinery. Several studies demonstrated that the depletion of mitochondrial ISC components results in severe defects in the maturation of cytosolic and nuclear Fe/S proteins (Kaut *et al.*, 2000; Kispal *et al.*, 1999; Lange *et al.*, 2000; Li *et al.*, 2001). In two cases, for Nfs1 and Isu1, experimental data indicated that these proteins need to be located inside mitochondria to be functional in the biogenesis of extra-mitochondrial Fe/S proteins (Gerber *et al.*, 2004; Mühlenhoff *et al.*, 2004). The displacement of Nfs1 or Isu1 into the cytosol leads to a strong impairment in the *de novo* assembly of Fe/S clusters into cytosolic and nuclear Fe/S proteins.

Due to the importance of mitochondria in the maturation of cytosolic and nuclear Fe/S proteins, it has been postulated that the ISC-assembly machinery produces a still unknown compound that is exported into the cytosol and required for the synthesis of Fe/S clusters and their insertion into cytosolic apoproteins (Figure 1.4).

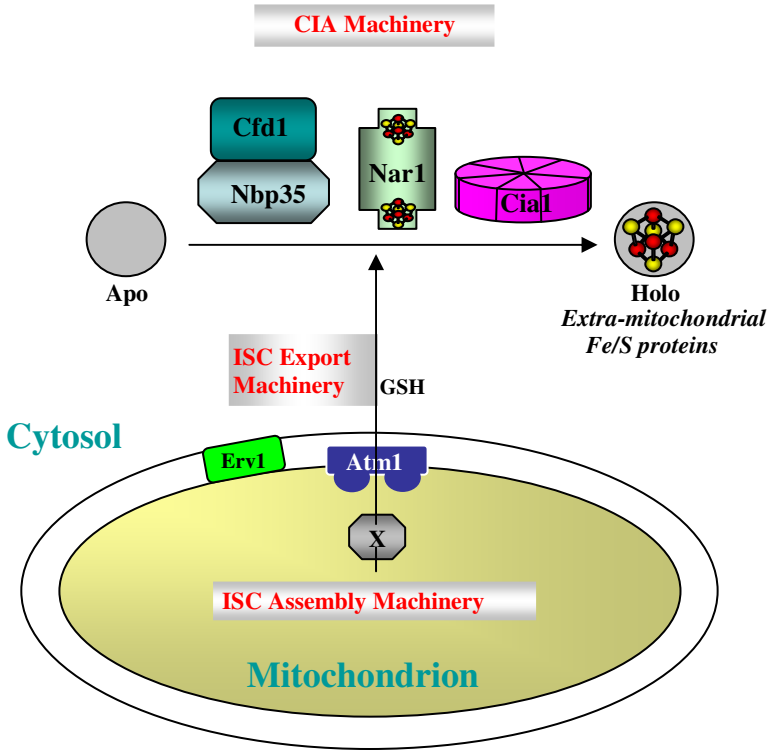


Figure 1.4 Model for the function of the CIA-machinery in the maturation of cytosolic and nuclear Fe/S proteins. The mitochondrial ISC-assembly machinery is required for the generation of a compound (X) that is exported to the cytosol by the ISC-export machinery comprised of the mitochondrial inner membrane ABC-transporter Atm1, the sulfhydryl-oxidase Erv1 of the intermembrane space and the tripeptide glutathione (GSH). The Fe/S cluster synthesis in the cytosol requires the CIA (cytosolic Fe/S protein assembly)-machinery encompassing at least four proteins: two P-loop NTPases, Cfd1 and Nbp35, the iron-only hydrogenase-like Nar1 and the WD40 repeat protein Cia1.

Components involved in the translocation of the substrate X (Figure 1.4) to the cytosol are known as the ISC-export machinery. So far, three members of the ISC-export machinery were identified. The central component is the mitochondrial ABC transporter Atm1 which is located in the inner membrane with its ABC domains facing the matrix indicating that it functions as an exporter. Depletion of Atm1 using a regulatable yeast mutant results in defects in the maturation of cytosolic/nuclear Fe/S proteins and accumulation of iron within mitochondria (Kispal *et al.*, 1997; Kispal *et al.*, 1999). The substrate of Atm1 is still

unknown. *In vitro* studies using reconstituted Atm1 into proteoliposomes showed that its ATPase activity is stimulated by compounds containing free sulfhydryl (SH) groups (Kuhnke *et al.*, 2006). Therefore, it might be possible that the substrate of Atm1 is a sulfur-containing compound.

Another component that is specifically required only for the maturation of extra-mitochondrial Fe/S protein is Erv1 (Lange *et al.*, 2001). Erv1 is located in the mitochondrial intermembrane space and is a FAD-dependent sulfhydroxyl oxidase that introduces disulfide bridges into target proteins, such as Mia40, a protein involved in the translocation of proteins into the inner membrane (Mesecke *et al.*, 2005). The precise role of Erv1 in the biogenesis of cytosolic and nuclear Fe/S proteins is still unknown. However, Erv1 may have an influence on the Atm1 substrate, as both proteins deal with sulfhydryl groups.

Glutathione (GSH) is the third component of the ISC-export machinery (Sipos *et al.*, 2002). The tripeptide serves as the major protective agent against oxidative stress in *Saccharomyces cerevisiae*. Depletion of GSH results in severe defects in cytosolic/nuclear Fe/S proteins and iron accumulation within mitochondria similar to those observed for Atm1 and Erv1 mutants, whereas the mitochondrial Fe/S protein biogenesis was not impaired.

Assembly of Fe/S proteins in the cytosol is assisted by a set of proteins with no relation to the components of mitochondrial ISC-machinery. To date, four components of the CIA-machinery have been identified to be involved in cytosolic Fe/S protein maturation. The first known component of the CIA-machinery was the P-loop NTPase Cfd1 (Roy *et al.*, 2003). Mutations in Cfd1 are associated with defects in virtually all cytosolic and nuclear Fe/S proteins, but not mitochondrial Fe/S proteins. Another P-loop NTPase protein of the CIA-machinery is Nbp35. Nbp35 shows sequence similarity to Cfd1 in the central and C-terminal parts. Nbp35 contains at its N-terminus a stretch of 50 amino acids which coordinate and Fe/S cluster via four conserved cysteines (Hausmann *et al.*, 2005). Recent *in vitro* and *in vivo* experiments showed that both Cfd1 and Nbp35 form a stable complex and both Cfd1 and Nbp35 can associate with a [4Fe-4S] cluster at their C-termini (Netz *et al.*, 2007). These C-terminal clusters are bound in a labile fashion and can be rapidly and efficiently transferred to apoproteins (apo-Leu1) *in vitro* leading to the activation of the Leu1 isopropylmalate isomerase function. Assembly of these clusters depends on the function of mitochondrial components Nfs1 and Atm1. The transient character of Fe/S cluster binding suggests that these proteins serve as scaffolds for the transient assembly and binding of Fe/S cluster before their transfer to apoproteins.

The other two components of the CIA-machinery are Nar1 and Cia1. Nar1 is highly conserved in eukaryotes and exhibits sequence similarity to the bacterial and algal iron-only hydrogenases (Horner *et al.*, 2002; Nicolet *et al.*, 2002). Nar1 contains two Fe/S clusters and their assembly depends on mitochondrial-ISC and export machineries (Balk *et al.*, 2004). Since Nar1 is an Fe/S protein and is essential for the maturation of target cytosolic and nuclear Fe/S proteins, it can be viewed as both component and target of the CIA-machinery. The fourth member, Cia1 has different characteristics from the other 3 components. Depletion of Cia1 in yeast cells results in a strong impairment of Fe/S cluster incorporation into target proteins as cytosolic Leu1 and Rli1 or nuclear Ntg2, but the Fe/S clusters of the CIA components Nbp35 and Nar1 are assembled normally. Hence, during Fe/S protein maturation, Cia1 acts after the assembly of the Fe/S cluster on Nbp35 and Nar1. Cia1 is a member of a large family of WD40 proteins known to act as a protein-interaction platform and may play a role in the final incorporation of the Fe/S clusters into true target Fe/S proteins.

1.7. Hydrogenases

As part of this study deals with the Nar1 protein which exhibits sequence similarities to iron-only hydrogenases, the following chapter gives a short introduction into the structure and function of bacterial iron-only hydrogenases.

Many microorganisms, such as methanogenic, nitrogen-fixing, photosynthetic, or sulfate-reducing bacteria metabolize hydrogen. Hydrogen plays a vital role in the metabolism of these organisms, where the reaction $2H^+ + 2e^- \leftrightarrow H_2$ is catalysed by metalloenzymes known as hydrogenases (Armstrong, 2004). Hydrogenases were also found in subcellular organelles of eukaryotes, namely hydrogenosomes of protozoa and chloroplasts of green algae (Vignais *et al.*, 2001). Hydrogenases are classified into two major families on the basis of the metals present in their catalytic centers: NiFe- and Fe-only hydrogenases (Frey, 2002). There is also a third type of hydrogenase, found so far only in methanogens, and it has been claimed that it does not contain any metal (Geierstanger *et al.*, 1998). Recent studies revealed the presence of mononuclear iron in these hydrogenases (Shima *et al.*, 2007). Evidence from sequence and structural data indicates that the NiFe- and Fe-only hydrogenases are phylogenetically distinct classes of proteins. The NiFe-hydrogenases are normally found in hydrogen-oxidizing microorganisms, whereas the Fe-only hydrogenases are most often found in hydrogen-producing microorganisms. The various functions are often associated with

different cellular localizations, e.g., hydrogen evolution is usually cytosolic, whereas hydrogen uptake is most often periplasmic or membrane-localized.

1.7.1. NiFe hydrogenases

The first presented structure of a hydrogenase was that of the hydrogen-oxidizing NiFe-hydrogenase from *Desulfovibrio gigas* (Volbeda *et al.*, 1995). This is a heterodimeric enzyme consisting of a small and a large subunit that interact with each other. The large subunit contains the NiFe catalytic center which is deeply buried inside the protein. The Ni atom is in the Ni^{2+} or Ni^{3+} oxidation states and is ligated by four conserved cysteine residues, two of these also bridge the Fe atom. One cysteine is sometime substituted by selenocysteine, as in the NiFeSe hydrogenase from *Desulfomicrobium baculatum* (Garcin *et al.*, 1999). The Fe is coordinated by three non-protein ligands: two CN^- and one CO (Pierik *et al.*, 1999). The smaller subunit contains two [4Fe-4S] clusters and a [3Fe-4S] cluster. The molecule also contains a large cavity and channels, lined with hydrophobic amino acid side-chains that interconnect the NiFe center and the molecular surface (Montet *et al.*, 1997); (Böck *et al.*, 2006).

1.7.2. Fe-only hydrogenases

Two Fe-only hydrogenases from *Clostridium pasteurianum* (CpIHase) and *Desulfovibrio desulfuricans* (DdHase) are the best characterized enzymes within this class of hydrogenases. Fe-only hydrogenases are found in both, monomeric (CpIHase) and dimeric (DdHase) forms. However, both the large and small subunits of the dimeric form share sequence similarities with regions of the monomeric forms. Extensive spectroscopic studies have indicated that Fe-only hydrogenases generally contain two [4Fe-4S] clusters (called F-clusters) in a ferredoxin-like domain (Figure 1.5 A). In addition, the X-ray crystallographic structures of CpIHase and DdHase showed that the catalytic site consists of an unusual Fe/S cluster, the H-cluster (Figure 1.5 B) (Nicolet *et al.*, 1999; Peters *et al.*, 1998).

In addition to the clusters mentioned above, *C. pasteurianum* Fe-only hydrogenase contains two Fe/S centers coordinated by domains found in the N-terminal part of the protein (Figure 1.5 A). One of these domains bears a striking structural similarity to [2Fe-2S] plant-type ferredoxins (Peters *et al.*, 1998). The H-cluster is an unusual type of Fe/S clusters which consists of a [4Fe-4S] linked by a cysteine residue to a [2Fe-2S] center (Nicolet *et al.*, 2002; Nicolet *et al.*, 1999). The unusual feature of the H-cluster is the presence of non-protein ligands at each Fe atom of the Fe bi-nuclear center in both, *C. pasteurianum* and *D.*

desulfuricans Fe-only hydrogenases. These ligands are most likely represented by CN^- and CO. The active site Fe1 and Fe2 ions are bridged by two S atoms, whereas the sulfur atoms in the [2Fe-2S] center are connected probably by a di(thiomethyl)amine in *D. desulfuricans* and a water molecule in *C. pasteurianum* (Nicolet *et al.*, 2002; Peters *et al.*, 1998).

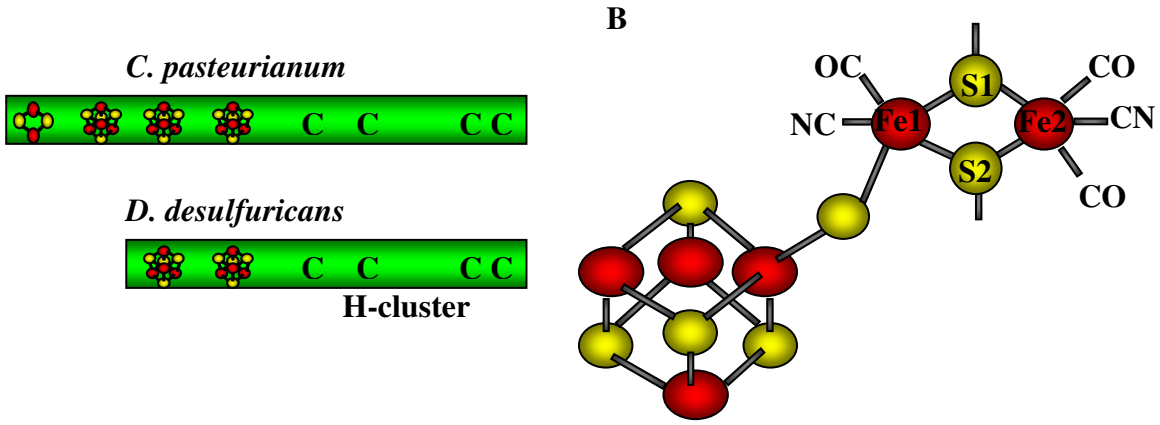


Figure 1.5 Representation of the A) sequence features of two bacterial Fe-only hydrogenases and B) catalytic site (H-cluster) in Fe-only hydrogenases. A) Conserved cysteines involved in coordination of the H-cluster and the Fe/S clusters bound at the N-terminal parts of these proteins are emphasized. **B)** A [4Fe-4S] cluster is linked by a cysteine residue to the [2Fe-2S] center. The Fe1 and Fe2 atoms are coordinated by non-protein ligands, such as CO and CN^-. Fe1 and Fe2 of the [2Fe-2S] center are also bridged by the two sulfur atoms (S1 and S2), which are connected to each other most likely by a di(thiomethyl)amine (not depicted) (Horner *et al.*, 2002; Nicolet *et al.*, 2002).

In Fe-only hydrogenases the set of Fe/S clusters is dispersed regularly between the bi-nuclear Fe1-Fe2 center and the molecular surface. The catalytic sites in both Fe-only hydrogenases are deeply buried inside the proteins. The components of the catalytic reaction have to shuttle between these sites and the molecular surface via the accessory Fe/S clusters. The X-ray structures of both Fe-only hydrogenases indicated the presence of a unique continuous hydrophobic channel running from the molecular surface to the active site, most likely to the Fe2 which has a vacant coordination site. The location of Fe-only hydrogenases in the cell reflects the enzyme's function. *D. desulfuricans* hydrogenase is a periplasmic protein whose physiological role is hydrogen uptake. Protons resulting from the oxidation of hydrogen by the Fe-only hydrogenase in the periplasm create a gradient across the membrane that is thought to be coupled to the ATP synthesis in the cytoplasm. Fe-only hydrogenase from *C. pasteurianum* is a cytoplasmic, hydrogen-producing enzyme. Ferredoxins transfer two electrons to Fe-only hydrogenase, which, in turn, uses protons as electrons acceptors to generate hydrogen (Nicolet *et al.*, 2000). A plausible proton transfer pathway for Fe-only

hydrogenases has been proposed for CpIHase starting with Cys 299 (Cys178 in DdHase) (Peters *et al.*, 1998). The residues and water molecules involved in this pathway are conserved in the DdHase crystal structure and, furthermore, the respective residues are conserved in all Fe-only hydrogenases (Nicolet *et al.*, 2002).

The assembly of the active site in Fe-only hydrogenases is assisted by a set of distinct proteins. Homologs of the *hyp* genes involved in the maturation of NiFe-hydrogenases were found in organisms expressing only Fe-hydrogenases (Vignais *et al.*, 2001). Accessory proteins HydEF and HydG were identified in *Chlamydomonas reinhardtii* (Posewitz *et al.*, 2004). These proteins are encoded in all genomes with Fe-only hydrogenase genes. HydE and HydG are members of the radical *S*-adenosylmethionone (SAM) superfamily. HydF is a GTPase and spectroscopic studies have indicated that it contains a [4Fe-4S] cluster coordinated by three cysteines and an exchangeable ligand (Brazzolotto *et al.*, 2006). Expression of all three proteins is necessary and sufficient for production of fully active Fe-only hydrogenase in *E. coli* (King *et al.*, 2006). The exact mechanism for the Hyd proteins in the production of the catalytic [Fe-Fe] center might be similar to that of lipoate synthase (LipA) and biotin synthase (BioB), two radical SAM enzymes that catalyse the formation of sulfur–carbon bonds, where likely an Fe/S cluster serves as a sulfur source (Cicchillo *et al.*, 2004; Ugulava *et al.*, 2001). HydE and HydG could catalyse the insertion of sulfur atoms from the additional clusters that are bound to the proteins converting dimethylamine (or propane) into the bridging compound (a dithiolate-bridged-cluster intermediate). The energy required to transfer this intermediate could be supplied by HydF-dependent GTP hydrolysis (Leach *et al.*, 2007; Peters *et al.*, 2006; Rubach *et al.*, 2005).

Fe-only hydrogenases are not only found in bacteria, they also exist in some hydrogen-producing anaerobic eukaryotes. The eukaryotic Fe-only hydrogenases are restricted to a few physiologically unrelated groups, trichomonads, cythrid fungi and green algae (Horner *et al.*, 2002). The enzymes are localized in hydrogenosomes in the case of chytrid fungi, trichomonads and anaerobic ciliates. In green algae (Chlorella, Chlamydomonas) the enzymes are in the chloroplast stroma and are linked to the photosynthetic electron transport chain (Horner *et al.*, 2002). Although there are some similarities between hydrogenases and the several subunits (NuoE, NuoF, NuoG and NuoI) of the NADH–ubiquinone oxidoreductase (complex I) of the respiratory chain (Vignais *et al.*, 2001), a surprising observation is the general presence of Fe-only hydrogenase homologues in eukaryotes. In mammals these proteins have been designated NARF (Barton *et al.*, 1999) and IOP1 (Huang *et al.*, 2007). NARF, IOP1, their yeast homologue Nar1 and Fe-only

hydrogenases show sequence similarity, especially in the region involved in the coordination of the H-cluster (Balk *et al.*, 2004). Human Narf is a nuclear protein that binds to prenylated lamin A in the nucleus (Barton and Worman, 1999). Recently, it has been shown that the second mammalian homologue, IOP1 regulates the expression of HIF1α (hypoxia inducible factor) that represents the global mediator of the mammalian response to hypoxia and that IOP1 and NARF do not possess hydrogenase activities (Huang *et al.*, 2007). The yeast NARF-member, Nar1, is an essential cytosolic protein required for the maturation of cytosolic and nuclear Fe/S proteins (Balk *et al.*, 2004). Thus, it seems possible that the eukaryotic Narf-like proteins have developed from their ancestors (Fe-only hydrogenases) to factors with distinct functions.

1.8. Aim of the present study

This study focuses on two different aspects with respect to the biogenesis of Fe/S proteins in *S. cerevisiae*. To better understand the mechanism of how the ISC machinery works inside mitochondria, in the first part of this work it was investigated whether the ISC-assembly machinery requires additional mitochondrial factors for its proper function in the biosynthesis of cellular Fe/S proteins. In contrast to the cytosolic CIA-machinery, the mitochondrial ISC-assembly machinery is well characterized. To date, more than 10 proteins were identified to play a role in the maturation of cellular Fe/S proteins. In this study, a new member of mitochondrial ISC-assembly machinery, designated Isd11, was identified and characterized. Systematic gene deletion and subcellular localization studies predicted that Isd11 is an essential, mitochondrial protein (Huh *et al.*, 2003; Sickmann *et al.*, 2003; Winzeler *et al.*, 1999). The project was performed in collaboration with the group of Dr. Nikolaus Pfanner from the Institute of Biochemistry and Molecular Biology in Freiburg. Many essential mitochondrial proteins are involved either in protein translocation into mitochondria or the biogenesis of Fe/S proteins. The goal of this study was to determine whether Isd11 is involved in one of these pathways. To this end, experiments employing protein import assays, measurement of the enzymatic activities of several mitochondrial and cytosolic Fe/S proteins and of the *de novo* incorporation of Fe/S clusters into reporter proteins, or experiments to verify the iron homeostasis in Isd11 mutant cells were performed. This work presents data that clearly establish an essential function of Isd11 in the biogenesis of Fe/S proteins. Hence, Isd11 can be regarded as a novel member of the mitochondrial ISC-assembly machinery.

In the second part of this thesis, the main goal was to gain insights into the molecular function of Nar1. Biogenesis of extra-mitochondrial Fe/S proteins requires the function of three systems, mitochondrial ISC-assembly and export systems and the cytosolic CIA-machinery. As outlined above, four members of the CIA-machinery were identified, Cfd1, Nar1, Nbp35 and Cia1 (Balk *et al.*, 2005a; Balk *et al.*, 2004; Hausmann *et al.*, 2005; Roy *et al.*, 2003). Nar1 is an essential protein in yeast and exhibits sequence similarities to the bacterial and algal Fe-only hydrogenases. Nar1 contains eight conserved cysteine residues that correspond to those that in hydrogenases coordinate the medial [4Fe-4S] cluster and the H-cluster. Previously it was demonstrated that Nar1 is an Fe/S protein, containing two magnetically interacting clusters (Balk *et al.*, 2005c). The goal of this part of the study was to test the role of these eight conserved cysteines as coordinating ligands for the two Fe/S clusters. All eight conserved cysteines were exchanged to alanine residues by site-directed mutagenesis and the resulting Nar1 protein mutants were analysed for their ability to complement the growth defect of Nar1-depleted Gal-NAR1 cells. It was further investigated whether the respective mutant proteins could associate the Fe/S clusters and perform their function in the maturation of extra-mitochondrial Fe/S proteins. Further, it was also interesting to determine the chemical nature of the bound Fe/S clusters. To analyse the type of clusters associated with Nar1, UV-VIS and EPR experiments were performed using recombinant *E. coli* wild-type and mutant Nar1 proteins. Previous work, (Balk *et al.*, 2005a) has indicated a physical interaction between Nar1 and Cia1. Therefore, a final goal was to analyse, using coimmunoprecipitation experiments, whether Nar1 binds to other components of the CIA-machinery. The answers to these questions will help to understand the molecular function of Nar1 as part of the CIA machinery.

2. Materials and methods

2.1. Bacteria and Yeast strains

2.1.1. Escherichia coli

Strain	Genotype	Reference
DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17 (r_K-, m_{K+}) supE44 relA1 deoR ΔlacU169(ϕ80 lacZ DM15)*	Promega
C41(DE3)	F⁻*ompT gal hsdS_B* ($r_B^-m_B^-$) *dcm lon λ*DE3 and an uncharacterized mutation	(Miroux *et al.*, 1996)
BMH 71-18 *mut*S	*thi, sup*E, Δ(*lac-proAB*), [*mut*S::Tn10] [F´, *pro*AB, *lac*lqZΔM15]	Promega

2.1.2. Saccharomyces cerevisiae

Strain	Genotype	Reference
W303-1A	*MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 can1-100 GAL*$^+$	DSM [1)]
Gal-NAR1	W303-1A, *NAR1::(GAL1-10)-HIS3*	(Balk *et al.*, 2004)
Gal-NBP35	W303-1A, *NBP35::(GAL1-10)-HIS3*	(Hausmann *et al.*, 2005)
Gal-ISD11	W303-1A *Yer048w-a::(GAL1-10)- HIS3*	(Wiedemann *et al.*, 2006)
YPH499	*Matα ade2-101 his3-200 leu2-1 trp 1-63 ura 3-52 lys2-801*	DSM [1)]
ISD11-ts	*MAT***a**, *ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63, lys2-801, isd11::*ADE2, pFL39-*fomp6*Ts1-CEN; *fomp* is the original genetic designation of *ISD11*	(Wiedemann *et al.*, 2006)

1)DSM, Deutsche Stammsammlung für Mikroorganismen, Braunschweig.

2.2. Growth conditions

2.2.1. E. coli: Culture and Media

E. coli cells were grown in LB medium as described in (Sambrook *et al.*, 2001), and depending on growth requirements the medium was supplemented with antibiotics (Ampicillin-100 µg/ml; Tetracycline -10 µg/ml or Chloramphenicol -34 µg/ml). The *E. coli* liquid cultures were incubated under aerobic or anaerobic conditions at 37°C and 250 rpm.

LB-medium:

1% Bacto-Tryptone
0.5% Yeast extract
1% NaCl

To prepare plates with solid media, 2% (w/v) bacto-agar was added. Bacto-agar and media were autoclaved separately. The appropriate antibiotics were added after the media were chilled to 50°C.

2.2.2. S. cerevisiae: Culture and Media

Yeast cells were grown at 30°C in rich medium (YP) or in synthetic minimal medium (SC) and depending on the auxothophic markers with amino-acids and supplements. Solid medium plates contained 2% (w/v) bacto-agar.

YP-medium:

1% (w/v) Yeast extract
2% (w/v) peptone

YPD-medium: YP-medium supplemented with 2% glucose;

YPGal-medium: YP-medium supplemented with 2% galactose;

SC-medium:

0.17% (w/v) Yeast Nitrogen Base
0.5% (w/v) ammonium sulphate

SD-medium: SC-medium supplemented with 2% glucose;

SGal-medium: SC-medium supplemented with 2% galactose;

Lactate medium:

0.3% (w/v)Yeast extract
0.05% (w/v) Glucose
0.05% (w/v) $CaCl_2 \cdot 2H_2O$
0.05% (w/v) NaCl
0.06%(w/v) $MgCl_2$
0.1% (w/v) KH_2PO_4
0.1% (w/v) NH_4Cl
2% (v/v)Lactate
0.75%(w/v) NaOH

(adjust the pH with 10 mM NaOH)

Fe-free medium:

2% Glucose (or Galactose)
50 ml/L 20X Salts
5 ml/L 200X Vitamins
1 ml/L 1000X Trace salts

20X Salts:	**200X Vitamins:**	**1000X Trace Salts:**
17 g/L KH_2PO_4	400 mg/L calcium pantothenate	500 mg/L H_3BO_3
3 g/L K_2HPO_4	400 mg/L Thiamine HCl	40 mg/L $CuCl_2.2H_2O$
2 g/L NaCl	400 mg/L Pyridoxine HCl	100 mg/L KJ
100 g/L NH_4SO_4	4 mg/L Biotine	500 mg/L $MnCl_2.4H_2O$
10 g/L $MgSO_4.6H_2O$	4 g/L Inositol	200 mg/L $Na_2MoO_4.2H_2O$
2 g/L $CaCl_2$		200 mg/L $ZnCl_2$

Following amino-acids and supplements were used:

Adenine	80 mg/L
Uracil	20 mg/L
L-Tryptophan	40 mg/L
L-Histidine	20 mg/L
L-Leucine	60 mg/L
L-Lysine	30 mg/L

2.3. Oligonucleotides :

The oligonucleotides used for PCR, cloning, subcloning and mutagenesis are listed below. Oligonucleotides were supplied by Metabion.

Primers used to exchange the endogenous promoter of ISD11 with the Gal1-10 promoter

1) Gal-ISD11-Promoter:

5´- GTC CAC AGT AAA GTA GAT AAA GCA ATA GCT ATT TCC ATC AAA TCC AAA CAA TCG - 3´

2) Gal-ISD11-ORF:

5´- GTA TAA AGA CAA CAC CTG CCT TCT TGT TGG AGC TGT AAA TCC AGG CAT CGA ATT -3´

Oligonucleotides used for site-directed mutagenesis (Cysteine codons are undelined and the nucleotides changed are depicted in bold-italics)

3) Nar1-C20A- forward:

5´- TCA GTC CCG CCC TTG CG ***GC***C G TCA AAC CTA CCC AGG - 3´

4) Nar1-C20A-reverse:

5´- CCT GGG TAG GTT TGA C G***GC*** CG CAA GGG CGG GAC TGA - 3´

5) Nar1-C59A- forward:

5´- ATT ACC CTA TCA GAC ***GC***C CTC GCG TGT TCT GG - 3´

6) Nar1-C59A- reverse:

5´- CCA GAA CAC GCG AG G***GC*** G TCT GAT AGG GTA AT - 3´

7) Nar1-C62A-forward:

5´- ACC CTA TCA GAC TGC CTC GCG ***GC***T TCT GGT TGT ATA ACG TCT AG- 3´

8) Nar1-C62A-reverse:

5´- CTA GAC GTT ATA CAA CCA GA A***GC*** C GCG AGG CAG TCT GAT AGG GT- 3´

9) Nar1-C65A-forward:

5´- CCT CGC GTG TTC TGG T ***GC***T AT AAC GTC TAG TGA AG - 3´

10) Nar1-C65A-reverse:

5´- CTT CAC TAG ACG TTA T A***GC*** AC CAG AAC ACG CGA GG - 3´

11) Nar1-C177A-forward:

5´- GCC TCT ATT ATC TGC TGTG ***GC***T CC GGG ATT TCT TAT ATA C - 3´

12) Nar1-C177A-reverse:

5´- GTA TAT AAG AAA TCC CGG A***GC*** CAC AGC AGA TAA TAG AGG C - 3´

13) Nar1-C231A-forward:

5´- CAT CTG TCG CTG ATG CCA ***GC***T TTT GAC AAG AAA CTA G - 3´

14) Nar1-C231A-reverse:

5´- CTA GTT TCT TGT CAAA A***GC*** TGGCA TCA GCG ACA GAT G - 3´

15) Nar1-C412A-forward:

5´- TCG AGG TAA ACG CG ***GC***T CCG GGT GCA TGC ATG - 3´

16) Nar1-C412A-reverse:

5´- CAT GCA TGC ACC CGG A***GC*** CGC GTT TAC CTC GA - 3´

17) Nar1-C416A- forward:

5´- TAA ACG CGT GTC CGG GTG CA ***GC***C ATG AAC GGT GGT GGC CTA C - 3´

18) Nar1-C416A- reverse:

5´- GTA GGC CAC CAC CGT TCA T G***GC*** TGC ACC CGG ACA CGC GTT TA - 3´

19) Nar1-C59S- forward:

5´- TAC CCT ATC AGA C T***CA*** CTC GCG TGT TCT GG- 3´

20) Nar1-C177S- forward:

5´- TAT TAT CTG CTG TG T***C***T CCG G GAT TTC TTA T - 3´

21) Nar1-C412S- forward:

5´- ATC GAG GTA AAC GC***A*** T***C***T CCG GGT GCA TGC AT - 3´

22) Nar1-R144A- forward:

5´- GGA ACG GAG ATG GGC ***GC***A ATC ATA TCG ATT AG -3´

23) Nar1-Q356E- forward:

5´- GTT TAG AAA CAT C ***G***AA AAT CTG GTG CG - 3´

Primers for deletion of residue W491

24) Nar1-C-term (ΔW491)-BamHI:

5´- GCA TCA GT GGATCC TTA GGT GCT CCC AAC AGA G - 3´

Primers used to replace the *MET25* promoter from the p416-MET25 plasmid with the endogenous *NAR1* promoter

25) Nar1-promoter-forward:

5´- GTG TCA CTC ACT ACC CGG CGA TTG CAT GCT - 3´

26) Nar1-ORF-reverse:

5´- GGA CTC ACG CGC GAT AGC GAG ACT CTC GAA - 3´

27) Nar1-promoter-*Sac*I:

5´- TCT AT GAGCTC CCCG TCG GCG ACT TGT CAG C - 3´

28) Nar1-promoter-*Xba*I:

5´- CAC C TCTAGA GC TTT CTT GTA CAC TTA TCT T - 3´

Flanking primers used for PCR-based C-A mutagenesis (C20A; C62A; C65A; C231A; C416A)

29) Nar1-*BamH*I-forward:

5´- GTA CA GGATCC **ATG** A GTG CTC TAC TGT CCG - 3´

30) Nar1-*Xho*I – reverse:

5´- TCA GCT CTCGAG CT TAC TTA CCA GGT GCT CCC – 3´

31) Nar1-*Xba*I – forward:

5´- GTA CAG TCTAGA **ATG** A GTG CTC TAC TGT CCG – 3´

32) Nar1-*BamH*I – reverse:

5´- GATCTA CCTAGG CT TAC TTA CCA GGT GCT CCC – 3´

The corresponding restriction sites are underlined.

2.4. Plasmids

2.4.1. Escherichia coli plasmids:

Plasmid	Marker	Description	Reference
pBluescript II KS +	*Amp*^{R}	no insert	Stratagene
pBluescript II KS-HA	*Amp*^{R}	contains the HA tag	Stratagene
pISC plasmid	*Tetracycline*^{R}	ISC gene cluster from *E. coli*	(Takahashi and Nakamura, 1999)
pET15b	*Amp*^{R}	no insert	Novagen
pET15b-NAR1	*Amp*^{R}	*NAR1* ORF	(Balk *et al.*, 2004)
pET15b-NAR1-C20A	*Amp*^{R}	*NAR1* ORF with indicated C to A mutation	This study
pET15b-NAR1-C59S	*Amp*^{R}	*NAR1* ORF with indicated C to S mutation	This study
pET15b-NAR1-C62A	Amp^R	*NAR1* ORF with indicated C to A mutation	This study
pET15b-NAR1-C65A	Amp^R	*NAR1* ORF with indicated C to A mutation	This study
pET15b-NAR1-C177S	Amp^R	*NAR1* ORF with indicated C to S mutation	This study
pET15b-NAR1-C412S	Amp^R	*NAR1* ORF with indicated C to S mutation	This study
pET15b-NAR1-C416A	Amp^R	*NAR1* ORF with indicated C to A mutation	This study
pET15b-NAR1-C177S-C412S	Amp^R	*NAR1* ORF with indicated C to S mutations	This study
pET-15b-NAR1-C231A	Amp^R	*NAR1* ORF with indicated C to A mutation	This study

2.4.2. Saccharomyces cerevisiae plasmids:

Plasmid	Marker, ARS/CEN, 2µ	Description	Reference
p414-MET25	(Amp^R, *TRP1*, ARS/CEN)	no insert	(Mumberg *et al.*, 1995)
p424-TDH3	(Amp^R, *TRP1*,2µ)	no insert	(Mumberg *et al.*, 1995)
p416-MET25	(Amp^R, *URA3*, ARS/CEN)	no insert	(Mumberg *et al.*, 1995)
p426-TDH3	(Amp^R, *URA3*, 2µ)	no insert	(Mumberg *et al.*, 1995)
pTL26	(Amp^R, *HIS3*, 2µ)	contains the GAL1-10 promoter and the *HIS3* cassette for promoter exchange	Lafontaine and Tollervey
p416-FET3-GFP	(Amp^R, *HIS3*, 2µ)	contains the GFP under control of *FET3* promoter	(Rutherford *et al.*, 2005)

Plasmids for overproducing proteins of the ISC machinery:

Plasmid	Marker, ARS/CEN, 2µ	Description	Reference
p416-NAR1-C20A	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to A mutation	This study
p416-NAR1-C59A	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to A mutation	This study
p416-NAR1-C59S	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to S mutation	This study
p416-NAR1-C62A	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to A mutation	This study
p416-NAR1-C65A	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to A mutation	This study
p416-NAR1-C20A-C62A	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to A mutations	This study
p416-NAR1-C20A-C65A	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to A mutations	This study
p416-NAR1-C177A	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to A mutation	This study
p416-NAR1-C177S	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to S mutation	This study
p416-NAR1-C231A	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to A mutation	This study
p416-NAR1-C412A	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to A mutation	This study
p416-NAR1-C412S	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to S mutation	This study
p416-NAR1-C416A	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to A mutation	This study
p416-NAR1-C177A-C416A	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to A mutations	This study
p416-NAR1-C231A-C416A	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to A mutations	This study

p416-NAR1-C177S-C412S	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to S mutations	This study
p416-NAR1-R144A	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated R to A mutation	This study
p416-NAR1-Q356E	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated Q to E mutation	This study
p416-NAR1-ΔW491	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with residue W491 deleted	This study
p416-ep-NAR1-C177S	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to S mutation under control of endogenous *NAR1* promoter	This study
p416-ep-NAR1-C177A	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to A mutation under control of endogenous *NAR1* promoter	This study
p416-ep-NAR1-C412A	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to A mutation under control of endogenous *NAR1* promoter	This study
p416-ep-NAR1-C412S	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated C to S mutation under control of endogenous *NAR1* promoter	This study
p416-ep-NAR1-R144A	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated R to A mutation under control of endogenous *NAR1* promoter	This study
p416-ep-NAR1-Q356E	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with indicated Q to E mutation under control of endogenous *NAR1* promoter	This study
p416-ep-NAR1-ΔW491	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with residue W491 deleted under control of endogenous *NAR1* promoter	This study
p416-NAR1-HA	(Amp^R, *URA3*, ARS/CEN)	*NAR1* ORF with a C-terminal HA tag under control of the *MET25* promoter	(Balk *et al.*, 2004)
p416-CFD1-HA	(Amp^R, *URA3*, ARS/CEN)	*CFD1* ORF with a C-terminal HA tag under control of the *MET25* promoter	(Balk *et al.*, 2005a)
p416-CIA1-HA	(Amp^R, *URA3*, ARS/CEN)	*CIA1* ORF with a C-terminal HA tag under control of the *MET25* promoter	(Balk *et al.*, 2005a)
p426-RLI1-HA	(Amp^R, *URA3*, 2µ)	*RLI1* ORF with a C-terminal HA tag under control of the *TDH3* promoter	(Lange *et al.*, 2001)
p424-RLI1-HA	(Amp^R, *TRP1*, 2µ)	*RLI1* ORF with a C-terminal HA tag under control of the *TDH3* promoter	N. Richhardt
p426-NTG2-HA	(Amp^R, *URA3*, 2µ)	*NTG2* ORF with a C-terminal HA tag under control of the *TDH3* promoter	(Balk *et al.*, 2004)
p426-ISU1	(Amp^R, *URA3*, 2µ)	*ISU1* ORF under control of the *TDH3* promoter	(Gerber *et al.*, 2004)
pYep352-ISD11-A	(Amp^R, *URA3*, 2µ)	*ISD11* ORF under control of the *MET25* promoter	B. Guiard
pYep352-ISD11-B	(Amp^R, *URA3*, 2µ)	*ISD11* ORF under control of the *ISD11* promoter	B. Guiard

2.5. Constructions of Gal-ISD11 strain

The W-303-1A was used to generate the Gal-ISD11 strain by PCR-mediated DNA replacement (Muhlenhoff *et al.*, 2002). Oligonucleotides Gal-ISD11-promoter and Gal-ISD11-ORF containing the start (-290) and the end (-1) of the *ISD11* promoter, the plasmid pFA6a-HIS3 carrying the *GAL1-10* promoter and the *HIS5* marker of *S. pombe* have been used for a PCR reaction to amplify the *HIS5* gene and the *GAL1-10* promoter. The PCR product flanked by 50 bp with homology to the region upstream of *ISD11* ORF was then transformed into the W303-1A yeast strain. The region -290 to -1 was replaced by homologous recombination with the *GAL1-10* promoter. Correct insertion of the *GAL1-10* promoter was verified by colony PCR.

2.6. Site-Directed Mutagenesis

For the introduction of the cysteine mutations into *NAR1*, three methods for site-directed mutagenesis were used.

2.6.1. GeneEditor™ in vitro Site-Directed Mutagenesis System

The GeneEditor™ *in vitro* Site-Directed Mutagenesis kit from Promega uses antibiotic selection to obtain the desired mutations. Selection oligonucleotides contain mutations that alter the ampicillin resistance gene thus creating a new additional resistance to the Antibiotic Selection Mix. The mutagenic oligonucleotide is annealed to the alkaline denaturated DNA template at the same time as the selection oligonucleotide. Then the mutant strain is synthesized using T4 DNA Polymerase and T4 DNA Ligase. The efficiency of mutagenesis is enhanced by an initial transformation into the competent BMH 71-18 *mut*S competent cells. This strain is used to avoid selection against the desired mutation. Then, a second transformation into *E. coli* strain JM109 is performed to allow segregation of mutant plasmids which are selected using ampicillin and the Antibiotic Selection Mix.

The following point mutations were obtained by this method using the corresponding oligonucleotides:

1. C59S primer C59S-forward
2. C177S primer C177S-forward
3. C412S primer C412S-forward
4. R144A primer R144A-forward
5. Q356E primer Q356E-forward

The plasmid pET15b-NAR1 served as DNA template. The *NAR1* mutant fragments were removed by digesting the pET15-b vector with *Xba*I and *Bam*HI, and cloned in p416 vector.

2.6.2. QuikChange® Site-Directed Mutagenesis Kit

The procedure (with minor changes from the original protocol-www.stratagene.com) utilizes a supercoiled double-stranded DNA vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended by *Pwo* DNA polymerase (Peqlab). Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with *Dpn* I. The *Dpn* I endonuclease is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from almost all *E. coli* strains is *dam* methylated and therefore susceptible to *Dpn* I digestion. The nicked vector DNA containing the desired mutations is then transformed into JM109 competent cells. Following the manufacturer´s protocol the *NAR1* mutations listed below were obtained:

1. pET15b-NAR1-C20A primers used: *NAR1*-C20A-forward & reverse
2. pET15b-NAR1-C62A primers used: *NAR1*-C62A-forward & reverse
3. pET15b-NAR1-C65A primers used: *NAR1*-C65A-forward & reverse
4. pET15b-NAR1-C231A primers used: *NAR1*-C231A-forward & reverse
5. pET15b-NAR1-C416A primers used: *NAR1*-C416A-forward & reverse

2.6.3. PCR-mediated Site-Directed Mutagenesis

The PCR-mediated site-directed mutagenesis involved two rounds of amplification cycles using two mismatch and two flanking primers (*Bam*HI-forward or *Xba*I-forward and *Xho*I-reverse or *Bam*HI-reverse). During the first round, 2 separate reactions were carried out with one of the flanking primers and the corresponding mismatch primer (Fig.2.1).

In two separate reactions (reactions I and II) the two “halves” of the area to be mutated are amplified by PCR. For the PCR reactions the *Pwo*-DNA-Polymerase (Peqlab) and the plasmid p416-NAR1 were used as DNA template. During the second step, the products of the first round were used as templates and the PCR was carried out using the two flanking primers.

Reaction I:
- 2 µl 10 mM $dNTP_s$
- 3 µl 10 pmol/µl mutagenic primer-forward
- 3 µl 10 pmol/µl Nar1-*Xho*I (*Bam*HI)-reverse
- 1 µl DNA template (200 ng)
- 41 µl H_2O

Reaction II:
- 2 µl 10 mM $dNTP_s$
- 3 µl 10 pmol/µl mutagenic primer-reverse
- 3 µl 10 pmol/µl Nar1-*Bam*HI (*Xba*I)-forward
- 1 µl DNA template (200 ng)
- 41 µl H_2O

To each reaction were added on ice:

-10 µl 10x *Pwo* buffer

-2.5 µl *Pwo*-DNA-Polymerase (2,5 U)

-37.5 µl H_2O

For PCR the following program was used:

1. Initial denaturation	2 min. at 94°C	
2. Denaturation	1 min at 94°C	x10
3. Annealing	1 min at 50-60°C	
4. Elongation	2 min at 72°C	
5. Denaturation	1 min at 94°C	x15
6. Annealing	1 min at 50-60°C	
7. Elongation	2 min at 72°C (+ 20 sec. each cycle)	
8. Elongation	10 min at 72°C	

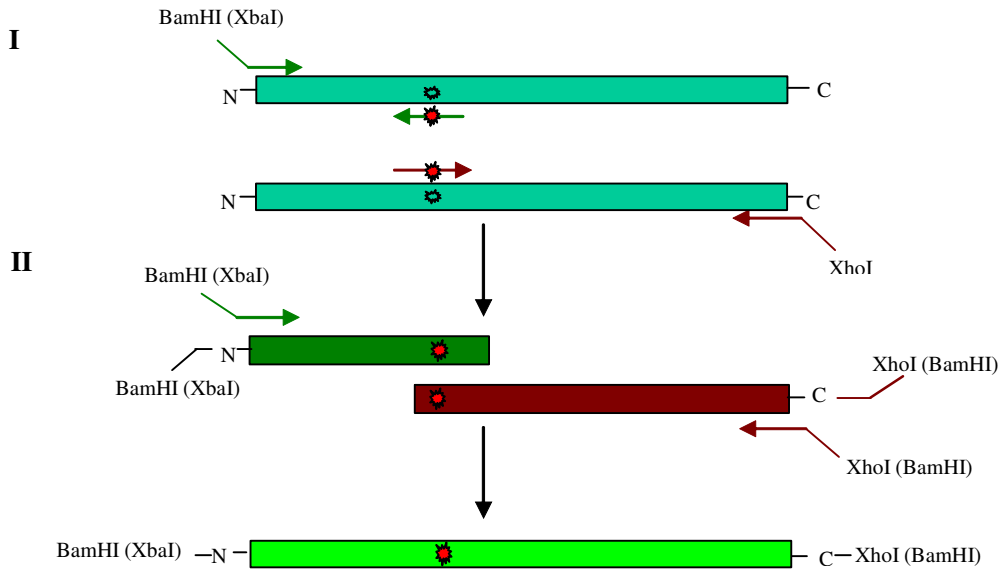
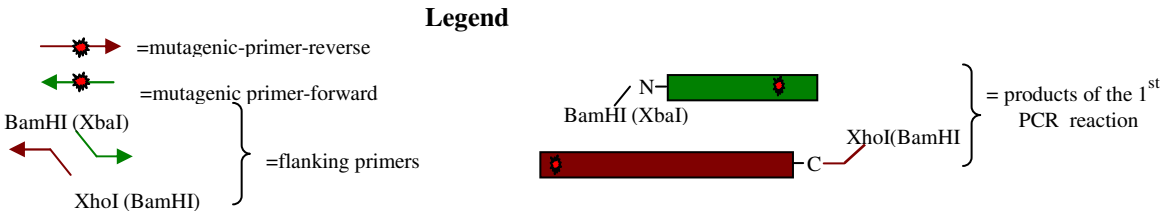


Figure 2.1 General scheme of PCR-based site-directed mutagenesis.



The PCR products were then digested with *Bam*HI / *Xho*I (*Xba*I / *Bam*HI) and cloned into p416 plasmid which has been cut with same restriction endonucleases. Using this procedure the following constructs were made:

1. p416-NAR1-C20A primers: NAR1-C20A-forward & reverse
2. p416-NAR1-C59A primers: NAR1-C59A-forward & reverse
3. p416-NAR1-C62A primers: NAR1-C62A-forward & reverse
4. p416-NAR1-C65A primers: NAR1-C65A-forward & reverse
5. p416-NAR1-C177A primers: NAR1-C177A-forward & reverse
6. p416-NAR1-C231A primers: NAR1-C231A-forward & reverse
7. p416-NAR1-C412A primers: NAR1-C412A-forward & reverse
8. p416-NAR1-C416A primers: NAR1-C416A-forward & reverse

Double Cysteine Mutants:

To obtain two simultaneous cysteine mutations we searched for restriction endonucleases that cut only once in the *NAR1* gene and in the p416 vector. The p416 plasmids coding for the single cysteine mutations were cut by the restriction enzymes and then a fragment containing one mutation was ligated into the vector coding for the other mutation. An example is illustrated in Figure 2.2 for the p416-NAR1-C177-416A.

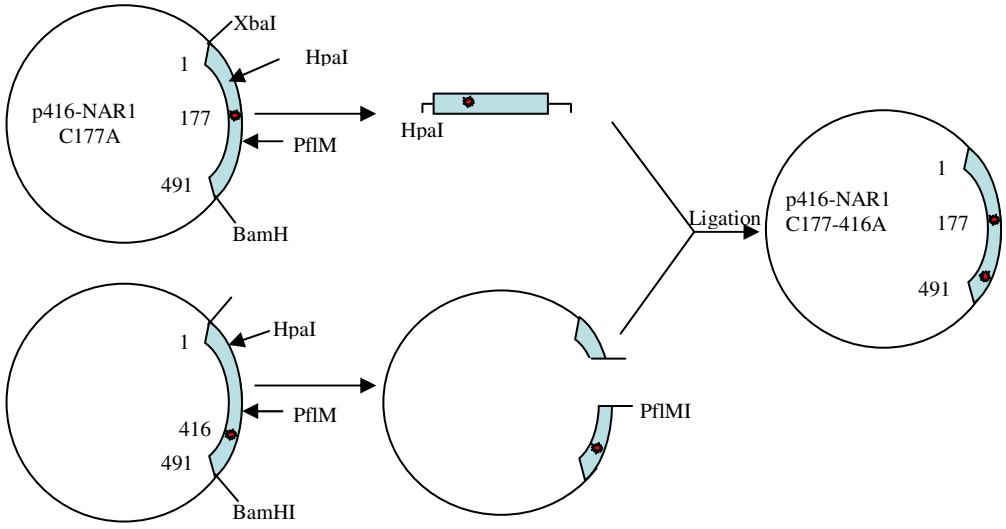


Figure 2.2 Scheme depicting the strategy for generating the double cysteine mutants.

All other double cysteine mutants were made in the same way using different restriction enzymes:

Double mutation	Plamids	Fragments used for ligations	Restriction enzymes
p416-NAR1-C20-62A	p416-NAR1-C20A p416-NAR1-C62A	p416-NAR1-C20A (X) NAR1-C62A (X)	*Hpa*I & *Xho*I
p416-NAR1-C20-62A	p416-NAR1-C20A p416-NAR1-C65A	p416-NAR1-C20A (X) NAR1-C65A (X)	*Hpa*I & *Xho*I
p416-NAR1-C177-412S	p416-NAR1-C177S p416-NAR1-C412S	NAR1-C177S (X) p416-NAR1-C412S (X)	*Xba*I & *PflM*I
p416-NAR1-C177-416A	p416-NAR1-C177A p416-NAR1-C416A	NAR1-C177A (X) p416-NAR1-C416A (X)	*Hpa*I & *PflM*I
p416-NAR1-C231-416A	p416-NAR1-C231A p416-NAR1-C416A	p416-NAR1-C231A (X) NAR1-C416A (X)	*Xba*I & *PflM*I

Table1: List of the Nar1p double cysteine mutants

Insertion for correct mutation was analysed by unique site restriction analysis and the positive clones were confirmed by DNA sequencing.

2.7. Molecular Biological Methods

2.7.1. Isolation of Plasmid-DNA from E. coli

The isolation of Plasmid-DNA from *E. coli* was performed by alkyline lysis (Maniatis *et al.*, 1982). The cells were grown over night in 3.5 ml LB-Amp medium at 37°C. In case of DNA sequencing, the plasmid-DNA was isolated using the NucleoSpin Plasmid-Kit from Macherey-Nagel following the manufacturer´s protocol.

2.7.2. Preparation of genomic DNA from S. cerevisiae (Hoffman et al., 1987)

To isolate DNA from *S. cerevisiae*, 5-20 ml YPD medium were inoculated with yeast cells and incubated over night at 30°C. The cells were harvested by centrifugation (3 min at 3,000 rpm) and resuspended in 200 µl TEST buffer. After adding 200µl Phenol/Chloroform/Isoamyl alcohol (25:24:1) and 0.3 g glass beads the samples were vigorously vortexed. Then 200 µl of TE-buffer were added and samples were centrifuged 5 min at 13.000 rpm. The supernatant (aqueous phase) was transferred to a new tube and a second Phenol/Chloroform extraction was performed. The DNA was ethanol precipitated (chapter 2.7.3.), resuspended in 50 µl TE-buffer containing RNase (20 µg/ml) and incubated for 30 min at 37°C. Ethanol precipitation was repeated and the DNA was resuspended in 50-500 µl TE-buffer.
The purified DNA was stored at -20°C.

TEST-buffer	10 mM Tris-HCl, pH 8,0
	1 mM EDTA
	100 mM NaCl
	2 % (v/v) Triton X-100
	1 % (v/v) SDS

TE-buffer	10 mM Tris-HCl, pH 8,0
	1 mM EDTA

2.7.3. Purification and Analysis of DNA

Phenol-Chloroform Extraction (Sambrook and Russel, 2001)

For separation of DNA from proteins, phenol/chloroform extraction was performed. DNA-containing solutions are first treated with an equal volume of phenol-chloroform solution (Phenol:Chloroform:Isoamyl alcohol=25:24:1) and vigorously mixed. The aqueous DNA-containing phase (upper phase) is separated by centrifugation for 5 min at 13,000 rpm and then washed with Chloroform:Isoamyl alcohol (24:1) to remove the phenol. After a second centrifugation step the DNA in the aqueous phase was precipitated with ethanol.

Ethanol Precipitation (Shapiro, 1981)

To precipitate DNA, the aqueous DNA-containing solutions were treated with 1/10 volumes of 3 M Na-Acetate (pH 5.2) and 3 volumes of 100% ethanol. After an incubation for 1 h at -80°C, DNA was pelleted by centrifugation for 30 min at 13,000 rpm (at 4°C), washed once with 70% ethanol, dried and resuspended in H_2O.

2.7.4. DNA Agarose Gel Electrophoresis (Sambrook and Russel, 2001)

1% to 2% agarose gels were routinely used to separate plasmid or linear DNA-fragments. The appropriate amount of agarose was dissolved in 1x TAE buffer and ethidium bromide was added to a final concentration of 1µg/ml. DNA samples were mixed with 1/10 volumes of 10x loading buffer and electrophoresis was achieved in TAE buffer by applying a voltage of 80-120 V. As size standard 1kb DNA marker from Fermentas was used. After completion of electrophoresis the gel was examined on a 302 nm UV transilluminator and documented using a thermoprinter.

2.7.5. Extraction of DNA from Agarose Gels

To extract and purify DNA from agarose gels the NucleoSpin Extract II Kit was used following the instruction of the supplier Macherey-Nagel.

2.7.6. Polymerase Chain Reaction (PCR)

All PCR reactions to amplify DNA fragments were performed in a 50 or 100 µl reaction volume. Genomic *S. cerevisiae* or plasmid DNA served as template and Combi*Zyme* DNA-Polymerase (Invitek, Berlin) as polymerizing enzyme.

100µl reaction volume:

0.5-1 µg DNA template
10 µl 10x OptiPerform buffer III (without Mg^{2+})
5 µl 50 mM $MgCl_2$ (end concentration
2 µl 10 mM dNTPs (end concentration
5 µl Primer 1 and 2 (end concentration
20 µl 5x OptiEnhancer buffer
1 µl Combi*Zyme* Polymerase (5 U)
ad 100 µl H_2O

For the PCR the following program was used (Thermocycler Biometra):

1. Initial denaturation	2 min at 94°C	
2. Denaturation	30 sec. at 94°C	35 cycles
3. Annealing	40 sec. at 50-60°C	
4. Elongation	1 min/kb at 72°C	
5. Elongation	10 min at 72°C	

2.7.7. Determination of Protein Concentration

To determine the DNA concentration 1-2 µl of DNA samples were loaded on an agarose gel. For comparison 1 kb DNA Ladder (1 µg/µl) (Invitrogen) was used. After agarose gel electrophoresis the fluorescence intensity from ethidium bromide of DNA samples was visually compared to that of DNA marker.

2.7.8. Digestion of DNA with Restriction Endonucleases

DNA samples (0.5-5 µg) were digested by restriction endonucleases using recomended reaction buffers. Generally, restriction digests were prepared in 20 µl (analytical purposes) or 40 µl (preparative purposes) final volume, with 2-3 Units (per µg DNA) and incubated at 37°C from 2 to 6 h (maximum over night). Finally the samples were analysed by agarose gel electrophoresis.

2.7.9. Ligation of DNA Fragments

For ligation of DNA fragments the T4 DNA –Ligase (Promega) was used. T4 DNA Ligase catalyses the joining of two strands of DNA between the 5´-phosphate and the 3´-hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt-ended configuration. The ligation reactions were performed in a 20 µl total volume with 1 µl T4 DNA Ligase (3 Units) and 2 µl of 10x Ligase buffer. Vector to insert ratio was 1:3. The ligation reactions were incubated over night at 16°C.

2.7.10. Preparation and Transformation of Competent E. coli Cells

Preparation of Competent Cells ($CaCl_2$ method)

For preparation of competent cells for transformation, a single colony of the appropriate *E. coli* strain was inoculated into 3 ml of LB medium and grown over night at 37°C. Then, 50 ml LB + 20 mM $MgSO_4$ were inoculated with the over night-grown *E. coli* cells. The cells were grown further until they reached the logarithmic growth phase (OD=0.4). After keeping them on ice for 10 min., the cells were harvested by centrifugation (10 min at 4,000 rpm, 4°C). The cell pellet was resuspended in 20 ml FSB solution followed by incubation on ice for 10 min. The cells were centrifuged at 4°C and the pellet resuspended in 4 ml FSB solution. After adding 140 µl DMSO the cells were mixed, incubated for 15 min. on ice and another 140 µl DMSO were added. Competent cells were aliquoted and stored at -80°C.

FSB solution:

	Final Concentration
K-acetate pH=7,5	10 mM
$MnCl_2 \cdot 4\ H_2O$	45 mM
$CaCl_2 \cdot 2\ H_2O$	10 mM
KCl	100 mM
Hexaminecobaltchloride	3 mM
Glycerol	10 %

-adjust the pH to 6.4 with 0.1 M HCl

Transformation of E. coli Competent Cells

The compentent cells (100 µl) were incubated on ice for 30 min with 50-100 ng plasmid DNA (or 10-20 µl ligation mixture). After a heat shock (90 sec. at 42°C) the cells were incubated on ice for 2 min. Then the cells were harvested by centrifugation, resuspended in 1 ml LB medium and incubated for 60 min at 37°C under moderate shaking conditions. The transformed cells were harvested by centrifugation and plated on LB-medium plates supplemented with the appropriate antibiotic. The plates were incubated over night at 37°C.

2.7.11. Transformation of Yeast Cells with Recombinant DNA

The *S. cerevisiae* cells were transformed using the lithium acetate method (Ito *et al.*, 1983). By this method the yeast cells were transformed with plasmid-DNA or with linear DNA- fragments used for genomic homologous recombination. The corresponding yeast strain was grown over night and diluted the next morning in 50 ml YPD medium to an OD_{600} of 0.2.The cells were further grown until they reached an OD_{600} = 0.6 - 1, and harvested by centrifugation (5 min, at 3,000 rpm). After washing with 20 ml of H_2O, the cells were pelleted under the same conditions and resuspended in 1 ml lithium-acetate solution. For each transformation 100 µl suspension of cells was mixed with 5 µl denaturated salmon sperm DNA and 5-10 µg of DNA to be transformed. The mix was incubated for 30 min, at 30°C, with moderate shaking followed by addition of 700 µl of PEG-solution and a heat shock for 15 min, at 42°C. The cells were pelleted, resuspended in 1 ml YPD medium and incubated for 60 min, at 30°C. After harvesting, the cells were washed in H_2O and spread on selective solid SC plates. The plates were incubated for 2-5 days at 30°C to recover transformants.

LiAc solution
100 mM Lithiumacetate
10 mM Tris-HCl, pH 7.5
1 mM EDTA, pH 8.0

PEG solution
40 % Polyethylenglycol 4000
100 mM Lithiumacetate
10 mM Tris-HCl, pH 7.5
1 mM EDTA, pH 8.0

2.8. Cell Biological Methods

2.8.1. Isolation of Mitochondria from S. cerevisiae (Daum et al., 1982; Diekert et al., 2001)

Yeast cells were cultivated either in reach or minimal medium at 30°C to OD_{600} of 1-1.5 and harvested by centrifugation (5 min, at 3000 rpm). The cells were washed once with H_2O and Tris-SO_4 buffer and the cells wet weight was measured. After washing in 1.2 M Sorbitol-buffer, the cells were resuspended in the same buffer (4 ml/g cells). To digest the cell wall (spheroplasting), Zymolyase T100 (1.5 mg/g cells) was added and the cell suspension was incubated for 30-60 min at 30°C and 150 rpm. To test the cell wall digestion (spheroplast formation), 50 µl cell suspension was diluted with 2 ml H_2O. The solution of spheroplasts in H_2O becomes clear because spheroplasts burst under these conditions. All subsequent steps were performed at 4°C. The spheroplasts were isolated by centrifugation (5 min, 3,000 rpm, 4°C) and washed twice in 1.2 M Sorbitol-buffer. The spheroplasts were then resuspended in 2x BB buffer containing 1mM PMSF and diluted with 1 volume of ice-cold water. After homogenization by 15 times in a Dounce-Homogenisator, the cell remnants and unopened cells were sedimented by double centrifugation (5 min, 4000 rpm, 4°C). The supernatant was centrifuged (12 min, 10,000 rpm, 4°C) to pellet the mitochondria. The supernatant was used for further experiments as post-mitochondrial supernatant (PMS). Pelleted mitochondria were washed once in 1x BB buffer to remove the remaining PMS and finally resuspended in a small volume of 1x BB buffer. Mitochondria and PMS were aliquoted, frozen in liquid nitrogen and stored at -80°C.

Tris-SO_4 buffer:
100 mM Tris-SO_4, pH 9,4
10 mM Dithiothreitol (DTT)

1.2 M Sorbito buffer:
1.2 M sorbitol
20 mM KPi, pH 7.4

2x BB :
1.2 M sorbitol
40 mM Hepes-KOH, pH 7.4
1 mM PMSF

1x BB:
0.6 M sorbitol
20 mM Hepes-KOH, pH 7.4

2.8.2. Crude isolation of Mitochondrial Fractions from S. cerevisiae

Yeast cells cultivated in 50-100 ml over night at 30°C were pelleted by centrifugation (5 min, 3,000 rpm) and washed once with Sorbitol-buffer. 0.3-0.5 g of cells were resuspended in 300-500 µl Sorbitol-buffer, 2 mM PMSF and 1/3 volume of glass beads were added. The tubes were placed upside down on ice and incubated for 5 min. Yeast cell were lysed by three bursts of 1 min each on a vortex with intermediate cooling. Cells debris were removed by centrifugation (5 min, 2,500 rpm, 4°C). The supernatant was transferred to a new tube and centrifuged (12 min, 9,000 rpm, 4°C) to separate the mitochondrial fraction (pellet) and the post-mitochondrial supernatant (PMS). The pelleted mitochondrial fraction was resuspended in 50-100 µl Sorbitol-buffer.

Sorbitol-buffer:
20 mM Tris pH 7,4
50 mM NaCl
0.6 M Sorbitol

2.8.3. Preparation of Yeast Cell Extract (Method of Rödel)

To prepare a cell extract, yeast cells were grown over night in 10-25 ml liquid media. 0.5 ml of over night preculture were transferred in a 1.5 ml Eppendorf tube and centrifuged for 3 min at 13,000 rpm (4°C). The pellet was washed once with 0.5 ml 10 mM Tris/HCl, 1 mM EDTA pH 8.0 and centrifuged again. The pellet was resuspended in 0.5 ml ice-cold H_2O, 75 µl of Mix-solution was added and incubated for 10 min on ice. After incubation, 575 µl 50 % cold TCA were added, followed by a short vortexing and incubation on ice for 10 min. The samples were centrifuged (10 min, 12,000 rpm, 4°C), supernatant removed, pellet washed twice with 1 ml cold acetone. The pellet was air-dried and resuspended in 100 µl 1x gel – loading buffer and 25 µl were loaded on SDS-polyacrylamide gels.

Mix-solution:
1.85 ml 10 N NaOH
740 µl mercapto-ethanol
6.91 ml H_2O
0.5 ml 200 mM PMSF

2.8.4. Enzyme Activities of Mitochondrial Proteins

Aconitase (Fansler et al., 1969)

The aconitase catalyses the following reversible reaction:

citrate <=>(cis-aconitate) <=> isocitrate

The intermediate product cis-aconitate formed in the aconitase reaction absorbs light at a wavelength of 240 nm because of its double bond. Lysis of mitochondria: in an Eppendorf tube on ice add 20 µg of mitochondria, 4 µl 3% dodecylmaltoside and up to 50 µl buffer. Add immediately to cuvette for measurement.

Buffer
50 mM Tris, pH 8.0,
50 mM NaCl.
20 mM isocitrate (fresh)

Assay
950 µl buffer
50 µl freshly lysed mitochondria

The increase of extinction is measured in a quarz cuvette for 2 min at 235 nm.
$\varepsilon_{235nm} = 4.9\ M^{-1}cm^{-1}$

Succinate Dehydrogenase (Complex II)

Complex II specific activity is measured by following the reduction of DCPIP (2,6-dichlorophenol-indophenol) by quinones at 600 nm for 2 min.($\varepsilon_{235nm} = 21\ M^{-1}\ cm^{-1}$). Malonate in the reference cuvette inhibits complex II activity.

Buffer
50 mM Tris, pH 8.0,
50 mM NaCl
1 mM fresh KCN
0.1 % Triton X-100

Stock solutions
10 mM Decylubiquinone
10 mM 2,6-Dichlorphenol-indophenol (DCPIP)
20 % Na-Malonate
20 % Na-Succinate

Assay:

	Sample cuvette	Reference cuvette:
Buffer	950 µl	950 µl
10 mM Decylubiquinone	7 µl	7 µl
10 mM DCPIP	10 µl	10 µl
20 % Succinate	12 µl	12 µl
20 % Malonate	-	12 µl
Mitochondria	25 µg	25 µg

Succinate-Cytochrome c Reductase (Complex III) (Robinson et al., 1995)

Succinate dehydrogenase oxidizes succinate and the electrons are transferred to Coenzyme Q, complex III and finally to cytochrome *c*, which is then reduced. Normally, cytochrome *c* would pass on the electrons to oxygen via cytochrome *c* oxidase (complex IV), however, this enzyme complex is inactivated by cyanide. The reduction of cytochrome *c* is measured by the increase in absorbance at 550 nm for 2 min. (ε_{550nm} = 20 $M^{-1}cm^{-1}$):

Buffer
50 mM Tris, pH 8.0,
50 mM NaCl
1 mM fresh KCN

Stock solutions
20% Na-Malonate
20 % Na-Succinate
20 mg/ml cytochrome *c*

Assay:

	Sample cuvette	Reference cuvette
buffer	920 µl	920 µl
20 % succinate	12 µl	12 µl
20 % malonate	-	12 µl
cytochrome *c*	50 µl	50 µl
	adjust spectrometer	
mitochondria	25 µg	25 µg

Malate dehydrogenase (Englard et al., 1969)

Malate dehydrogenase catalyses the last reaction of TCA cycle, namely the NADH-dependent reduction of oxaloacetate to malate. The enzyme activity is determined by measuring the oxidation of NADH for 2 min at $\lambda = 340$ nm. $\varepsilon_{340nm} = 6.22 \times 10^3\ M^{-1}cm^{-1}$

Buffer
50 mM Tris, pH 8.0
50 mM NaCl

Stocks
10 mg/ml NADH
5 mg/ml oxaloacetate in buffer

Lysis of mitochondria (on ice):

20 µg	Mitoprep
4 µl	3 % dodecylmaltoside
50 µl	buffer

Assay:

Buffer	930 µl
NADH (freshly prepared)	10 µl
Oxaloacetate	10 µl
Lysed mitochondria	50 µl

Mitochondrial Cysteine Desulfurase

The enzymatic desulfurase activity was measured in yeast mitochondria using cysteine as a substrate. 100 µg of mitochondria were diluted in 30 µl 1x buffer, 2 µl of 3% dodecylmaltoside were added and the samples incubated for 2 min on ice. After adding 100 µl 2x buffer the samples were diluted with H_2O to a final volume of 200 µl and 8 µl of 0.1 M cysteine were added. The samples were mixed and incubated for 30 min at 30°C. The reaction was stopped by adding 20 µl of 30 mM $FeCl_3$ in 1.2 M HCl and 20 µl of 40 mM *N,N*-dimethyl-*p*-phenylenediamine in 7.2 M HCl. After further incubation for 20 min in the dark, the samples were centrifuged and the absorption of methylene blue was measured at 667 nm.

2x buffer
40 mM Tris, pH 8,0
10 mM DTT
100 mM NaCl
10 mM Pyridoxal-phosphate
0.4 M sucrose

Stock solutions
0.1 M Cysteine
3% Dodecylmaltoside
30 mM $FeCl_3$
40 mM *N,N*-DMPD

2.8.5. Enzyme Activities of Cytosolic Proteins

Isopropylmalate -Isomerase-Leu1p

Isopropylmalate-isomerase is a cytosolic enzyme and therefore its activity was measured in yeast cell extracts. Due to high instability of the protein, the enzyme activity was measured immediately after preparing the cell extracts. Isopropylmalate-isomerase catalyses the following reversible reaction:

α-Isopropylmalate <=> (Dimethylcitraconate) <=> β-Isopropylmalate

The enzyme activity is measured by the increase in extinction at 235 nm of the double bond of the intermediate product dimethylcitraconate ε_{235} = 4.53 M^{-1} cm^{-1} (Kohlhaw, 1988). To prepare the cell extracts a 50 ml overnight yeast cell culture is washed once in H_2O, harvested by centrifugation and finally resuspended in TNETG-buffer. After adding 2mM PMSF and ½ volumes of glass beads, the cells are lysed by three bursts of 1 min each on a vortex with intermediate cooling. Cell debris was removed by centrifugation (5 min, 13,000 rpm, 4°C). The supernatant (cell lysate) was used in the assay.

Buffer
50 mM Tris, pH 8.0
50 mM NaCl

Stock solutions
10 mM β-Isopropylmalate

Assay:

Buffer	970 µl
10 mM β-Isopropylmalate	20 µl
Zero the photometer	
Cell lysate	10µl

Alcohol-Dehydrogenase-Adh1p

The alcohol-dehydrogenase activity was measured in cell extracts as Leu1p. The assay is based on absorbance of NADH at 340 nm. (ε =6.22 $mM^{-1}cm^{-1}$ at 340 nm.)

Buffer
50 mM Tris, pH 8,0
50 mM NaCl

Stock solutions
50 mM NAD^+
100 % Ethanol

Assay:

Buffer	930 µl
Ethanol	30 µl
50 mM NAD^+	30 µl
Zero the photometer	
Cell lysate	10 µl

2.8.6. Determination of de novo Fe/S cluster biogenesis by ^{55}Fe radiolabelling

Yeast cells were grown over night in 50 ml SC-medium containing the required carbon sources. In the next morning the cells were pelleted (5 min, 3,000 rpm), washed once in water, resuspended in 100 ml of "iron-free SC-medium" (containing either galactose or glucose) to an OD_{600} of 0.2 and incubated over night at 30°C. Cells were harvested (5 min, 3,000rpm) and washed once in 20 ml H_2O. 0.5 g cells were resuspended in 10 ml iron-free medium and incubated with $^{55}FeCl_3$ (10 µCi) and 1 mM ascorbate for 2 h at 30°C. The labelling reaction was transferred into a 15 ml tube and cells were pelleted by centrifugation for 5 min at 3000 rpm. The radiolabelled yeast cells were washed once in citrate buffer (50 mM Citrate, 1 mM EDTA, pH 7.0) and once in 20 mM HEPES-KOH, pH 7.4. All following steps were performed on ice. The cells was resuspended in 500 µl TNETG buffer + 2 mM PMSF and ½ volumes of glass beads and lysed by three bursts of 1 min each on a vortex with intermediate cooling. The cell debris were removed by centrifugation (5 min, 3,000 rpm, 4°C), the supernatant transferred into a new 1.5 ml Eppendorf tube and centrifuged (10 min, 14,000 rpm, 4°C). The supernatant was transferred to a fresh tube avoiding to transfer any of the membrane pellets. 5 µl of the cell lysate were mixed with 1 ml scintillation cocktail to quantify the iron uptake into the cells. The remaining supernatant (^{55}Fe radiolabelled) was

used also to quantify *de novo* Fe/S cluster biogenesis. For this, 250 µl cell lysate were mixed with 10-40 µl protein A-sepharose beads (see 2.9.8.). Depending on the experimental conditions, antisera against Nar1p, Leu1p, Isu1p, Aco1p or HA-tag were used and the mix was incubated for 1 h at 4°C. The sepharose beads were pelleted by centrifugation (5 min, 3,000 rpm, 4°C), washed 3 times in TNETG buffer. Finally, 50 µl of H_2O and 1 ml scintillation cocktail were added and the radioactivity associated with the beads was counted in a scintillation counter. The principle of the assay is depicted in Figure 2.3.

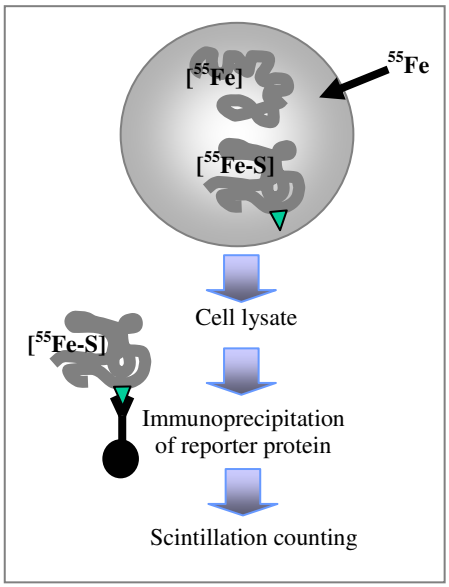


Figure 2.3 Schematic illustration of the ^{55}Fe *in vivo* radiolabelling assay.

2.8.7. Determination of Mitochondrial Iron Content (Li et al., 1999; Tangeras et al., 1980)

The mitochondrial iron content (not heme-bond iron) was measured photometrically with bathophenanthroline-disulfonate, which can bind iron leading to a complex that absorbs light at 540 nm. $\varepsilon_{540} = 23.5\ M^{-1}\ cm^{-1}$

Buffer
1 M Tris, pH 7.4

Stock solutions
10 % SDS
1 M Na-dithionite
100 mM Bathophenantroline-disulfonate

Assay:

	Sample cuvette	Reference cuvette
buffer	100 µl	100 µl
10 % SDS	60 µl	60 µl
1 M Na-dithionite	20 µl	20 µl
100 mM Bathophenantroline	100 µl	100 µl
H_2O	720-x µl	720 µl
Mitochondria (0,2 mg)	x µl	-

2.9. Biochemical Methods

2.9.1. Determination of Protein Concentration

Protein concentration was determined according to Bradford (Bradford, 1976) using the Bio-Rad protein assay (Bio-Rad, München). This measurement is based upon Coomassie® Brilliant Blue G-250 dye-binding assay. 200 µl Bio-Rad Protein Dye (Bio-Rad) were added to samples and standard (BSA, bovine serum albumine, 0-100 µg), and H_2O to a final volume of 1 ml. Afterwards they were gently mixed to avoid bubbles and after 10 min, the measurement was carried out at 595 nm.

2.9.2. TCA (Trichloroacetic Acid) Protein Precipitation

To concentrate proteins for analysis by SDS PAGE, an equal volume of 25% TCA was added to the protein sample and incubated for 30 min on ice. The samples were centrifuged at 4°C for 15 min (14,000 rpm) and the supernatant was carefully removed, leaving protein pellet intact. After washing the pellet with 1 ml cold acetone, the samples were centrifuged for 5 min at 4°C (14,000 rpm), the supernatant removed and the pellet dried for 5-10 min at room temperature. The samples were resuspended in 1x SDS gel-loading buffer and boiled for 10 min at 95°C before loading sample onto polyacrylamide gel. If the sample color turned yellow (acidic pH), 1-5 drops of 1 M Tris-HCl, pH 8.0 were added until the sample turns blue again.

3x SDS gel-loading buffer
187.5 mM Tris-HCl, pH 6,8
6 % SDS
15 % Mercaptoethanol
30 % Glycerol
0.006 % Bromphenol Blue

2.9.3. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The denatured polypeptides bind SDS and become negatively charged. Because the amount of SDS bound is usually proportional to the molecular mass of the polypeptide and is independent of its sequence, the mobility of protein-SDS complexes in polyacrylamide gels is inverse proportional to the size of the protein. By using markers of known size it is therefore possible to estimate the molecular mass of a protein.

For most purposes a 10 -15 % resolving gel were prepared.

Stacking gel:

H_2O	5.7 ml
30 % Acrylamide:Bisacrylamide (29:1)	2 ml
0.6 M Tris/HCl pH 6,8	2.1 ml
10 % SDS	100 µl
10 % APS (Ammoniumpersulfate)	100 µl
TEMED	10 µl

Resolving gel:

	10 %	12 %	15 %
H_2O	8.03 ml	6.73 ml	4.7 ml
30 %Acrylamide:Bisacrylamide (29:1)	6.7 ml	8 ml	10 ml
1.5 M Tris/HCl pH 8.8	5 ml	5 ml	5 ml
10 % SDS	200 µl	200 µl	200 µl
10 % APS	100 µl	100 µl	150 µl
TEMED	10 µl	10 µl	15 µl

Samples were prepared in 1x SDS gel-loading buffer and boiled for 5 min at 95°C. Electrophoresis was performed at 35 mA constant power until the bromphenol blue dye had reached the bottom of the gel.

2.9.4. Coomassie Blue Staining of Proteins

This method was performed to verify the purity of recombinant purified proteins. The proteins separated in SDS-polyacrylamide gels were stained over night in Coomassie solution (0.25 % Coomassie Brilliant-Blue R-250, 50 % ethanol, 10 % acetic acid). Distaining was done in 30 % ethanol and 10 % acetic acid. The stained gels were then incubated in 10 % glycerol for 30 min and dried using a gel-drying system (Promega) for 48 h at 4°C.

2.9.5. Transfer of Proteins to Nitrocellulose-Membrane (Western-Blot)

Proteins separated via SDS-PAGE were transferred onto nitrocellulose membrane using the semi-dry blotting method (Kyshe-Andersen, 1984).The gel, membrane, and four sheets of Whatman filter paper were incubated in transfer buffer (25 mM Tris, 200 mM glycine, 20 % (v/v) methanol, 0.02 % (w/v) SDS). Two sheets of filter paper were placed on the anode electrode followed by the membrane and the gel. This was covered with another two filter papers and with the cathode electrode. The transfer was performed at 1 mA/cm^2 for 1 h. To verify transfer efficiency, the nitrocellulose membranes were reversibly stained with Ponceau S solution (0.2 % (w/v) Ponceau S in 3 % (w/v) TCA).

2.9.6. Immunostaining

Protein bands immobilized on the nitrocellulose or PVDF membranes could be visualized via decoration with specific antibodies. The non-specific protein binding sites were blocked during membrane incubation with 5 % (w/v) skim milk in TBS buffer at RT for 1 h or over night at 4°C (in case of HA-tagged proteins the blocking step was performed in 1 % BSA). The immunodecoration was done for 1 h at RT or longer at 4°C, with specific antiserum (1:200 to 1:1000 dilutions in milk/TBS). After washing 3 times (each 5-10 min in TBS + 0.1 % Tween 20), the membrane was incubated (1 h at RT) with the secondary antibody (peroxidase coupled anti-rabbit-IgG or anti-mouse-IgG) (diluted 1:10.000 in milk/TBS). The membrane was washed again as described above and treated with ECL detection solution. After incubating for 3 min, excess detection reagent was drained off. The

membrane was placed into a film cassette and exposed to a sheet of X-ray film (Kodak) in a dark room. The film was immediately developed.

TBS-buffer
50 mM Tris-HCl, pH 7,5
0.9 % (w/v) NaCl

2.9.7. Quantification of Protein Levels after Immunostaining

The protein levels were quantified using the TINA 2.0 program. The values obtained for each protein band (from independent experiments) were taken and the average calculated. The experimental results (from ^{55}Fe *in vivo* labeling experiments – cpm/0.25 g cells) were divided by average of protein levels.

2.9.8. Coupling of Antibodies to Protein-A Sepharose

50 mg of Protein-A-Sepharose were resuspended in cold 500 µl TNETG buffer. The beads were swollen by incubation for > 30 min in the cold room and mixed occasionally. The beads were pelleted by a 5 min spin at 3,000 rpm. After adding 500 µl of antibody serum the beads were incubated in the cold room on a shaker for > 1 hr. They were pelleted by centrifugation for 5 minute at 3,000 rpm and washed 5 times in 500 µl TNETG buffer with centrifugation for 5 min at 3,000 rpm in between the washes. Finally, the beads were resuspended in 500 µl TNETG and stored at 4°C.

TNETG buffer
20 mM Tris-HCl, pH 7,4
150 mM NaCl
2.5 mM EDTA
10 % (v/v) Glycerol
0.5 % (v/v) Triton X-100

2.9.9. Pull-Down Assay

To demonstrate a direct interaction between 2 proteins a pull-down assay was performed. To show an interaction between Nar1p and Nbp35p both proteins had to be overproduced in the cells as HA-tagged proteins. The assay was performed using yeast cell extracts. To prepare the cell extracts a 50 ml overnight culture is washed once in H_2O,

harvested by centrifugation and 0.5 g of cells were resuspended in 500 µl TNETG-buffer. After adding 2 mM PMSF and ½ volumes of glass beads, the cells are lysed by three bursts of 1 min each on a vortex with intermediate cooling. Cell debris was removed by centrifugation (5 min, 13,000 pm, 4°C). The supernatant (cell lysate) was used in the assay. To 250 µl cell lysate 10 µl of HA-coupled beads (or 25 µl anti-Nar1 beads) were added and the incubated in the cold room on a rotating shaker for > 3 hr. The beads were centrifuged for 5 min at 3000 rpm (4°C), washed 3 times in 500 µl TNETG-buffer and centrifuged again. Pelleted beads were resuspended in 50 µl 1x SDS gel-loading buffer and boiled for 5 min at 95°C. Usually 15-20 µl were loaded on a SDS gel. Also, 25 µl of total cell lysate were TCA-precipitated and resuspended in 50 µl 1x SDS gel-loading.

2.9.10. GFP-Reporter Assay

Yeast cells carrying plasmids p415-FET3-GFP or p416-FET3-GFP were grown in minimal medium supplemented with 200 µM ferric ammonium citrate. In the next morning over night cultures were diluted to an OD_{600} = 0.2 and incubated further until the OD_{600} = 0.5. The cells were collected by centrifugation (5 min at 3,000 rpm) and resuspended in 3 ml of H_2O to get a final OD_{600} = 1. The fluorescence emission of the cell suspension was measured at 513 nm (excitation 480 nm) using a fluorescence spectrophotometer.

2.9.11. Overexpression and Purification of Recombinant His-tagged Proteins

The plasmids pET15b encoding the wild type *NAR1* gene or the *NAR1* cysteine mutants (see *2.4.1.)* were transformed into *E. coli* C41-DE3 competent cells that already contained the plasmid pISC (coding for the *E. coli* ISC operon). One colony was used to inoculate 50 ml LB-medium with ampicillin (100 µg/ml) and tetracycline (34 µg/ml) and the cells were grown over night at 37°C (250 rpm). The over night culture served to inoculate 2 L of LB medium (+ ampicillin and tetracycline) and the cells incubated at 37°C until the culture reached an OD_{600} of 0.5-0.6. To induce overexpression, 1 mM IPTG (final concentration) was added and the cells were further incubated at 30°C for another 4 h. The *E. coli* cells were harvested by centrifugation (10 min, 5,000 rpm, 4°C) and the cell pellet was washed once in lysis buffer. The cells were lysed at 4°C in 30-50 ml lysis buffer by applying a pressure of 1,0 x 10^8 Nm^{-2} using an high pressure homogenisator (EmulsiFlex-C3). The unopened cells and cell debris were removed by centrifugation (30 min, 100.000 x *g*, 4°C). The overexpressed recombinant His-tagged proteins were purified by Ni-NTA affinity chromatography. Ni-NTA

agarose (≈ 10 ml) was first equilibrated in lysis buffer, mixed with the cell lysate (supernatant) and incubated for 1 h at 4°C in a rotary shaker. The lysate-Ni-NTA mixture was loaded into a column. The Ni-NTA agarose was washed with 80 ml washing buffer to remove the unspecifically bound proteins. The His-tagged protein bound to the Ni-NTA agarose was eluted with ≈ 10 ml elution buffer. Immediately after elution, the purified protein was desalted on a previously equilibrated PD-10 column, frozen in liquid nitrogen and stored at -80°C.
Aliquots from each purification step were taken, mixed with 1x SDS loading buffer, boiled for 5 min at 95°C and used for analysis by SDS-gel electrophoresis.

Lysis buffer	*Wash buffer*	*Elution buffer*
50 mM NaH_2PO_4	50 mM NaH_2PO_4	50 mM NaH_2PO_4
300 mM NaCl	300 mM NaCl	300 mM NaCl
10 mM imidazole	25 mM imidazole	150 mM imidazole

PD-10 buffer
25 mM Tris-HCl, pH 8,0
150 mM NaCl

2.9.12. Electron Paramagnetic Resonance (EPR) Spectroscopy

Electron Paramagnetic Resonance (EPR) is a powerful technique for the structural investigation of Fe/S proteins. The iron centers in Fe/S proteins can be prepared in a redox state in which their ground state is paramagnetic. This state is therefore characterized by its spin *S* and its magnetic moment $\vec{\mu} = -ß\vec{g}\,\vec{S}$. The *g* is the so-called *g* tensor that is defined by its principal values (g_x, g_y, g_z). Wild-type Nar1 protein is EPR inactive in oxidized form and in order to see an EPR signal the protein must be in the reduced form. Reduction of wild-type Nar1 protein and its cysteine mutants was performed under anaerobic conditions using two methods.

a) Chemical reduction by sodium-dithionite: to 180 µl protein 20 µl of 20 mM sodium-dithionite were added, samples incubated for 4 min and then frozen in liquid nitrogen.

b) Photoreduction: 160 µl protein were reduced by adding 40 µl *5´- deazaflavin* (50 mM final concentration) and light (5 min slide projector). Samples were then frozen in liquid nitrogen. Low temperature X-band EPR spectra were recorded with a Bruker ESP 300E cw spectrometer, equipped with a helium flow cryostat ESR910 (Oxford Instruments); (Pierik *et al.*, 1992).

2.9.13. Determination of Iron Content of Purified Fe/S Proteins (Hennessy et al., 1984)

The iron complexed by the protein is liberated by treatment with hydrochloric acid. Excess acid is neutralized with ammonium acetate, Fe^{3+} is converted to Fe^{2+} by reduction with ascorbic acid. Precipitated protein is complexed with SDS and finally iron chelator (ferene) is added to form a blue complex.

Reagents:
1% (w/v) HCl
7.5% (w/v) Ammonium acetate
2.5 %(w/v) SDS
4% (w/v) Ascorbic acid (fresh)
1.5% (w/v) 3-(2-pyridyl)-5,6-bis(5-sulfo-2-furyl)-1,2,4-triazine, disodium salt (ferene)
0.2 mM $(NH_4)_2Fe(SO_4)_2.6H_2O$

Three samples of the unknown (p, q and r µl), two blanks and four samples of iron standard (10, 25, 50 and 100 µl, i.e. 2-20 nmol Fe) were diluted to 100 µl with H_2O in Eppendorf reaction vessels. Subsequently 100 µl 1 % HCl were added, the samples were mixed by gentle shaking and incubated for 10 min at 80°C. Then, the samples were treated with 500 µl 7.5 % ammonium acetate, 100 µl 4 % ascorbic acid, 100 µl 2.5 % SDS and 100 µl ferene with vortexing after each addition. The samples were centrifuged for 5 min, 9000 x g and the absorbance of the solutions at 593 nm measure against water.

2.9.14. Determination of Sulfide Content of Purified Fe/S Proteins (Pierik et al., 1992)

The iron-sulfur protein is denatured in an alkaline medium containing zinc hydroxide. Released sulfide is coprecipitated with $Zn(OH)_2$ as ZnS. After acidification, H_2S condenses with two molecules of *N,N'*-dimethyl-p-phenylenediamine to form methylene blue.

Reagents:
1% (w/v) Zn acetate
7% (w/v) NaOH
0.1% (w/v) N,N'-dimethyl-p-phenylene-diamine (DMPD), in 5 M HCl
10 mM $FeCl_3$ in 1 M HCl
2 mM $Na_2S.9H_2O$ (sulfide standard)

Sulfide standard (≈ 2mM) solution.

A crystal of appropriate size (≈ 0.5 g) was blotted on filter paper, rapidly weighed and added to a 1 liter volumetric flask containing 10 mM NaOH which has been purged of air with argon. The flask was closed immediately and the solution was stirred magnetically.
Three protein samples (x, y and z µl), two blanks and five sulphide standards (5, 10, 15, 20, and 25 µl i.e. 10-50 nmol S^{2-}) were prepared in Eppendorf reaction tubes and made up to a volume of 0.20 ml with H_2O. After addition of 0.6 ml of 1% Zn-acetate and 0.05 ml of 7% NaOH the samples were mixed and incubated for 15 min at room temperature. Then, the samples were centrifuged for a few seconds at 1,000 rpm. The next step of the procedure was performed on one Eppendorf tube at a time to minimize loss of sulfide. 0.15 ml DMPD solution were carefully added by putting the end of the tip at the bottom of the vessel, the liquid was gently released and mixed by slowly rotating the pipette. Then, 0.15 ml $FeCl_3$ solution were quickly added and the samples were vortexed for 30 sec. After treatement with DMPD/$FeCl_3$, all samples were centrifuged for 5 min, 9000 x g and the absorbance at 670 nm against water after 20 minutes was measured.

2.10. Structural modelling

The sequence alignment of *S. cerevisiae* Nar1 and *C. pasteurianum* Fe-only hydrogenase was performed using the Multialin program (Corpet, 1988). This alignment was used to calculate the structural model of yeast Nar1 using the CPH 2.0 server (Lund *et al.*, 2002). All structure Figures were prepared with PyMOL (DeLano, 2002).

2.11. Equipment

	Model, Manufacturer
Agarose gel chambers	Owl Separation Systems
Anaerobe chamber	COY, Laboratory Products Inc.
Autoclave	Systek V-150
Blot chambers	PeqLab Biotechnologie GmbH
Fluorescence Spectrophotometer	FP-6300, Jasco, Groß-Umstadt
High-Pressure Homogenisator	EmulsiFlex-C3, Avestin
Incubators	Function line, Heraeus instruments Steri Cult, Forma Scientific
Power supply units	EPS600, Pharmacia Biotech
pH-Meter	CG 840, Schott
UV-VIS Spectrophotometer	V-550, Jasco, Groß-Umstadt
Pipetman	Gilson
Shackers	Multitron, HT INFORS
Clean bench	NU-437-600E, Nuaire
Sterilisator	Modell 700, Memmert
Thermocycler	UNO Thermoblock, Biometra
Thermomixer	Thermomixer 5436, Eppendorf
UV-Transilluminator/Kamera	CN6 1.4 Raytest, Isotopenmessgeräte GmbH
Ultracentrifuge	Combi Plus, OPTIMA TL, Sorvall,Beckman
Ultracentrifuge-Rotors	SW-41 Ti, TLA 45, Beckman
Scales	SBC 22, PT 1500, SCALTEC, SARTORIUS
Centrifuges	Biofuge pico, Heraeus instruments 3K30, Sigma Megafuge 1.0R, Heraeus instruments J2-HS, Beckman Avanti^{TM} J-20 XP, Beckman
Centrifuge-Rotors	JA-10, JA-20, JLA 8.1000, JLA 16.250, Beckman

2.12. Chemicals

Chemicals	Manufacturer
1 kb Marker	MBI Fermentas
Acrylamid-Stock solution (Rotiphorese Gel 30)	Roth
Amino-acids	Merck, Sigma
Bacto Peptone, Bacto Tryptone	DIFCO
Blocking Reagent	Roche Diagnostics GmbH
Desoxyribonucleosidtriphosphate (dNTPs)	BD Biosciences Clontech
Dithiothreitol (DTT)	GERBU
$^{55}FeCl_3$, 10 mCi/ml, 30 mCi/mg	NEN
HA-Antibodies, HA-Sepharose	Santa Cruz
Yeast Nitrogen Base	Gibco
Yeast-Extract	ICN
HEPES	GERBU
Salmon sperm-DNA	Gibco
Lambda-DNA	MBI Fermentas
NADH	GERBU
NucleoSpin® Plasmid-Kit	Macherey-Nagel
NucleoSpin® Extract II Kit	Macherey-Nagel
Peroxidase-coupled Antibodies	Bio-Rad Laboratories GmbH
Peroxidase anti-Peroxidase Antibodies	Sigma
Phenol	ICN
Phenylmethylsulfonylfluorid (PMSF)	Roth
Polymerase (Combi-Pol)	Invitek
Protein A-Sepharose™ CL-4B	GE Healthcare
Solution for protein concentration determinations	Bio-Rad Laboratories GmbH
Protein size marker: DALTON MARK VII-L,	Sigma
Restriction Endonucleases	Gibco, Boehringer Mannheim, MBI Fermentas
RNase A	Calbiochem
Ni-NTA Agarose	Qiagen
T4 DNA-Ligase	Promega
IPTG	Roth
Zymolyase 100T	Seikagaku Corporation

Chemicals, not listed above were purchased from Sigma (München), Merck (Darmstadt), Serva (Heidelberg) und Roth (Karlsruhe).

3. Results

3.1. Isd11 plays an essential role in the biogenesis of mitochondrial and cytosolic Fe/S proteins

3.1.1. Isd11 is an essential mitochondrial matrix protein

The open reading frame *YER048w-a*, now termed *ISD11*, was found to be essential for cell viability in *Saccharomyces cerevisiae* and encodes an 11 kDa protein with mitochondrial localization (Huh *et al.*, 2003; Winzeler *et al.*, 1999). BLAST searches identified homologs of Isd11 throughout the eukaryotic kingdom but not in prokaryotes (Figure 3.1).

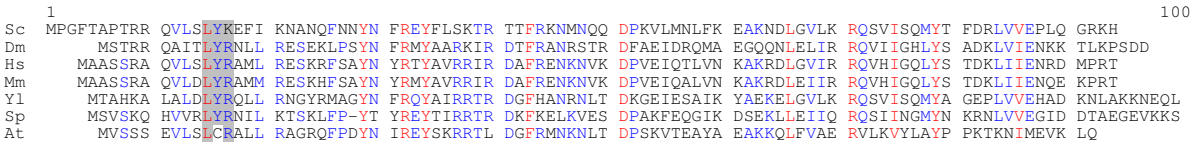


Figure 3.1 Sequence alignment of Isd11 proteins from *Saccharomyces cerevisiae* (Sc), *Drosophila melanogaster* (Dm), *Homo sapiens* (Hs), *Mus musculus* (Mm), *Yarrowia lipolytica* (Yl), *Schizosaccharoyces pombe* (Sp), and *Arabidopsis thaliana* (At). The alignment was performed using the Multalin program (Corpet, 1988). The conserved tripeptide LYR (or LYK) is marked in grey.

A limited similarity of Isd11 is observed with the eukaryotic accessory subunits B22 and B14 of mitochondrial complex I of the respiratory chain. Subunits B22 and B14 have been grouped into the LYR family, named after the conserved tripeptide LYR (LYK) present in the N-terminal part of these proteins. As the molecular functions of B22 and B14 are not known, assignment of Isd11 to the LYR family did not provide any information on its function. In addition, recent bioinformatic analysis indicated that Isd11 evolved during or shortly after endosymbiosis that gave rise to mitochondria, hydrogenosomes and mitosomes (Richards *et al.*, 2006).

To initiate functional studies on Isd11, we constructed a regulatable mutant of *ISD11*. To this end, 290 nucleotides upstream of the *ISD11* start codon were replaced with the *GAL1-10* promoter by homologous recombination to yield the strain Gal-ISD11. When the Gal-ISD11 cells were grown on glucose-containing SD plates, we observed no colonies, showing that *ISD11* is indispensable for viability of yeast cells (Figure 3.2). To show that the observed growth defect of Gal-ISD11 cells is due to depletion of Isd11, the *ISD11* gene was expressed from the plasmids Yep352 under the control of either the *MET25* promoter or the endogenous

ISD11 promoter in Gal-ISD11 cells. Expression of *ISD11* restored the growth defect of Gal-ISD11 cells on glucose-containing media, indicating that these cells specifically lack Isd11.

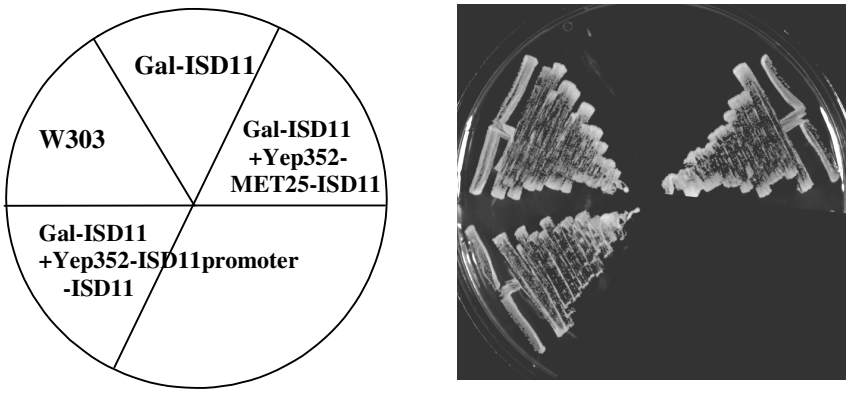


Figure 3.2 Isd11 is essential for yeast cell viability. Wild-type (W303), Gal-ISD11 and Gal-ISD11 cells carrying plasmids coding for the *ISD11* gene (Yep352-MET25-ISD11, Yep352-ISD11promoter-ISD11) were grown on SD plates for 2 days at 30°C.

Next, the subcellular localization of Isd11 was investigated. Upon differential centrifugation of yeast cell extracts, Isd11 cofractionated with the mitochondrial matrix protein Mge1 (Figure 3.3 A), confirming the mitochondrial localization observed with a green fluorescent protein (GFP)-tagged Isd11 (Huh *et al.*, 2003). To determine the submitochondrial localization of Isd11, isolated yeast mitochondria were treated with proteinase K. Isd11 remained protected, whereas the outer membrane receptor Tom70 was degraded (Figure 3.3 B). Upon swelling of mitochondria, Isd11 remained intact like Mge1, whereas the intermembrane space-exposed Tim23 was degraded. When the matrix was opened by sonication, Isd11 was accessible to the protease (Figure 3.3 B), indicating that Isd11 is a mitochondrial matrix protein.

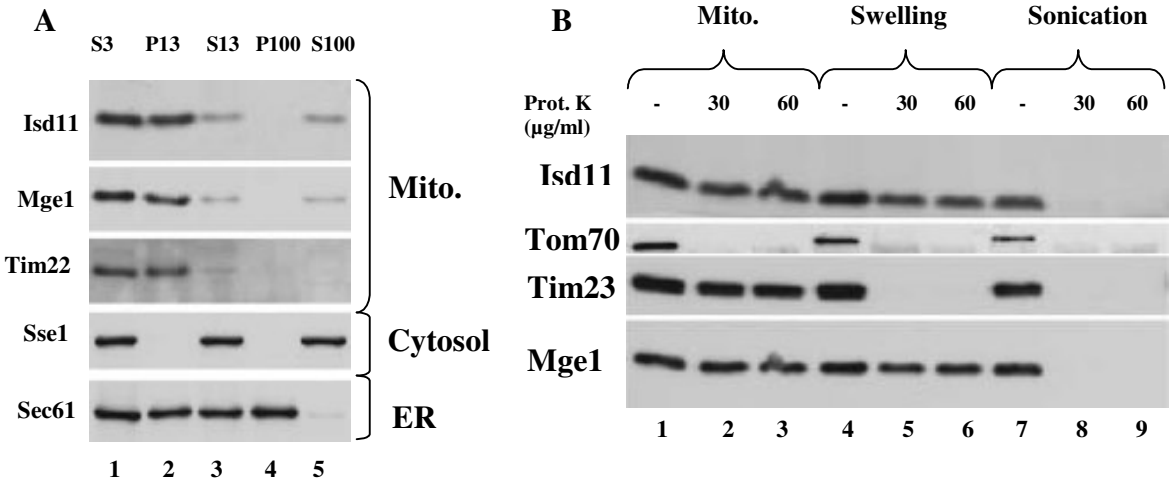


Figure 3.3 Isd11 is located in the mitochondrial matrix. **A)** Yeast cells were disrupted by removal of the cell wall and homogenization in the presence of protease inhibitors followed by differential centrifugation. Pellet (P) and supernatant (S) fractions (multiples of 1000 x g) were analysed by SDS-PAGE, Western blotting and immunostained for Isd11 or marker proteins of known cellular localizations (mitochondrial matrix protein Mge1; mitochondrial inner membrane Tim22; cytosolic Sse1 or endoplasmic reticulum translocon subunit Sec61). **B)** Mitochondria (Mito.) were left untreated or subjected to hypotonic swelling leading to the rupture of outer mitochondrial membrane or sonicated in high-salt buffer (0.5 M NaCl; 10 mM Tris-Cl, pH 7.2) to open the mitochondrial matrix. Treatment with proteinase K was performed as indicated followed by SDS-PAGE and Western blotting. Antibodies against Isd11, outer membrane protein Tom70, inner membrane Tim23 and matrix protein Mge1 were used for immunostaining (Wiedemann *et al.*, 2006).

3.1.2. A conditional yeast mutant of Isd11 reveals a function in Fe/S protein maturation but not in mitochondrial protein import

We generated conditional yeast mutants of *ISD11* by low fidelity PCR and selected the strain *isd11-1* that was strongly impaired in growth on non-fermentable medium at 37°C (Figure 3.4). Previous studies with temperature-sensitive yeast mutants of mitochondrial proteins revealed that mitochondria isolated from such cells often showed kinetic deficiencies in specific processes that pointed to the function of the mutated protein (Gabriel *et al.*, 2003; Truscott *et al.*, 2002). As the majority of essential mitochondrial proteins are required for protein import, first the import of radiolabelled preproteins into mitochondria was analysed. The matrix-targeted precursors of F_1-ATPase subunit β and Su9-DHFR fusion protein were imported and processed to the mature forms with similar efficiency in *isd11-1* and wild-type mitochondria (not shown). Similar rates were observed for the inner membrane protein cytochrome c_1 and the outer membrane protein porin (not shown).

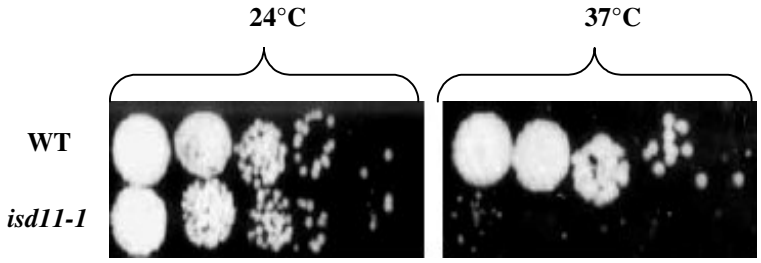


Figure 3.4 A conditional yeast mutant of *ISD11* (*isd11-1*) show impaired growth at 37°C. Wild-type (WT) and *isd11-1* yeast cells were spotted on YPGlycerol (YPG) agar plates and grown for 3 days at 24°C and 37°C (Wiedemann *et al.*, 2006).

As no major effects on protein import were observed, we analysed the maturation of Yah1 (Yeast adrenodoxin homolog) that contains an Fe/S cluster. The radiolabelled precursor of

Yah1 was first imported into isolated mitochondria and then the membrane potential Δψ across the inner membrane was dissipated to prevent further import of new precursors. The formation of processed, mature-sized Yah1, as analysed by SDS–PAGE, occurred with similar efficiency in wild-type and *isd11-1* mitochondria (Figure 3.5 A, lanes 1 and 5). To analyse the maturation of Yah1 to the holo-form containing the Fe/S cluster, we used the fact that holo-Yah1 migrates as a defined species on a native gel (Lutz *et al.*, 2001). The formation of the holo-form was significantly diminished in *isd11-1* mitochondria compared to wild-type mitochondria (Figure 3.5 B, lanes 1 and 5). In a second incubation (chase), further maturation of Yah1 was allowed for up to 30 min; however, the holo-Yah1 levels in *isd11-1* mitochondria remained far below the wild-type levels (Figure 3.5 B). A reduction of the mature-sized Yah1 was observed in *isd11-1* mitochondria (Figure 3.5 A, lanes 6 and 7), suggesting that the apo-form of imported mature-sized Yah1 is not stable without the Fe/S cluster. These results indicate a role of Isd11 in the formation of the holo-form of Yah1 but not in preprotein import.

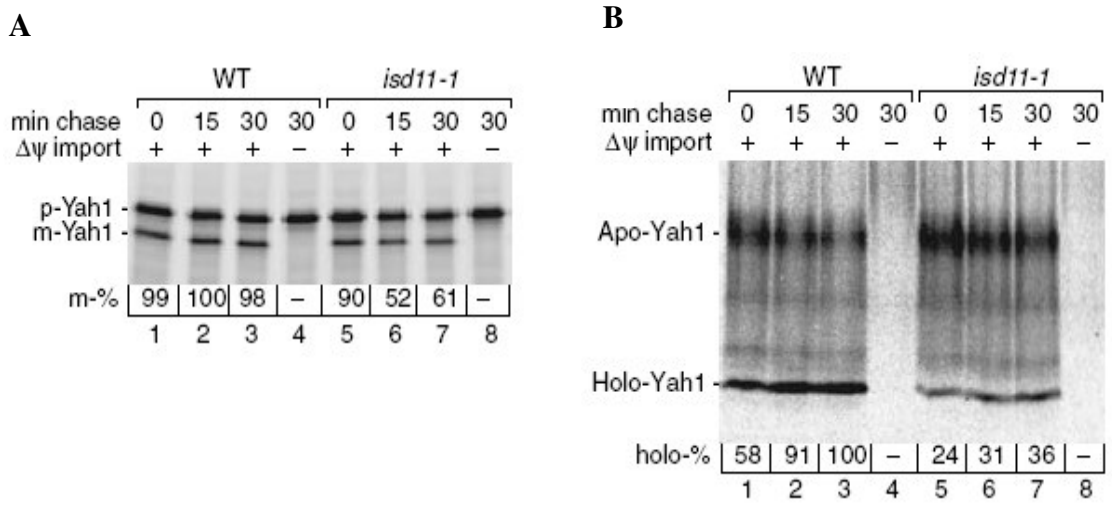


Figure 3.5 *isd11-1* mutant mitochondria are impaired in maturation of Yah1. A) ^{35}S-labelled precursor of Yah1 was imported into isolated mitochondria for 10 min, followed by dissipation of Δψ and a further incubation (chase). Imported mature-sized (m) Yah1 was analysed by SDS–PAGE and digital autoradiography. The maximal amount formed was set to 100 %. **B)** The experiment was performed as described for panel A except that apo-Yah1 and the Fe/S-containing holo-Yah1 were analysed by native PAGE (Lutz *et al.*, 2001; Wiedemann *et al.*, 2006).

3.1.3. Isd11 is required for the stability of the cysteine desulfurase Nfs1

To obtain evidence for the role of Isd11 *in vivo*, cells were grown under two conditions and the steady-state protein levels of isolated mitochondria were compared. One

set of cells was grown at permissive temperature, whereas the other set was first grown at low temperature (19°C) and then shifted to 37°C on non-fermentable medium. Upon growth of *isd11-1* cells at low temperature, the mitochondrial levels of all marker proteins tested were similar as those of wild-type mitochondria (Figure 3.6 A and data not shown). The mutant protein *isd11-1* migrated faster on SDS–PAGE than the wild-type protein (the lower signal intensity of *isd11-1* can be due to two reasons, a reduction of the amount of Isd11 in the mutant or a lower reactivity of the polyclonal antiserum against the mutant protein) (Figure 3.6 A, lower panel). After shift of *isd11-1* cells to 37°C, the level of Jac1 of the ISC assembly machinery was moderately affected (Figure 3.6 B).

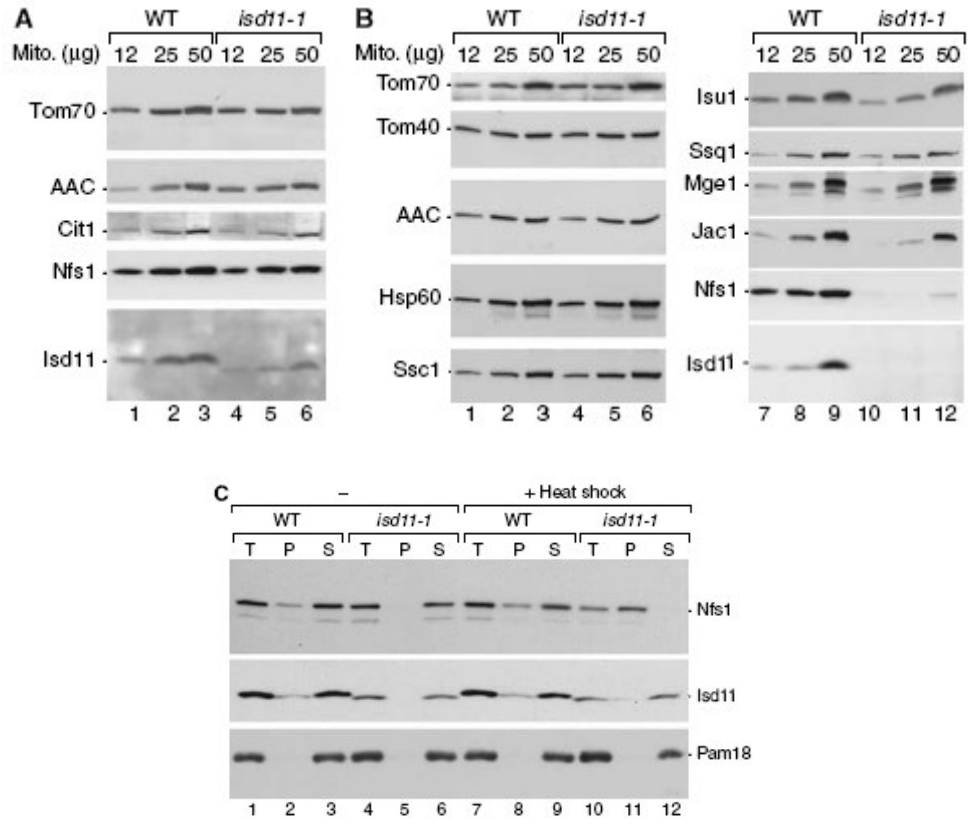


Figure 3.6 Reduced mitochondrial levels of Nfs1 in *isd11-1* cells grown under non-permissive conditions. A) Wild-type (WT) and *isd11-1* cells were grown at 19°C. Isolated mitochondria (mg of protein) were analysed by SDS–PAGE and Western blotting. Antibodies against mitochondrial proteins Tom70; ADP/ATP carrier, AAC; citrate synthase, Cit1, Nfs1 and Isd11 were used for immunostaining. **B)** Cells were grown at 19°C and shifted to 37°C for 8 h on non-fermentable medium followed by SDS-PAGE Western blotting and immunostaining against various mitochondrial proteins. **C)** Mitochondria isolated from cells grown at 19°C were either kept on ice or heat shocked for 15 min at 37°C. After lyses with digitonin, mitochondria were separated into pellet (P) and supernatant (S) by centrifugation (T, total mitochondrial fraction) (Wiedemann *et al.*, 2006).

A striking defect was observed for Nfs1 that was strongly diminished in *isd11-1* mitochondria, similar to the strong reduction of the mutant protein *isd11-1* itself (Figure 3.6 B). To obtain further insight into the role of Isd11, mitochondria isolated from cells grown at low temperature were shifted to non-permissive conditions *in vitro*. Nfs1 was still detectable (Figure 3.6 C, lane 10). Mitochondria were solubilized by digitonin and subjected to centrifugation. Isd11 from both wild-type and *isd11-1* mitochondria was mainly found in the solubilized fraction like a control protein, the inner membrane protein Pam18 (Figure 3.6 C). In wild-type mitochondria, the majority of Nfs1 was released to the supernatant, whereas a small fraction remained in the non-solubilized pellet (Figure 3.6.C, lanes 2, 3, 8 and 9). In *isd11-1* mitochondria, Nfs1 was released to the supernatant at low temperature but was fully found in the pellet after the heat shock (Figure 3.6.C, lanes 6 and 11). We conclude that after a heat shock of isolated mitochondria, Nfs1 aggregates in *isd11-1* mitochondria. After a prolonged shift of cells to non-permissive conditions, the aggregated Nfs1 is apparently degraded. Thus, Isd11 is involved in maintaining the stability of Nfs1.

3.1.4. Isd11 is required for the biogenesis of mitochondrial, cytosolic and nuclear Fe/S proteins

As shown above (Figure 3.5), Isd11 is involved in the formation of the holo-form of Yah1, an Fe/S protein. To analyse a possible function of Isd11 in Fe/S protein biogenesis, we first measured the enzyme activities of mitochondrial and cytosolic Fe/S proteins. Mitochondria were isolated from wild-type and *isd11-1* cells grown at 24–30°C. The activities of aconitase, succinate dehydrogenase (complex II) and cytochrome c reductase (complex III) were diminished by 75–95% in *isd11-1* mitochondria (Figure 3.7 A). Defects in the activities of mitochondrial Fe/S enzymes were observed even at the permissive-temperature of 24°C. In contrast, the activity of malate dehydrogenase as a non-Fe/S control enzyme was not impaired. The steady-state levels of the Fe/S proteins aconitase, subunit 2 of succinate dehydrogenase and Rieske Fe/S protein of complex III were moderately diminished in the *isd11-1* mitochondria, whereas those of Nfs1, Isu1 and control proteins like porin were not reduced at these growth conditions (Figure 3.7 B). The strong reduction of the enzyme activities of Fe/S proteins supports the view that Isd11 plays an important role in mitochondrial Fe/S protein assembly. In agreement with previous observations in various ISC-assembly mutants, Fe/S apoproteins are often sensitive to degradation when Fe/S protein maturation is impaired (see, e.g., (Balk *et al.*, 2004)).

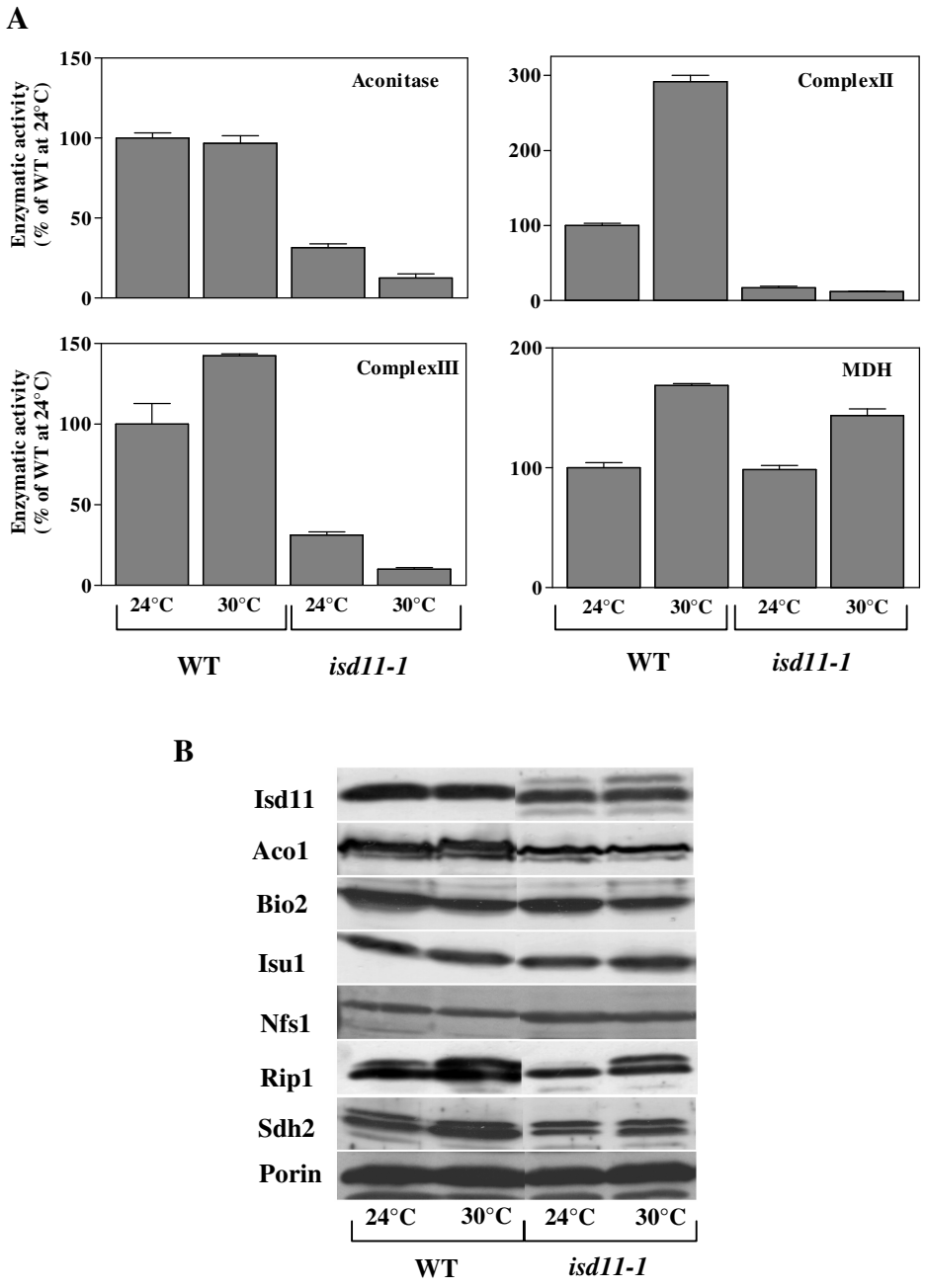


Figure 3.7 *isd11-1* cells show decreased activities of mitochondrial Fe/S proteins. A) Mitochondria were isolated from wild-type (WT) and *isd11-1* cells grown for 24 h in rich medium supplemented with glucose at 24°C and 30°C. The enzymatic activities of aconitase, respiratory complexes II and III and malate dehydrogenase (MDH) were measured. **B)** Levels of mitochondrial proteins detected by Western blotting. Aco1, aconitase; Rip1, Rieske Fe/S protein; Sdh2, subunit 2 of succinate dehydrogenase.

Next, a possible involvement of Isd11 in the biogenesis of cytosolic Fe/S proteins was investigated. To address this possibility the enzyme activity of isopropylmalate isomerase (Leu1p), a cytosolic Fe/S protein was analysed (Kispal *et al.*, 1999). The activity of cytosolic

Leu1p was two-to three-fold decreased in extracts of *isd11-1* cells, whereas the activity of the control enzyme alcohol dehydrogenase did not change significantly (Figure 3.8 A). The protein levels of Leu1 in *isd11-1* cells were comparable to those in wild-type cells (Figure 3.8 B), indicating a requirement of Isd11 for Leu1 function.

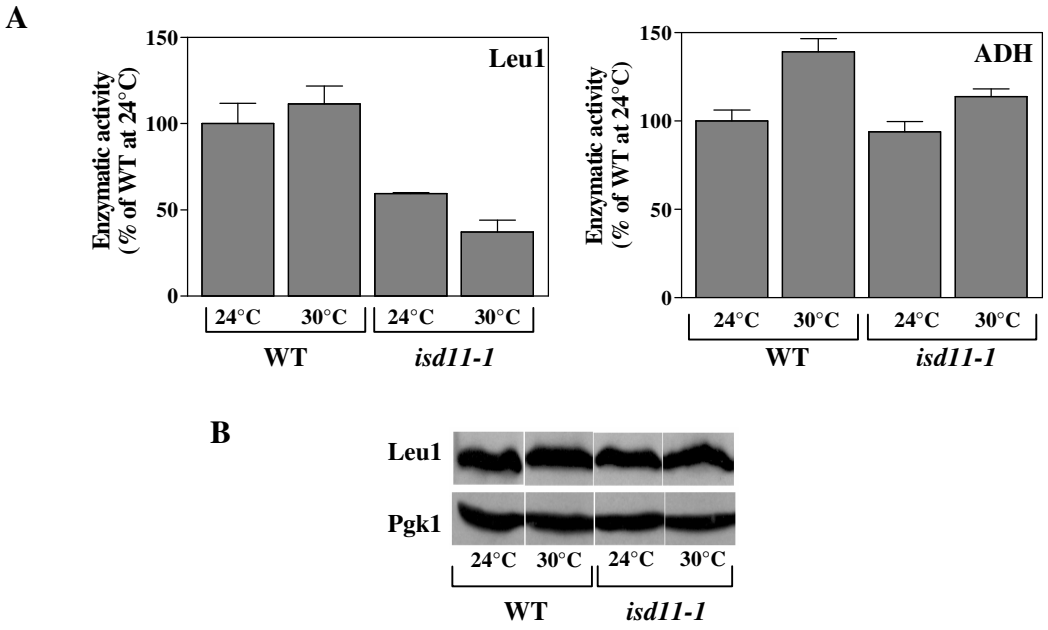


Figure 3.8 *isd11-1* cells show decreased activities of cytosolic Fe/S proteins. Wild-type (WT) and *isd11-1* cells were grown in minimal medium supplemented with glucose at 24°C or 30°C for 24 h. Extracts were prepared by disruption of cells with glass beads, and aliquots were analysed **(A)** for the enzymatic activities of Leu1 and alcohol dehydrogenase (ADH), or **(B)** for the protein levels of Leu1 and the cytosolic marker protein Pgk1 by Western blotting.

Similar results were observed when the regulatable Gal-ISD11 mutant strain was analysed. After depletion of Isd11 upon growth of the Gal-ISD11 cells in glucose-containing medium, Leu1 activity was diminished four-fold compared to Isd11-containing cells, whereas the alcohol dehydrogenase activity was unchanged (Figure 3.9 A). The protein levels of Leu1 were not significantly altered upon depletion of Isd11 (Figure 3.9 B).

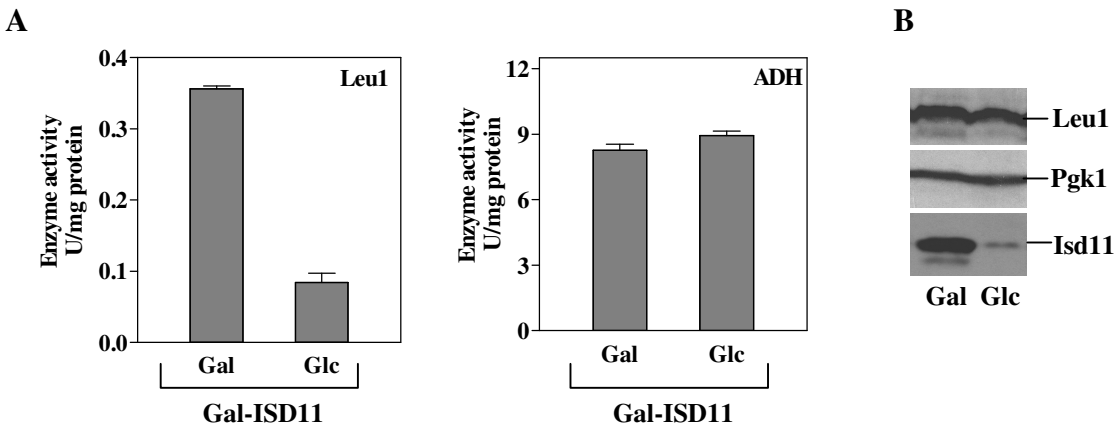


Figure 3.9 Gal-ISD11 cells show decreased activities of cytosolic Leu1. Gal-ISD11 cells were cultured for 18h in minimal medium containing galactose (Gal) or glucose (Glc) to induce or repress, respectively, synthesis of Isd11. Extracts were prepared by disruption of cells with glass beads, and aliquots were analysed **(A)** for the enzymatic activities of Leu1 and alcohol dehydrogenase (ADH) or **(B)** for the protein levels of Isd11, Leu1 and the cytosolic marker protein Pgk1 by Western blotting.

To analyse whether Isd11 was involved in the *de novo* maturation of cellular Fe/S proteins, the incorporation of ^{55}Fe into various Fe/S proteins was determined (Kispal *et al.*, 1999; Muhlenhoff *et al.*, 2002). Wild-type and *isd11-1* cells were grown in iron-poor minimal medium and incubated with ^{55}Fe in the presence of ascorbate for 2 h. A cell extract was prepared, Fe/S proteins were immunoprecipitated using specific antibodies. Finally, the amounts of incorporated Fe/S clusters were estimated by scintillation counting. As the first Fe/S proteins, the mitochondrial proteins aconitase (Aco1) and biotin synthase (Bio2) were analysed. The amounts of ^{55}Fe associated with Aco1 and Bio2 were dramatically decreased, when the *isd11-1* cells were cultivated at the non-permissive temperature of 37°C (Figure 3.10 A, C). Likewise decreased levels of ^{55}Fe incorporated into aconitase were observed, when the Isd11 was depleted by growing the Gal-ISD11 cells in the presence of glucose. The protein levels of Aco1 or Bio2 (Fig.3.10 B; C inset) were also diminished in *isd11-1* cells, a characteristics that has been previously observed for Fe/S proteins in their apo-form (Balk *et al.*, 2004). Together with the data from mitochondrial enzyme activities, these results indicate that Isd11 is required for the biogenesis of mitochondrial Fe/S proteins.

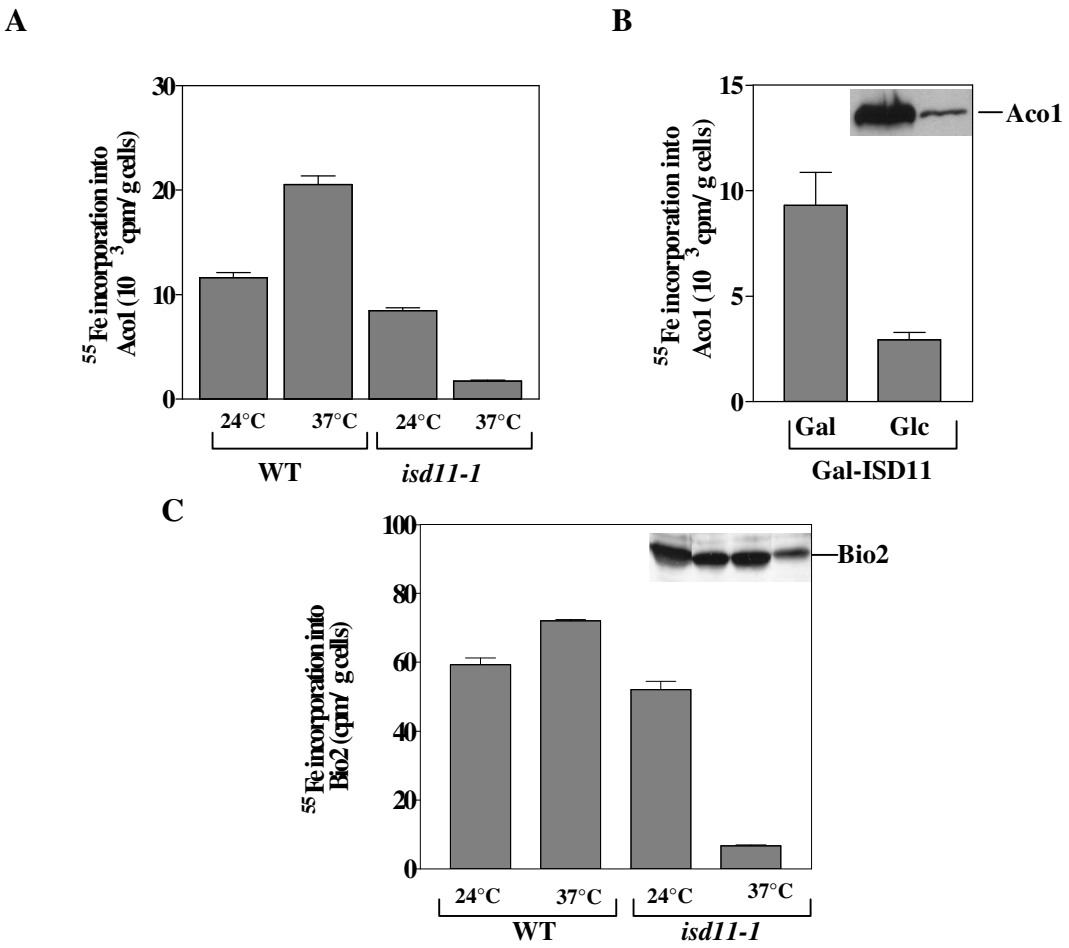


Figure 3.10 *isd11-1* cells are defective in the *de novo* formation of mitochondrial Fe/S proteins. Wild-type (WT) and *isd11-1* (A and C) and Gal-ISD11 (B) cells were grown in iron-free minimal medium supplemented with glucose at 24°C or 37°C for 24 h (A and C) and at 30°C (B). Cells were labelled with ^{55}Fe and a cell extract was prepared using glass beads. Endogenous Aco1 and Bio2 overexpressed from a plasmid (p426-MET25-BIO2) were immunoprecipitated. The radioactivity associated with the immuno-beads was quantified by liquid scintillation counting. Protein levels (insets) of aconitase (Aco1) and biotin synthase (Bio2) were visualized by immunoblot analysis using specific antibodies.

Fe/S protein assembly was significantly impaired in cytosolic Leu1 and in the nuclear DNA repair enzyme Ntg2 in *isd11-1* cells (Figure 3.11 A). An almost quantitative loss in Fe/S cluster incorporation into the cytosolic Rli1 (Kispal *et al.*, 2005) and the nuclear Ntg2 was found using Isd11-depleted Gal-ISD11 cells (Figure 3.11 B). The levels of the Fe/S apoproteins were decreased, but not as strongly as the assembly of the Fe/S clusters (Figure 3.11 A and B, insets). These results indicate that Isd11 is required for the formation of Fe/S proteins in mitochondria, cytosol and nucleus, identifying Isd11 as a novel component of the mitochondrial ISC-assembly machinery.

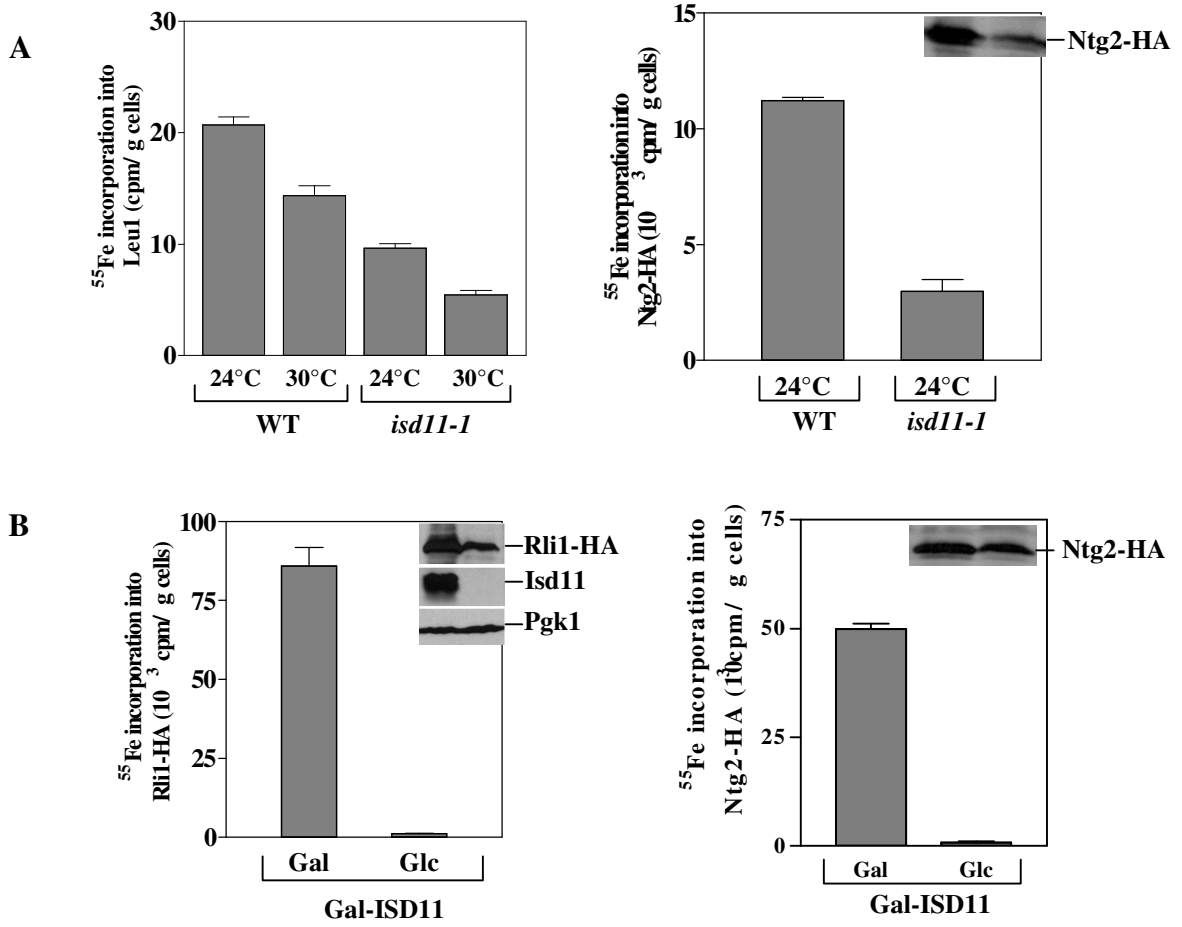


Figure 3.11 *isd11-1* cells are impaired in the *de novo* formation of cytosolic and nuclear Fe/S proteins. Wild-type (WT) and *isd11-1* **(A)** or Gal-ISD11 **(B)** cells were grown in iron-free minimal medium supplemented with glucose at 24°C or 30°C for 24 h. Cells were labelled with ^{55}Fe and a cell extract was prepared using glass beads. Endogenous Leu1, Ntg2-HA and Rli1-HA overexpressed from the plasmid (p426-MET25) were immunoprecipitated using specific anti-Leu1 or anti-HA immunobeads. The radioactivity associated with the immunobeads was quantified by liquid scintillation counting. The insets show the immunostaining of Ntg2-HA, Rli1-HA, Isd11 and cytosolic 3-phosphoglycerate kinase (Pgk1).

An impairment of the mitochondrial ISC-assembly or ISC-export systems is generally accompanied by an increased uptake of iron into the yeast cells owing to the activation of the iron-dependent transcription factors Aft1/Aft2 (Chen *et al.*, 2004; Rutherford *et al.*, 2001; Rutherford *et al.*, 2005)These proteins sense the cellular iron concentration, and, under iron-limiting conditions or upon Fe/S protein maturation defects, they induce the expression of genes of the 'iron regulon' resulting in increased iron uptake. The induction of the Aft1-dependent *FET3* gene, encoding a copper-dependent ferroxidase, was analysed in *isd11-1*

cells, using a fusion construct of the *FET3* promoter and the GFP gene. A plasmid encoding the *FET3-GFP* fusion gene was transformed into wild-type and *isd11-1* cells, and the GFP fluorescence was determined. A strongly increased fluorescence intensity under iron-replete conditions was observed in *isd11-1* cells as compared to wild-type cells (Figure 3.12 A). These data clearly show that *isd11-1* cells display a defect in cellular iron homeostasis. The next question was whether cells defective in Isd11 accumulate iron within mitochondria, as this is seen for mutants in the ISC-assembly and ISC-export systems (Kispal *et al.*, 1999; Lange *et al.*, 2000; Li *et al.*, 2001; Schilke *et al.*, 1999). The content of non-heme and non-Fe/S iron was quantified in isolated mitochondria. The *isd11-1* mitochondria contained a three-fold higher amount of iron (Figure 3.12 B). The dramatic effect of the *isd11* mutation on cellular iron uptake and mitochondrial iron accumulation supports the notion that Isd11 is a novel component of the ISC-assembly machinery.

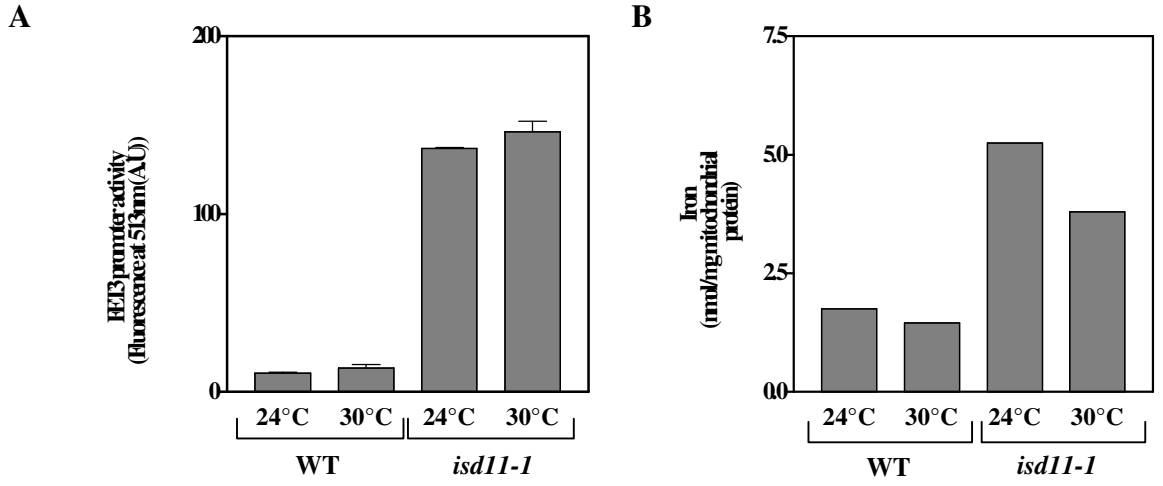


Figure 3.12 Cellular iron homeostasis is impaired in *isd11-1* cells. A) Wild-type (WT) and *isd11-1* cells carrying plasmid p415-FET3-GFP were grown in minimal medium supplemented with 200 mM ferric ammonium citrate at 24 or 30°C. Cells were harvested at an optical density of 0.5 and the transcriptional activity of the FET3 promoter was determined by recording the fluorescence emission of the cell suspension at 513 nm (excitation at 480 nm). The signal of cells lacking plasmid p415-FET3-GFP was subtracted. **B)** Mitochondrial iron accumulation in *isd11-1* cells. Mitochondria were isolated from the indicated cells grown in rich medium containing glucose, and the iron content was determined by the bathophenanthroline method (Li *et al.*, 1999).

3.1.5. An active cysteine desulfurase complex containing Nfs1 and Isd11

The stabilization of Nfs1 by Isd11 (see Fig. 3.6 C) suggested an interaction between these two proteins. To test the physical of Isd11 to Nfs1, a yeast strain expressing Isd11 with a tandem-affinity purification (TAP) tag was used and an IgG affinity purification after lysis

of mitochondria was performed under native conditions. A prominent protein band of about 50 kDa and several smaller bands were detected in the eluate of the purification procedure. These bands were not found with control mitochondria lacking a TAP tag. A mass spectrometric analysis of these bands led to the identification of numerous peptides derived from Nfs1 and Isd11 (Figure 3.13 A). The predicted size of 51 kDa of mature-sized Nfs1 (Kispal *et al.*, 1999; Li *et al.*, 1999) agrees with the size of the most prominent band.

To analyse whether Nfs1 and Isd11 can form a stable complex, BN-PAGE was used. Radiolabelled Isd11 was imported into wild-type mitochondria that were lysed with non-ionic detergent. In addition to Isd11 running in the front of the gel, a time-dependent formation of a complex migrating at about 230 kDa was observed. This species was termed the ISD complex (Figure 3.13 B, lanes 1–4). When mitochondria were lysed by SDS, the ISD complex was dissociated (Figure 3.13 B, lane 7). The 230 kDa ISD complex contains both, Isd11 and Nfs1 (not shown). In conclusion, these data show that Nfs1 and Isd11 interact and form a stable complex.

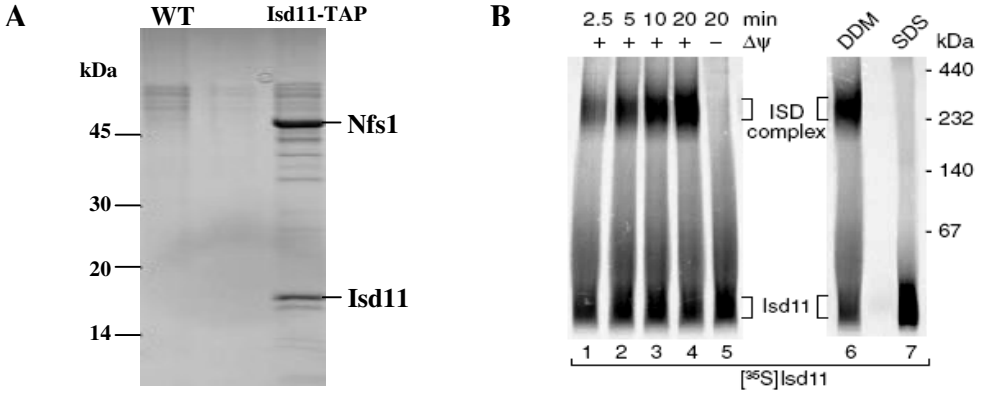


Figure 3.13 Nfs1 and Isd11 form a stable complex. A) Mitochondria were lysed in 0.1 % *n*-dodecyl-β-D-maltoside (DDM) and incubated with IgG-Sepharose. After washing of the beads and incubation with TEV protease, eluates were separated by SDS–PAGE, stained with colloidal Coomassie and analysed by tandem mass spectrometry. **B)** The [^{35}S]-labelled Isd11 was imported into wild-type (WT) mitochondria, followed by lysis with 0.1% DDM (samples 1–6) or 0.1% SDS (sample 7) and BN-PAGE analysis (Schagger *et al.*, 1991; Wiedemann *et al.*, 2006).

Nfs1 was previously shown to interact with Isu1/Isu2 (Gerber *et al.*, 2003). It was therefore important to analyse whether the 230 kDa ISD complex contained the Isu proteins. To test this $Nfs1_{ProtA}$ was purified from mitochondria that were lysed under non-denaturing conditions by IgG affinity purification and, a Western blot with antibodies against Isd11 and

Isu1 was performed. As expected from the stable association in BN-PAGE, Isd11 was efficiently co-purified with Nfs1 (Figure 3.14). Isu1 was also specifically found in the eluate, but the yield was about 10-fold lower than that of Isd11. Apparently Nfs1 and Isd11 represent the major components of the ISD complex. The Isu scaffold proteins are not stoichiometric subunits of the ISD complex, but can interact with the complex.

Does the ISD complex observed on BN-PAGE represent a functional desulfurase complex? To address this question, it was investigated whether Nfs1 in the ISD11 complex can form a protein-bound persulfide from added cysteine. During the desulfurase action of Nfs1, the sulfur derived from cysteine is bound to Nfs1 in the form of a persulfide intermediate (Zheng *et al.*, 1994b). Isolated mitochondria were treated with chloramphenicol to inhibit protein synthesis in the matrix. Mitochondrial lysates were generated with non-ionic detergent and incubated with [^{35}S]cysteine or [^{35}S]methionine as a control. Only in the presence of [^{35}S]cysteine, a radiolabelled complex of the size of the ISD complex was observed on BN-PAGE (Figure 3.15 A, lanes 1 and 3). In mitochondria containing TAP-tagged Isd11, this complex was shifted, confirming that it represented the ISD complex (Figure 3.15 A, lane 2). These data indicate that the ISD complex is formed only in the presence of the Nfs1 substrate, cysteine.

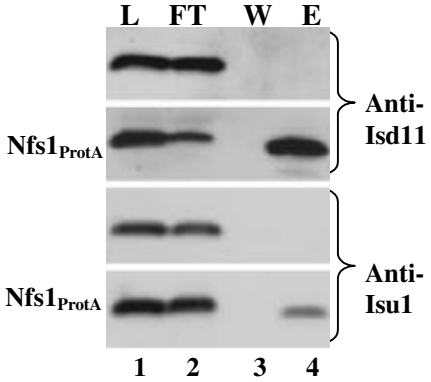


Figure 3.14 Nfs1 interacts with both Isd11 and Isu1. Wild-type (WT) and Nfs1$_{ProtA}$ mitochondria were lysed under non-denaturing conditions (1 % digitonin in 20 mM Tris, pH 7.4, 0.1 mM EDTA, 10 % glycerol, 0.25 M NaCl, 1 mM PMSF) and Nfs1$_{ProtA}$ was purified by IgG affinity chromatography. The load (L), flow-through (FT), wash (W) and eluate (E) fractions were subjected to SDS-PAGE and Western blotting. Immunostaining was performed with antibodies against Isd11 and Isu1 (Wiedemann *et al.*, 2006).

To obtain evidence that the [^{35}S] label observed in the ISD complex was present in a persulfide, dithiothreitol (DTT) was added before analysing the samples by BN-PAGE. After this treatment, the [^{35}S]cysteine-labelled ISD complex was no longer observed by digital autoradiography (Figure 3.15 B, lane 2). To exclude that the ISD protein complex itself was

dissociated by DTT, we used mitochondria with imported [^{35}S]methionine-labelled Isd11. In this case, DTT did not dissociate the ISD complex (Figure 3.15 B, lane 4). In conclusion ISD complex observed on BN-PAGE is functional in forming a persulfide intermediate. As total mitochondrial lysates were used in the labelling reaction with [^{35}S]cysteine, the preferential labelling of the ISD complex points to its major role as a mitochondrial desulfurase.

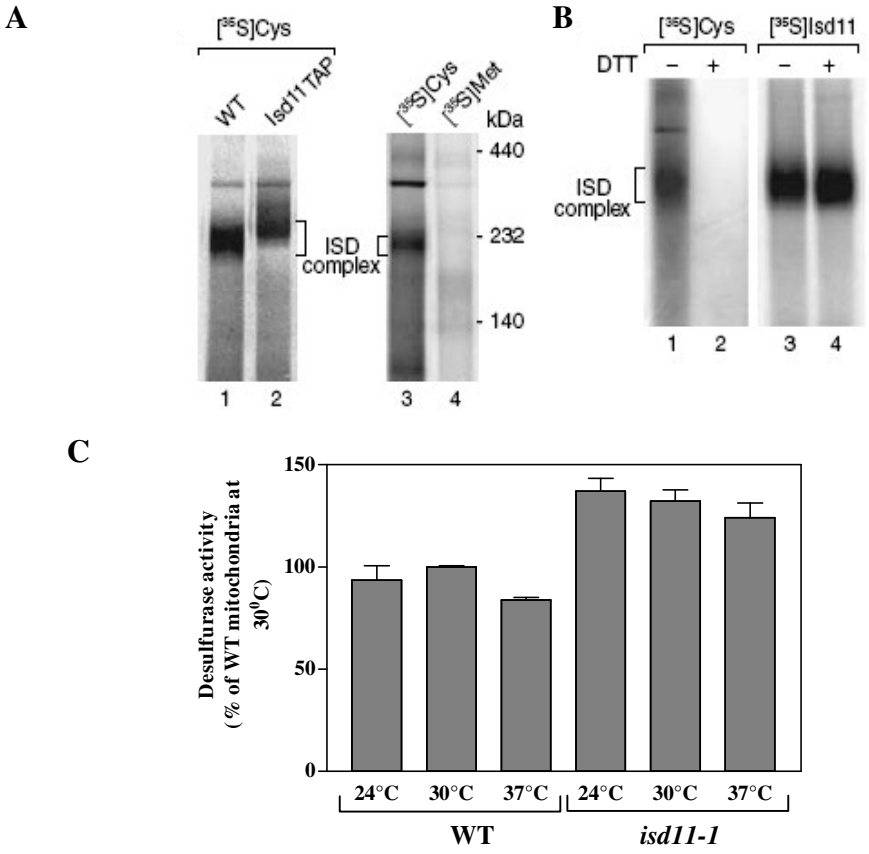


Figure 3.15 Isd11 is part of the active desulfurase complex. A) Wild-type (WT) and Isd11$_{TAP}$ mitochondria were treated with chloramphenicol. Mitochondria were lysed, incubated with [^{35}S]cysteine or [^{35}S]methionine and subjected to BN-PAGE (see Material and Methods sections 2.9.10 and 2.9.11) and digital autoradiography. **B)** Samples 1 and 2, wild-type mitochondrial lysate was incubated with [^{35}S]cysteine as described for panel A. Samples 3 and 4, [^{35}S]Isd11 was imported into wild-type mitochondria. Before BN-PAGE analysis, half of the samples were treated with 10 mM DTT (Wiedemann *et al.*, 2006). **C)** *isd11-1* cells are not impaired in cysteine desulfurase activities. Mitochondria isolated from cells grown at 24°C, 30°C and 37°C were lysed and incubated in the presence of 4 mM cysteine and 1 mM DTT for 20 min. The amount of sulfide formed from cysteine by Nfs1 was determined (Mühlenhoff *et al.*, 2004). The signal recorded in samples lacking cysteine was subtracted.

In *isd11-1* mutant mitochondria, the [^{35}S]cysteine-derived labelling of the ISD complex was blocked (not shown). However, the ISD protein complex itself was dissociated in *isd11-1* mitochondria (not shown) and thus it could not be decided whether functional Isd11 was required for formation of the persulfide intermediate at Nfs1 (the released Nfs1 did

not migrate as a defined band on BN-PAGE, a behavior previously observed with several Tom or Tim proteins upon release from translocase complexes; (Chacinska *et al.*, 2005; Chacinska *et al.*, 2003). Therefore the enzyme activity of the cysteine desulfurase Nfs1 was directly determined by following the sulfide production in extracts of isolated mitochondria (Mühlenhoff *et al.*, 2004). Surprisingly, the Nfs1 enzyme activity was even moderately increased in *isd11-1* cells (Figure 3.15 C). Thus, functional Isd11 and the formation of the ISD complex are not required for the activity of Nfs1 in sulfide production *in vitro*, yet both Isd11 and the ISD complex are needed for the formation of the sulfur present in Fe/S cluster-containing proteins.

3.1.6. The ISD complex is the functional desulfurase to generate Fe/S clusters on the Isu scaffold

All biochemical data indicated a role of Isd11 in the maturation of mitochondrial, cytosolic and nuclear Fe/S proteins. Isd11 forms a stable complex with the cysteine desulfurase Nfs1 but, that Isd11 is not required for the activity of Nfs1 in sulfur production.
A previously developed *in vivo* assay can discriminate whether ISC components are required early in the synthesis of the transient Fe/S cluster on Isu1 or whether they perform their function after cluster assembly on Isu1 (Mühlenhoff *et al.*, 2003). Using this assay it has been shown that depletion of the cysteine desulfurase Nfs1 by regulated gene expression caused a strong decrease in the *de novo* synthesis of Fe/S clusters on scaffold protein Isu1. Therefore it was interesting to know at which stage of mitochondrial Fe/S protein biogenesis Isd11 might be necessary.

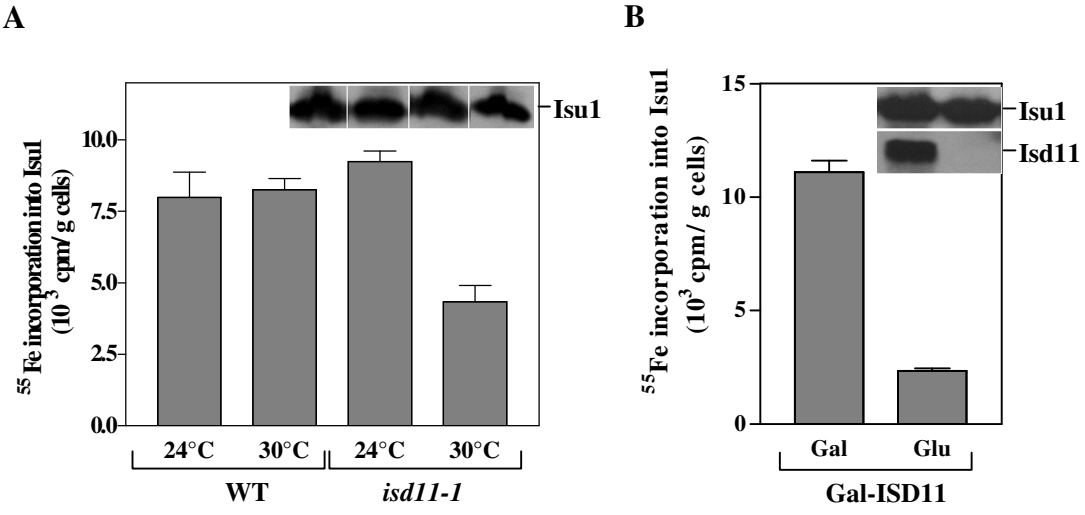


Figure 3.16 Isd11 is required for Fe/S cluster formation on the scaffold protein Isu1. A) Wild-type (WT) and *isd11-1* cells overexpressing *ISU1* from vector p426-GPD were grown for 24 h at 24°C and 30°C. **B)** Gal-ISD11 cells overexpressing *ISU1* were grown in the presence of galactose (Gal) or glucose (Glu). Fe/S cluster assembly on Isu1 was determined by ^{55}Fe labelling and the immunoprecipitation assay described in Figure 3.10.

Wild-type (WT) and *isd11-1* cells were transformed with a plasmid overproducing Isu1. After radiolabelling the cells with ^{55}Fe, Isu1 was immunoprecipitated from a cell extract and the associated radioactivity was quantified by scintillation counting. Fe/S cluster formation on Isu1 was significantly diminished in *isd11-1* cells (Figure 3.16 A). A five-fold reduction in Fe/S cluster assembly on Isu1 was seen after depletion of Isd11 in Gal-ISD11 cells (Figure 3.16 B). The protein levels of Isu1 in these experiments were hardly altered (insets). These data suggest that Isd11 is necessary at an early stage of Fe/S protein biogenesis. Like Nfs1, Yah1, Yfh1 (Mühlenhoff *et al.*, 2003) the protein is required for Fe/S cluster formation on Isu1. In summary, in this study it has been shown that the essential mitochondrial matrix protein Isd11 is a novel component of the mitochondrial ISC-assembly machinery for Fe/S protein biogenesis. Isd11 interacts with Nfs1 and Isu1, and is required for the sulfur transfer from Nfs1 to Isu1 scaffold protein to assist the Fe/S cluster formation.

3.2. Identification of residues important for function and Fe/S coordination of Nar1

3.2.1. Nar1 is an essential Fe/S protein with homology to Fe-only-hydrogenases

The *S. cerevisiae* protein Nar1 is highly conserved in all eukaryotes and is phylogenetically related to bacterial and algal iron-only hydrogenases (Horner *et al.*, 2002). The sequence alignment of Nar1-like proteins (Figure 3.17) indicates that the most conserved regions are located in the middle and in the C-terminal parts of the proteins with an overall amino-acid identity of 25-30% and sequence similarity of 40-50% (Balk *et al.*, 2004).

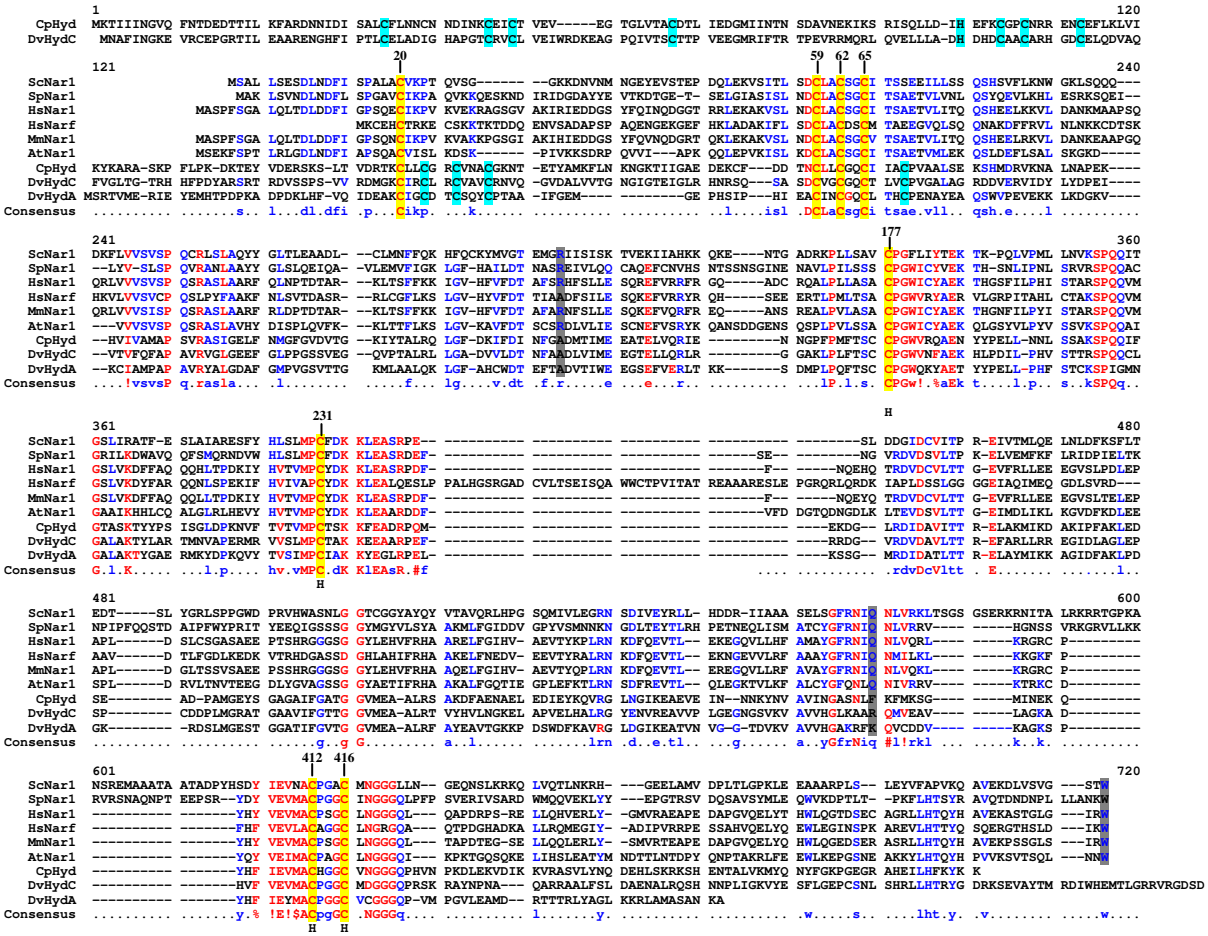


Figure 3.17 Sequence alignment of Nar1-like proteins and iron-only hydrogenases. The multisequence alignment of Nar1-like proteins, *Desulfovibrio* HydA and HydC, and clostridial CpI hydrogenases was performed using the Multalin program (Corpet, 1988). Conserved cysteine residues are highlighted in yellow and cyan (hydrogenase-specific). The cysteine residues coordinating the H-cluster in iron-only hydrogenases are indicated (H). Residues that are unique to the Nar1-family and analysed in this study are highlighted in grey. Abbreviations for organisms: Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Hs, *Homo sapiens*; Mm, *Mus musculus*; At, *Arabidopsis thaliana*; Cp, *Clostridium pasteurianum*; Dv, *Desulfovibrio vulgaris*.

The yeast Nar1 contains eight conserved cysteine residues. Four cysteines are concentrated at the N-terminus and resemble a ferredoxin-like domain ($CX_{38}CX_2CX_2C$) that may bind a [4Fe-4S] cluster. In *Clostridium pasteurianum* and *Desulfovibrio vulgaris* Fe-only hydrogenases these cysteine residues coordinate the medial [4Fe-4S] cluster (FS4A domain). The other four conserved cysteine residues are scattered in the middle and C-terminal parts of the protein and correspond to cysteines coordinating the so called "H-cluster" in iron-only hydrogenases (Nicolet *et al.*, 2002). As the yeast genome does not encode homologous genes to those responsible for H-cluster biogenesis in bacteria (*HydEF and HydG)*, it seems unlikely that Nar1 could also bind an H-cluster at the C-terminus (Posewitz *et al.*, 2004). The Nar1-protein family also shows some other unique features such as the presence of a tryptophan residue at the extreme C-terminus and the proposed proton-donating cysteine (Nicolet *et al.*, 2002) in hydrogen-producing hydrogenases to be not conserved in Nar1 protein.

As the function and nature of the Fe/S clusters in Nar1 are still unknown, in this study the importance of several residues for Nar1 function and Fe/S cluster coordination was analysed. For this purpose, several amino acid residues that are conserved only in the Nar1-protein family (R144, Q356 and W491) and the eight conserved Nar1 cysteine residues were exchanged by site-directed mutagenesis. To gain a rough idea where these residues might be located in the three-dimensional structure, first a theoretical model structure was calculated on the basis of the Fe-only hydrogenase from *Clostridium pasteurianum*.

3.2.2. Structural modelling of Nar1

The yeast Nar1 is phylogenetically related to bacterial Fe-only hydrogenases, whose structures, have been determined (Horner *et al.*, 2002); (Nicolet *et al.*, 1999; Peters *et al.*, 1998). We therefore tried to gain insight into the structure of Nar1 by modeling the *S. cerevisiae* Nar1 sequence into the structure of the hydrogenase from *Clostridium pasteurianum*. The alignment between the yeast Nar1 and the Fe-only hydrogenase from *C. pasteurianum* showed an overall sequence identity of 28%. This alignment was used to calculate the structural model of Nar1 using the CPH 2.0 server (Figure 3.18 A) (Lund *et al.*, 2002).

The alignment of the calculated Nar1 structure with the X-ray structure of Fe-only hydrogenase from *C. pasteurianum* (PDB code 1feh) (Peters *et al.*, 1998) shows that there is a significant level of structural similarity between the yeast Nar1 and the bacterial Fe-only hydrogenase with a root mean square deviation (RMSD) of 1.27. Another similarity feature is

the presence in the Nar1 structure of a Rossmann-type motif which consists of four parallel β-strands flanked by α-helices. This motif is found in all Fe-only hydrogenases (Figure 3.18 C) (Rees, 2002). The Fe/S clusters shown in Figure 3.18 C were imported into the calculated Nar1 structure from the published X-ray structure of Fe-only hydrogenase from *C. pasteurianum* (PDB code 1feh) (Peters *et al.*, 1998). With the exception of Cys62, all other conserved cysteine residues in Nar1 (Figure 3.19 A, B, in yellow) showed an almost similar orientation as those coordinating the Fe/S clusters in *C. pasteurianum* Fe-only hydrogenase (Figure 3.19 A, B, in red). The orientation of the N-terminal cysteine residues (C20, C59, C62 and C65) suggests that they could be involved in the coordination of one Fe/S cluster, whereas the other conserved cysteine residues (C177, C231, C412 and C416) may bind a second Fe/S cluster at the C-terminus. Cys62 was rotated in the calculated structure by almost 180° compared to the corresponding cysteines in hydrogenase. This rotation gave rise to the formation of a disulfide bridge between Cys62 and Cys412. Since the presence of two Fe/S clusters at the N- and C-terminus of Nar1 is incompatible with a disulfide bridge, this might represent a calculation artifact.

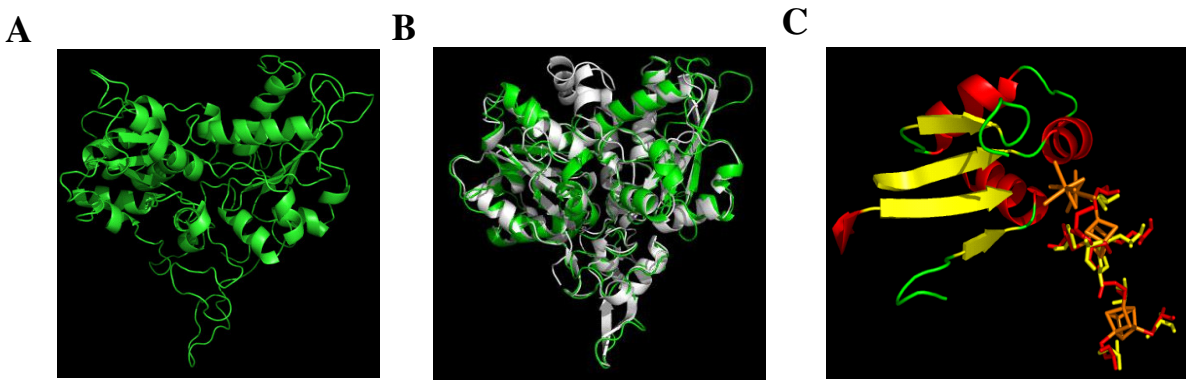


Figure 3.18 Structural model of the *S. cerevisiae* Nar1 protein. Nar1 was modeled using the Protein Data Bank coordinates of *Clostridium pasteurianum* iron-only hydrogenase (PDB code 1FEH). **A)** Nar1 structural model **B)** Nar1 (red) and *C. pasteurianum* (grey) structures were superimposed. **C)** The Rossmann motif conserved in all metalloproteins is found also in Nar1 protein. The Fe/S clusters and the cysteine residues depicted in red were imported from the published X-ray structure of Fe-only hydrogenase from *Clostridium pasteurianum*. Figure was prepared with PyMol (DeLano, 2002).

The cluster that might be coordinated by the C-terminal Nar1 cysteines (C177, C231, C412 and C416) is buried in the Nar1 protein, whereas the Fe/S cluster at the N-terminus is more exposed to the protein surface (Figure 3.19 C). The orientation of all eight cysteine residues in Nar1 may favour the binding of two Fe/S clusters.

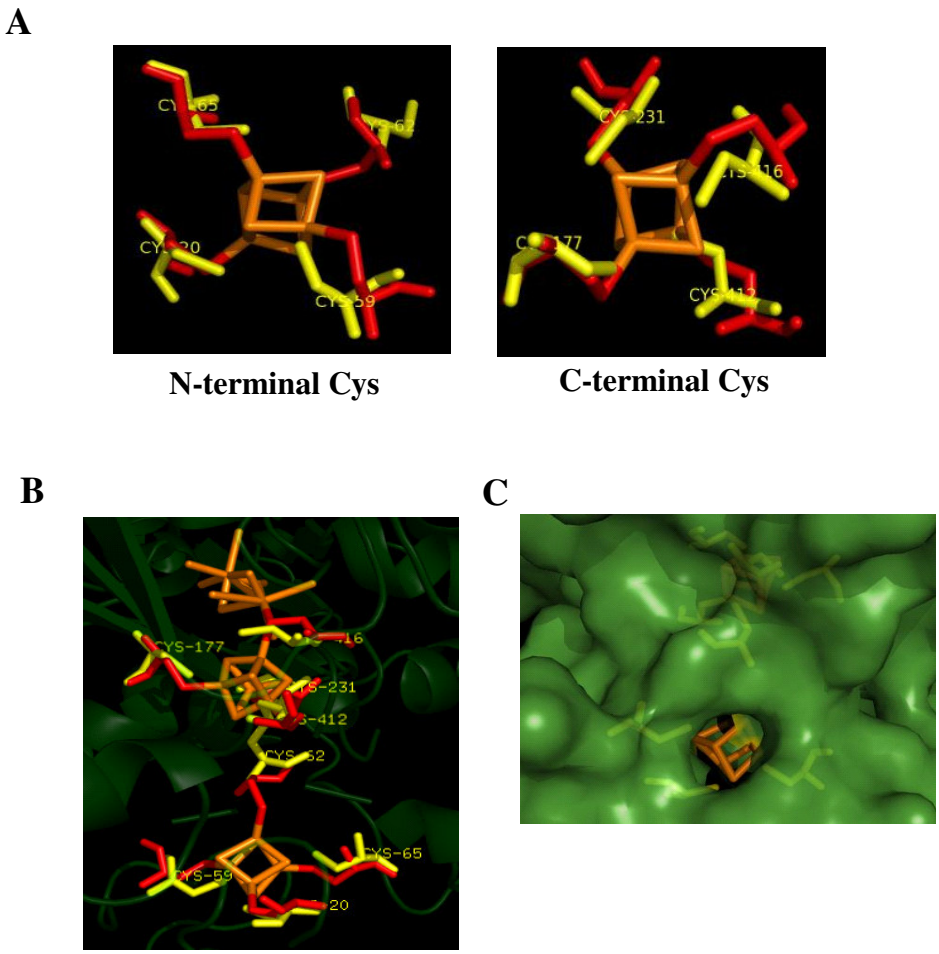


Figure 3.19 Close-up view of the Fe/S cluster binding sites of the modelled structure of Nar1. The Fe/S clusters were imported from the X-ray structure of *C. pasteurianum* hydrogenase. Cysteine residues were drawn as sticks and those from Nar1 are coloured in yellow and those from the hydrogenase in red. **A)** Position of Nar1 cysteines with respect to the hydrogenase cysteines. **B)** Overview of the possible Fe/S cluster binding sites in Nar1. **C)** Close-up view of the N-terminal Fe/S cluster of Nar1. Figure was prepared with PyMol (DeLano, 2002).

3.2.3. Characterization of the importance of the conserved cysteine residues of Nar1 for cell viability

Previously, it has been shown that Nar1 is an Fe/S protein playing an essential role in the maturation of cytosolic and nuclear Fe/S proteins (Balk *et al.*, 2005c; Balk *et al.*, 2004). Thus, Nar1 is both a component and a target of the CIA machinery. Assembly of the Fe/S clusters on Nar1 depends on mitochondrial ISC and export machineries and on two other cytosolic members of the CIA machinery, namely Nbp35 and Cfd1 (Balk *et al.*, 2004; Hausmann *et al.*, 2005). Electron paramagnetic resonance experiments indicate that Nar1 holds two Fe/S clusters that are magnetically coupled. To get insights into the molecular

function of Nar1, the chemical nature of the two Fe/S clusters was characterized and their coordination ligands were assigned. Using site-directed mutagenesis, each of the eight conserved cysteine residue was changed to alanine residues (and for some cysteines also to serine residues). Based on the homology to hydrogenases these positions were grouped into N- and C-terminal cysteines. All Nar1 site-directed mutants used in this study are schematically depicted in Figure 3.20.

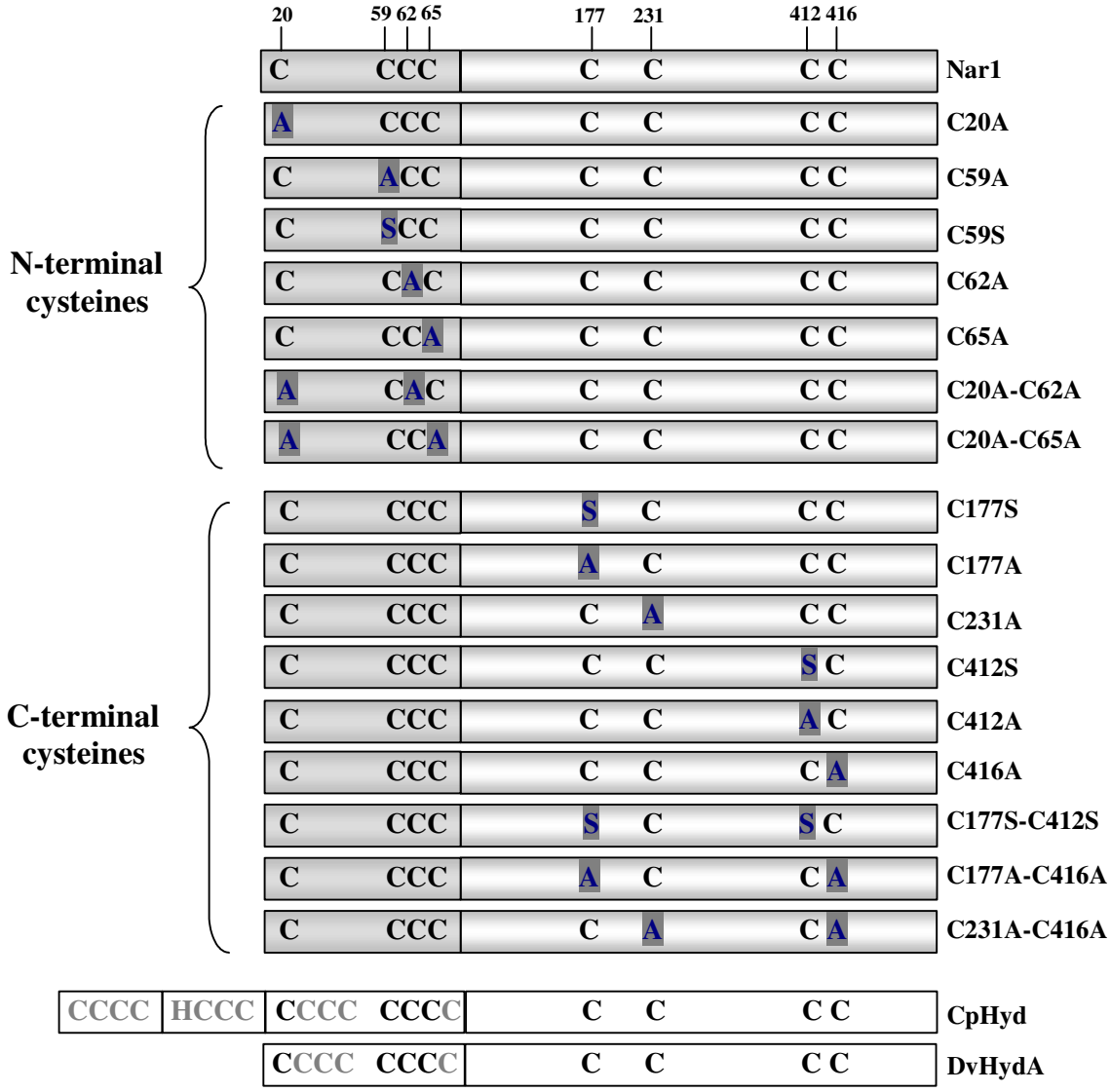


Figure 3.20 Schematic representation of point-mutations introduced in Nar1. Numbers indicate the amino-acid positions in the protein. CpHyd and DvHydA indicate two iron-only hydrogenases from *Clostridium pasteurianum* and from *Desulfovibrio vulgaris*, respectively. These proteins show considerable sequence similarities to Nar1. Residues depicted in light grey are involved in coordination of Fe/S clusters in hydrogenases and not conserved in Nar1.

First, it was investigated whether these eight conserved cysteine residues are essential for cell viability. Gal-NAR1 cells were transformed with plasmids p416-MET25 coding for wild-type Nar1, the Nar1 mutants or no gene (-). The functionality of mutant Nar1 proteins was tested by depleting the nuclear-encoded wild-type Nar1 by growing the cells on minimal medium lacking galactose. The wild-type *NAR1* restored the growth defect of Gal-NAR1 cells carrying the empty plasmid indicating that these cells specifically lack Nar1. When the N-terminal Nar1 cysteine mutants were analysed, two single-point mutant proteins (C59A and C62A) were not able to restore the wild-type growth. One Nar1 mutant -C65A- showed only partial complementation, whereas the C20A mutation had no effect on cell growth. However, the C20 residue is important because the double mutant C20A-C65A Nar1 can not complement the Gal-NAR1 cell growth deficiency (Figure 3.21 A and Table 3.1). This cumulative effect of C20 was not observed in the double mutant C20A-C62A because the single C62A mutation led to cell death (Figure 3.21 A and Table 3.1).Thus, three cysteine residues at the Nar1 N-terminus (C59, C62 and C65) are essential for the yeast cell viability. The C20 is also an important residue due to its synergetic effects.

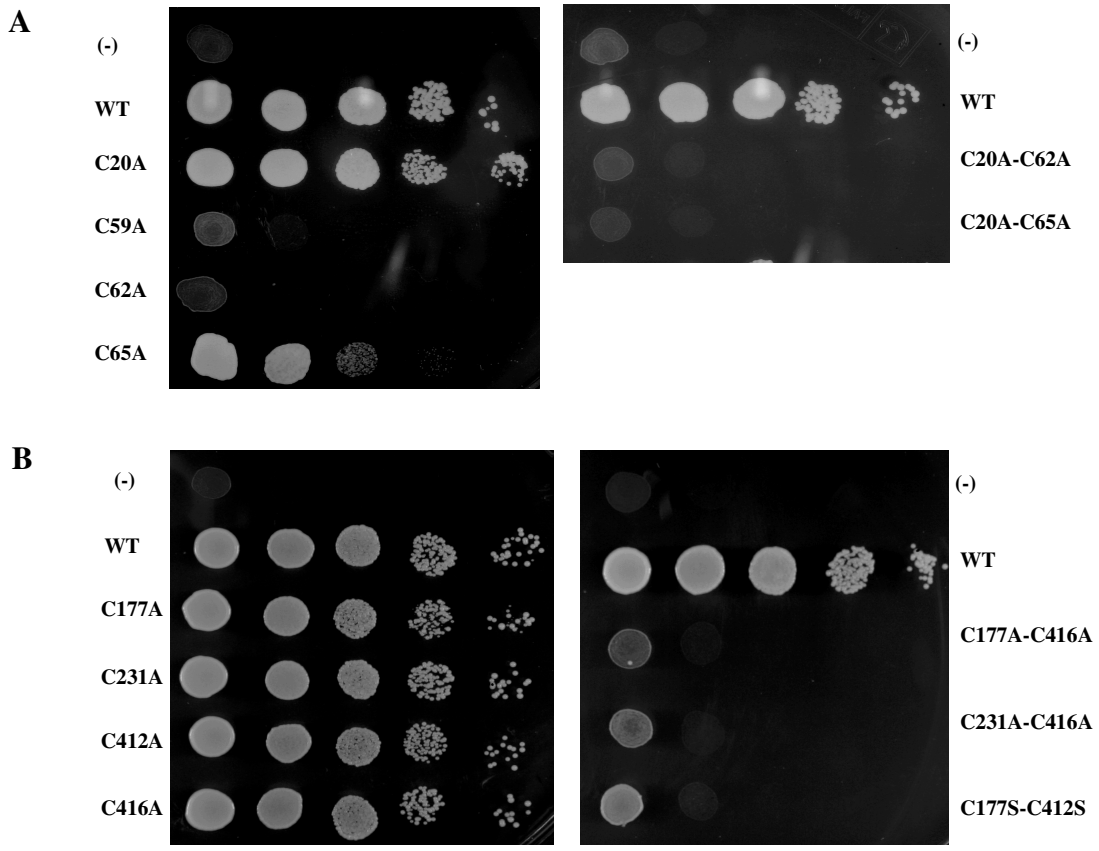


Figure 3.21 The importance of conserved cysteines in Nar1 for cell viability. Gal-NAR1 cells were transformed with plasmid p416MET25 containing either no insert (-), the wild-type (WT) *NAR1* gene, or the indicated **(A)** N-terminal and **(B)** C-terminal cysteine mutants. The cells were grown on SD medium for 2x2 days at 30°C. Ten-fold serial dilutions are shown.

Single-point mutants of Nar1 C-terminal cysteines residues (C177A, C231A, C412A, and C416A) showed wild-type growth. In contrast, double C-A mutant proteins were not able to rescue the lethal growth phenotype of Gal-NAR1 cells (Figure 3.21 B and Table 3.1). The cysteine to serine mutants C177S, C412S and C177S-C412S showed growth rates similar to the corresponding C-A mutants (Figure 3.22). These results suggest also an important function of the C-terminal residues for the Nar1 function. However, it apparently needs two simultaneous mutations to impact on Nar1 function. This demonstrates a synergetic role of the C-terminal cysteine residues.

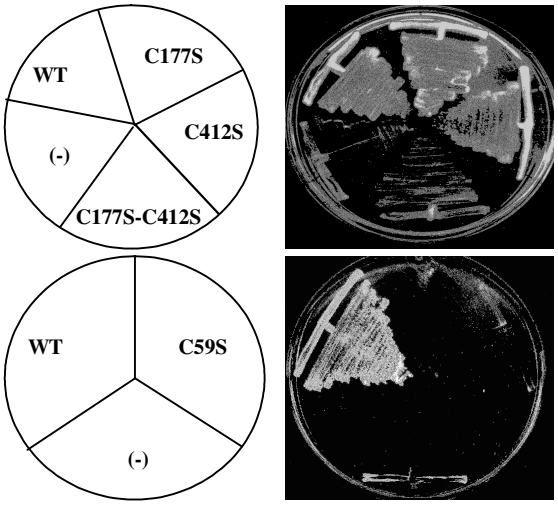


Figure 3.22 Cysteine residues 177 and 412 are not essential for cell viability. Gal-NAR1 cells were transformed with plasmids (p416-MET25) encoding the wild-type Nar1, the indicated cysteine to serine Nar1 mutants or no gene (-). The cells were grown on SD plates for 2x2 days.

All growth phenotypes obtained with the Nar1 mutants are summarized in Table.3.1:

N-terminal Nar1 cysteine mutants

empty	-
WT	++
C20A	++
C59S	-
C59A	-
C62A	-
C65A	+
C20A-C62A	-
C20A-C65A	-

C-terminal Nar1 cysteine mutants

empty	-
WT	++
C177S	++
C177A	++
C231A	++
C412S	++
C412A	++
C416A	++
C177A-C416A	-
C231A-C416A	-
C177S-C412S	-

Table 3.1 Summary of growth phenotypes of the Nar1 cysteine mutants. Gal-NAR1 cells were transformed with plasmid p416MET25 containing either no insert (empty), the wild-type *NAR1* gene, or the indicated cysteine mutants. Cells were grown as described in Figure 3.21. ++ indicates wild-type growth; +, impaired growth; -, no growth.

In the experiments above, the cysteine Nar1 mutant proteins were overproduced from vector p416-MET25 under the control of the strong constitutive *MET25* promoter. To rule out the possibility that the full growth complementation by some single-cysteine mutants (C177A(S), C412A(S)) was not due to high copy suppression, the *MET25* promoter in the p416 plasmid carrying the C177S, C177A, C412S and C412A Nar1 mutants was exchanged for the endogenous *NAR1* promoter. The growth rates of the Gal-NAR1 cells expressing these Nar1 mutants under the control of endogenous *NAR1* promoter were compared to those of cells transformed with plasmids containing either the wild-type *NAR1* gene or no insert (Figure 3.23). Under these conditions only one mutant protein -C412A- showed partial complementation, whereas all others could fully rescue the growth defect indicating that the full complementation seen for the C412A (Figure 3.21) is due to a partial overexpression effect. These Nar1 mutants that are expressed under the control of *NAR1* promoter were not used for further experiments because for immunoprecipitations overproduced mutant proteins were needed.

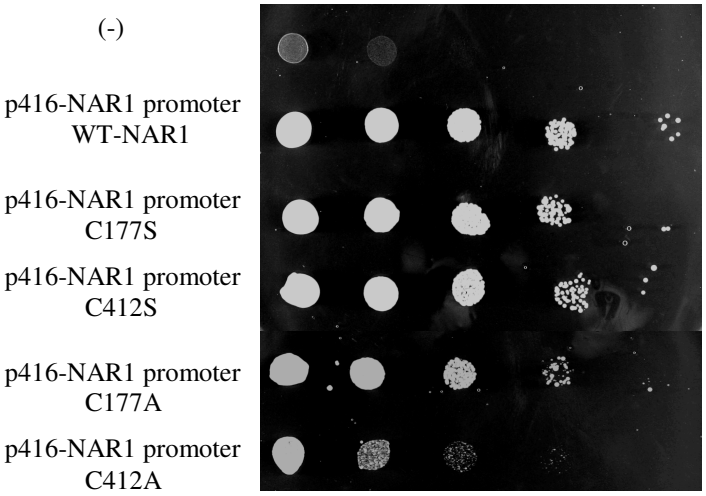


Figure 3.23 Nar1 Cys-Ala or Cys-Ser mutants can functionally replace wild-type Nar1 when expressed under the control of endogenous *NAR1* promoter. Gal-NAR1 cells were transformed with plasmid p416-NAR1-promoter containing either no insert (-), the wild-type (WT) *NAR1* gene, or the indicated mutants. The cells were grown as described in Figure 3.21. Ten-fold serial dilutions are shown.

Taken together, three of four cysteines at the N-terminus (C59, C62 and C65) are essential, whereas all four C-terminal cysteine residues are not. Although these C-terminal single-point cysteine mutant proteins can complement the growth phenotype of Gal-NAR1 cells lacking the nuclear encoded Nar1, the simultaneous exchange of two cysteines led to mutants proteins that cannot longer functionally replace the wild-type Nar1 protein. This clearly indicates that the conserved cysteines play an important role for Nar1 function, and that they cooperate in their function.

3.2.4. The requirement of N-terminal and C-terminal cysteine residues for the maturation of cytosolic Fe/S proteins

Further, the consequences of the Nar1 N-terminal and C-terminal cysteine residues mutations on the *de novo* synthesis of cytosolic Fe/S proteins were investigated by following the incorporation of radioactive iron ($^{55}FeCl_3$). The cytosolic Leu1 and Rli1 served as Fe/S reporter proteins. Gal-NAR1 cells carrying plasmids coding for wild-type Nar1, N-and C-terminal cysteine mutants, or no gene were grown in iron-poor minimal medium containing glucose and radiolabelled with $^{55}FeCl_3$ in the presence of ascorbate for 2 h. Yeast cells were lysed using glass beads and the endogenous Leu1 and Rli1-HA overexpressed from a "high-copy number" plasmid (p424-TDH3) were immunoprecipitated from cell extracts with specific anti-Leu1 antiserum and anti-HA antibodies, respectively. The amount of ^{55}Fe incorporated into Leu1 and Rli1 was measured by scintillation counting (Kispal *et al.*, 1999).

First, the N-terminal Nar1 cysteine mutants were analysed. In almost N-terminal cysteine mutants analysed (C59A(S), C62A, C65A, C20A-C62A and C20A-C65A) the amounts of ^{55}Fe incorporated into endogenous Leu1 were significantly decreased (to background levels) (Figure 3.24 A). Similar results were observed when a second cytosolic Fe/S protein (Rli1) was analysed (Figure 3.24 B). The only exception was for the C20A mutant which still showed ≈40 % of ^{55}Fe associated with Leu1 or Rli1 compared to wild-type levels.

The C20A mutant incorporated ^{55}Fe amounts that were above the threshold level sufficient to form functional Leu1 and Rli1 proteins and, therefore to complement the phenotype of Nar1-depleted Gal-NAR1 cells (Figure 3.21 and Figure 3.24 A, B). An important observation is that the amounts of ^{55}Fe associated with Leu1 and Rli1 in the C65A mutant were further diminished by the C20A mutation in the C20A-C65A double mutant. Leu1 and Rli1 protein levels detected for Nar1mutant cells were comparable to those present in the wild-type cells (Figure 3.24 C).

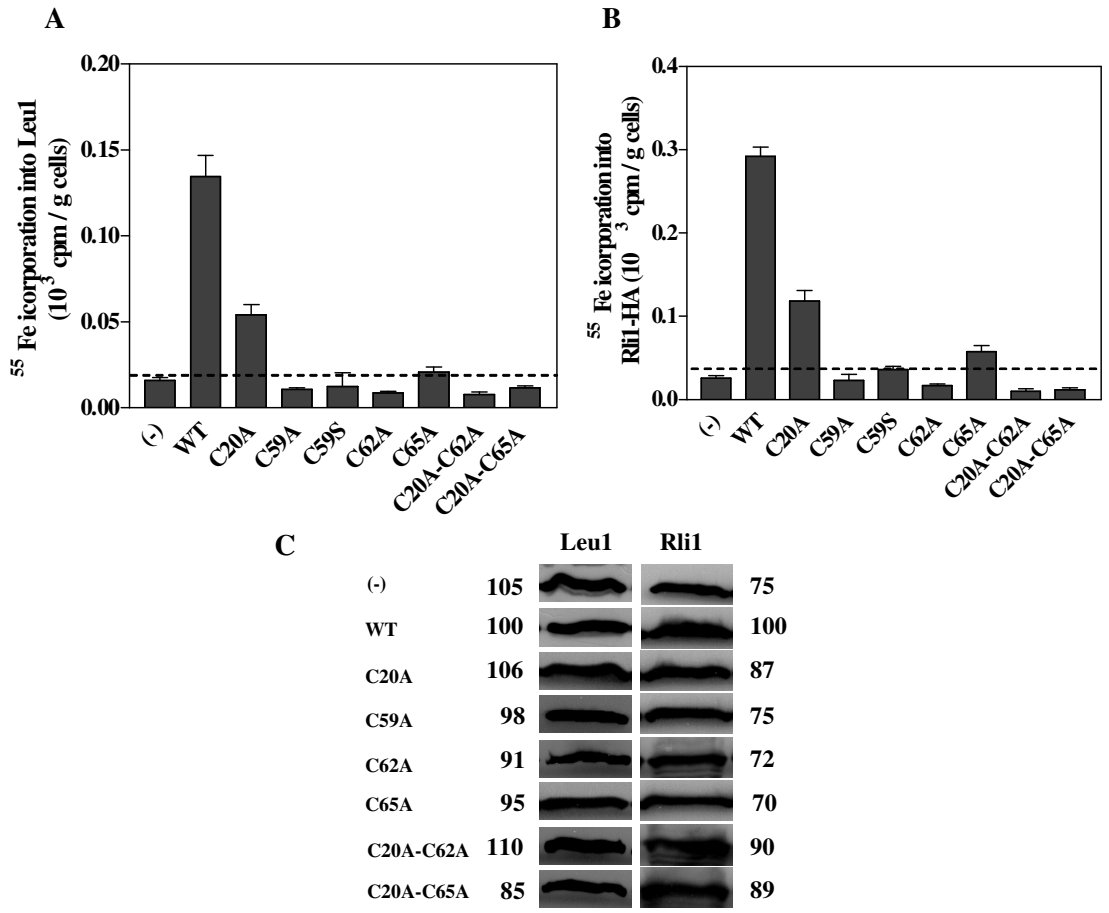


Figure 3.24 Three N-terminal Nar1 cysteines are essential for maturation of cytosolic Fe/S proteins. Galactose-regulatable Gal-NAR1 cells were transformed with plasmid p416-MET25 coding for wild-type Nar1, the indicated Nar1 N-terminal cysteine mutants or no gene (-). Cells were grown in SC-medium for 24 h and in iron-poor medium supplemented with glucose for 16 h. Endogenous Leu1 **(A)** and Rli1-HA **(B)** expressed from high copy number vector (p424-TDH3) were immunoprecipitated from cell extracts using Leu1 specific or anti-HA antibodies. The amount of ^{55}Fe was quantified by scintillation counting and corrected for the Leu1 and Rli1 protein levels. The dashed lines indicate the background levels of ^{55}Fe incorporation. **C)** The protein levels of Leu1 and Rli1 detected by immunostaining and quantified by densitometry. The levels of Leu1 and Rli1 in wild-type Nar1 were set to 100%.

Next, it was investigated whether also the conserved cysteines at the C-terminus of Nar1 are required for its function in the maturation of cytosolic Fe/S proteins. Nar1 with single mutations in the C-terminal conserved cysteines supported the incorporation of wild-type amounts of ^{55}Fe into Leu1 and Rli1. In contrast, when the double cysteine Nar1 mutants were analysed ≈ 6-fold lower amounts of ^{55}Fe incorporated into Leu1 and Rli1 were detected (Figure 3.25 A, B). As observed for the N-terminal cysteine mutant proteins, the Leu1 and

Rli1 protein levels were not significantly affected in cells carrying the C-terminal Nar1 cysteine mutants (Figure 3.25 C).

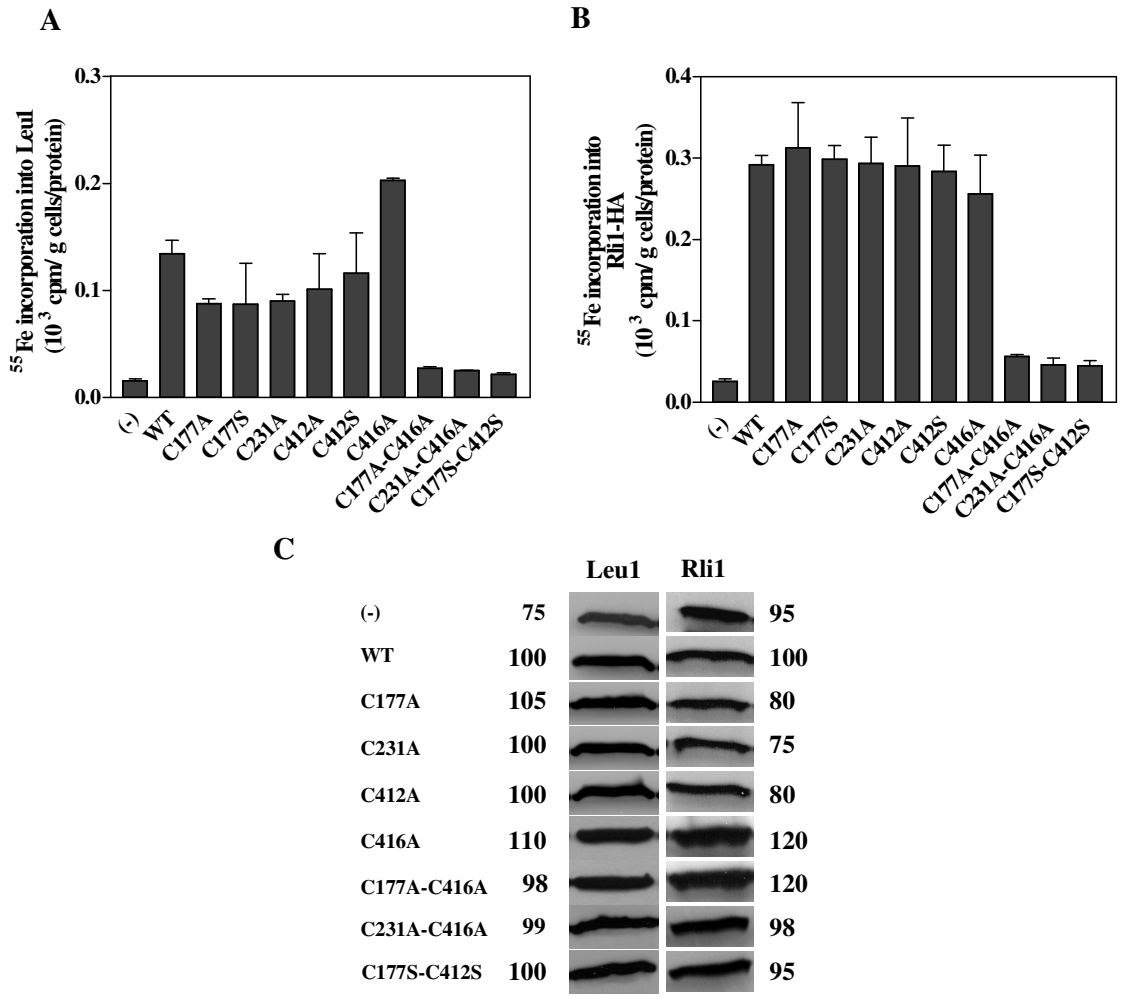
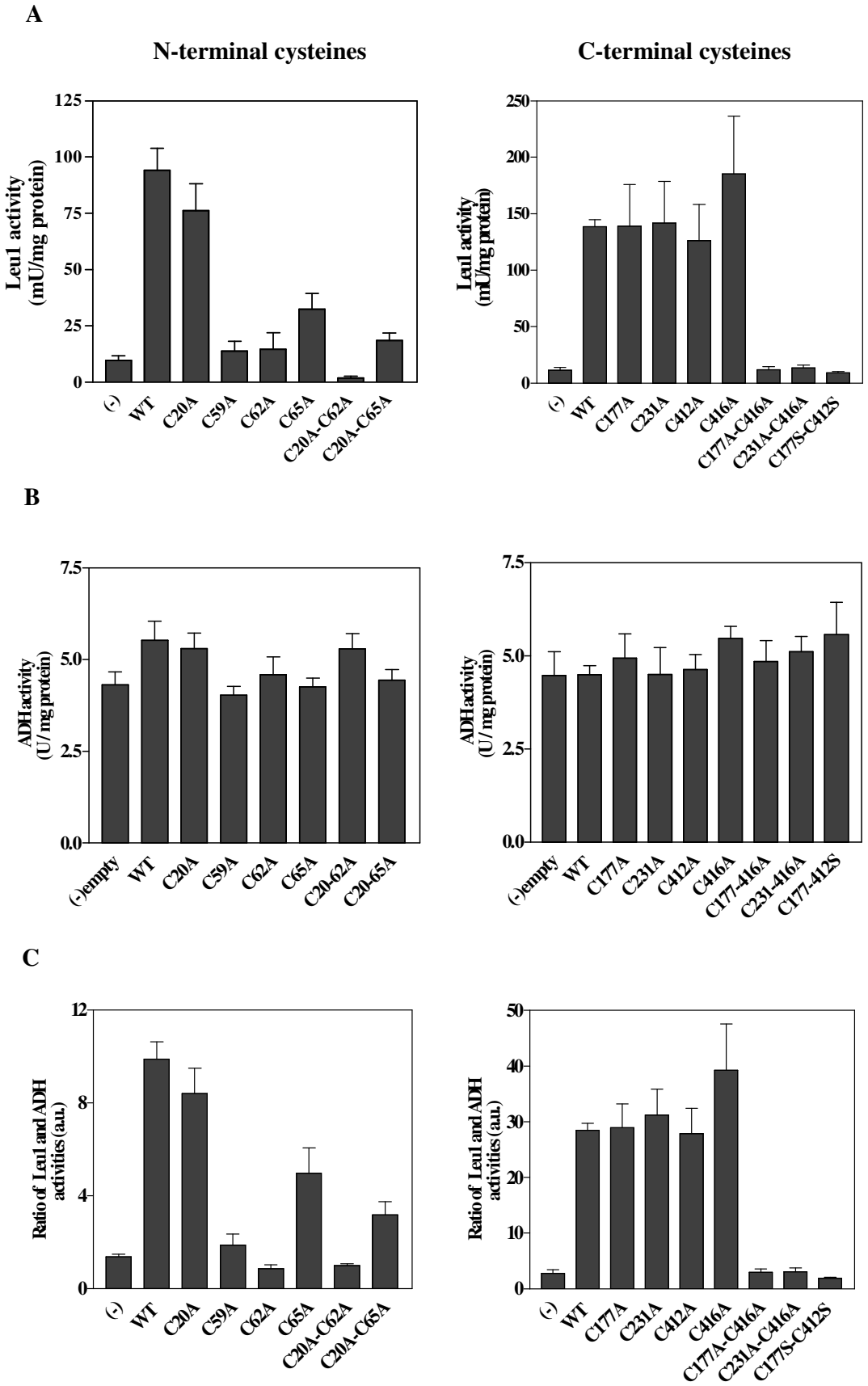


Figure 3.25 The role of the C-terminal cysteines of Nar1 in the maturation of cytosolic Fe/S proteins. Galactose-regulatable Gal-NAR1 cells were transformed with plasmid p416-MET25 coding for either wild-type (WT) Nar1, the indicated Nar1 C-terminal cysteine mutants or no gene (-). Cells were grown as described in Figure 3.24. Endogenous Leu1 **(A)** and Rli1-HA **(B)** expressed from high copy number vectors (p424-TDH3) were immunoprecipitated from cell extracts using Leu1-specific or anti-HA antibodies. The amount of ^{55}Fe was quantified by scintillation counting and corrected for the Leu1 and Rli1 protein levels. **C)** The protein levels of Leu1 and Rli1 detected by immunostaining and quantified by densitometry. The levels of Leu1 and Rli1 in wild-type Nar1 were set to 100%.

Together, these results demonstrate that all four cysteine residues at the N-terminus are important for the maturation of cytosolic Fe/S proteins. Three cysteines (C59, C62 and C65) are essential, whereas the C20A had only a cumulative effect on that observed for the C65A in the double C20A-C65A mutant. This cumulative effect of C20 was not observed in the double mutant C20A-C62A because already the single C62A mutation led to no Fe/S

cluster insertion into cytosolic Fe/S proteins. The single C-terminal cysteine mutants showed no defect in Fe/S cluster assembly, but the simultaneous exchange of two of these cysteines led to strongly impaired Fe/S cluster incorporation into Leu1 and Rli1 suggesting that also these residues play an important function. These data explain the inability of these mutants to complement the wild-type Nar1. Taking into consideration that these cysteines coordinate two Fe/S cluster, it was concluded that both Fe/S cluster are required for Nar1 function in cytosolic Fe/S protein biosynthesis.

The requirement of N- or C-terminal Nar1 cysteines for cytosolic iron-sulfur proteins maturation was further supported by determination of the enzyme activity of isopropylmalate isomerase (Leu1) (Kispal *et al.*, 1999). The Leu1 activity of the various Nar1 mutants showed similar profiles to those obtained by ^{55}Fe incorporation assay. Only a minor decrease in the activity in comparison to the wild-type activity was observed for the C20A Nar1 mutant. In contrast, all other N-terminal cysteine mutants indicated a significant 4-5 fold decrease in the Leu1 activity (C59A, C62A and C65A). The still detectable Leu1 activity in the C65A mutant was further diminished to almost background levels, when C20 and C65 were simultaneously changed to alanine residues (C20A-C65A). The isopropylmalate isomerase activity of single-point C-terminal cysteine mutants was found to be similar to that of the wild-type strain, even a little higher for the C416A mutant. The Leu1 activity was strongly diminished, when double cysteine mutants were analysed (C177A-C416A, C231A-C416A and C177S-C412S) (Figure 3.26 A). As a control, the activity of a non-Fe/S cytosolic protein (alcohol dehydrogenase-ADH) was also measured. ADH activity did not change significantly in any of the N- or C-terminal cysteine mutants when compared to wild-type ADH activity (Figure 3.26 B). For a better visualization of the effects caused by cysteine mutations on Leu1 activity the ratio of these two enzyme activities is depicted in Figure 3.26 C. The Leu1 protein levels were similar to those of the cytosolic control protein Pgk1 (Figure 3.26 D).


A
N-terminal cysteines
C-terminal cysteines
Leu1 activity (mU/mg protein)
125
100
75
50
25
0
(-)
WT
C20A
C59A
C62A
C65A
C20A-C62A
C20A-C65A
250
200
150
100
50
0
C177A
C231A
C412A
C416A
C177A-C416A
C231A-C416A
C177S-C412S
B
ADH activity (U/mg protein)
7.5
5.0
2.5
0.0
(-)empty
WT
C20A
C59A
C62A
C65A
C20-62A
C20-65A
C177A
C231A
C412A
C416A
C177-416A
C231-416A
C177-412S
C
Ratio of Leu1 and ADH activities (a.u.)
12
8
4
0
50
40
30
20
10

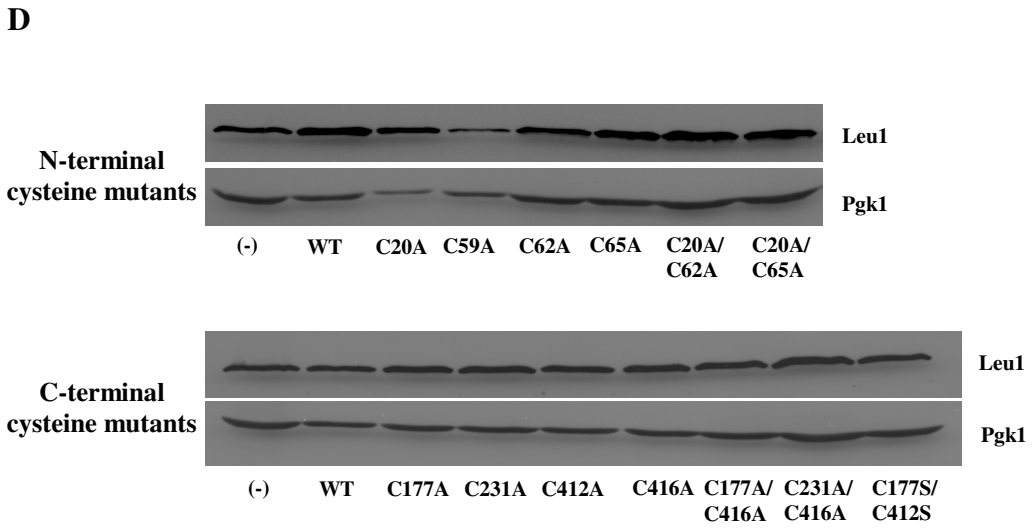


Figure 3.26 Nar1 conserved cysteine residues in the N- and C-terminus are required for the maturation of Leu1. Gal-NAR1 cells were transformed with plasmid p416-MET25 coding for wild-type (WT) Nar1, the indicated Nar1 N- and C-terminal cysteine mutants or no gene (-). Cells were grown in SC-medium for 40 h, and the isopropylmalate isomerase (Leu1) **(A)** and alcohol dehydrogenase (ADH) **(B)** activities were measured in cell extracts. **C)** shows the ratio of the Leu1 and ADH enzyme activities, **(D)** Leu1 and Pgk1 protein levels were visualized by immunoblotting with specific anti-Leu1 and anti-Pgk1 antibodies.

Together, these results indicate that three cysteines (C59, C62 and C65) from the N-terminus of Nar1 are essential for Nar1 function in the maturation of Fe/S proteins in the cytosol. The other conserved N-terminal cysteine residue (C20) is not essential. Yet, combination of a mutation of this residue with that of C65 (in C20A-C65A mutant) further decreased the low maturation activity observed in the C65A mutant. The C-terminal single cysteine mutants do not show any defects in the maturation of Leu1 and Rli1, but the double cysteine mutants led to a severe loss in the Fe/S incorporation of cytosolic Fe/S proteins suggesting that all four C-terminal cysteines are important for the maturation of cytosolic Fe/S proteins.

3.2.5. N- and C-terminal cysteine residues are essential for Fe/S cluster assembly on Nar1

The results presented above make it likely that at least those cysteine residues of Nar1 that are essential for viability and function are directly involved in the coordination of the two Fe/S clusters of Nar1 itself. However, also those residues which do not exhibit any detrimental effects on Nar1 upon mutation may still coordinate the Fe/S centers. Double mutations of non-essential residues resulted in non-functional Nar1 proteins indicating that all

eight cysteine residues, in particular all four C-terminal ones, are important for Nar1 function in the maturation of cytosolic Fe/S proteins. In the case of non-essential single cysteine mutations, the clusters might remain stably bound to Nar1 with only three coordination sites or other residues may replace the function of the exchanged cysteines.

To directly investigate the consequences of the cysteine mutations on Fe/S clusters binding to Nar1 itself, the ^{55}Fe radiolabelling assay described above was employed. Gal-NAR1 cells containing the plasmids encoding the wild-type Nar1, Nar1 cysteine mutants or no gene (-) were grown and radiolabelled with $^{55}FeCl_3$. Nar1 was immunoprecipitated from the cell extracts using a Nar1-specific antiserum and the amount of ^{55}Fe incorporated into Nar1 was measured by scintillation counting (Kispal *et al.*, 1999). When the N-terminal Nar1 cysteine mutants were analysed for their influence on Fe/S cluster binding, the ^{55}Fe associated with the C20A mutant was slightly diminished to ≈75-80 % of the wild-type levels, whereas in C59A(S) and C62A mutants the amount of incorporated ^{55}Fe was significantly decreased to 10-20 % of wild-type levels. The C65A Nar1 mutant still could incorporate ≈40 % of ^{55}Fe wild-type amounts, but the double C20-65A mutation lead to a further decrease in radiolabelled iron incorporation to ≈20 % (Figure 3.27 A). The Nar1 protein levels were almost similar (Figure 3.27 B). For more accurate results these levels were densitometrically quantified and used to correct the ^{55}Fe incorporation for protein levels. These results are in accordance with the functional complementation data indicating that the growth defects associated with some of the Nar1 N-terminal cysteine mutations correlate well with the amounts of ^{55}Fe incorporated into these Nar1 cysteine mutants. In case of C59A(S), C62A, C20A-C62A, and C20A-C65A Nar1 mutants, the low ^{55}Fe incorporation levels may indicate the simultaneous loss of both Fe/S clusters leading to an un-functional Nar1 protein and consequently to cell death (see Figure 3.21 A), whereas in the Nar1 C20A mutant the ≈80 % of incorporated radioactive iron, compared to wild-type indicates that this mutant still can bind the Fe/S clusters. This suggests that C20 is not a ligand or not an essential ligand for Fe/S cluster binding. Alternatively, ligand exchange might take place which is not an unusual observation for Fe/S proteins (Moulis *et al.*, 1996).

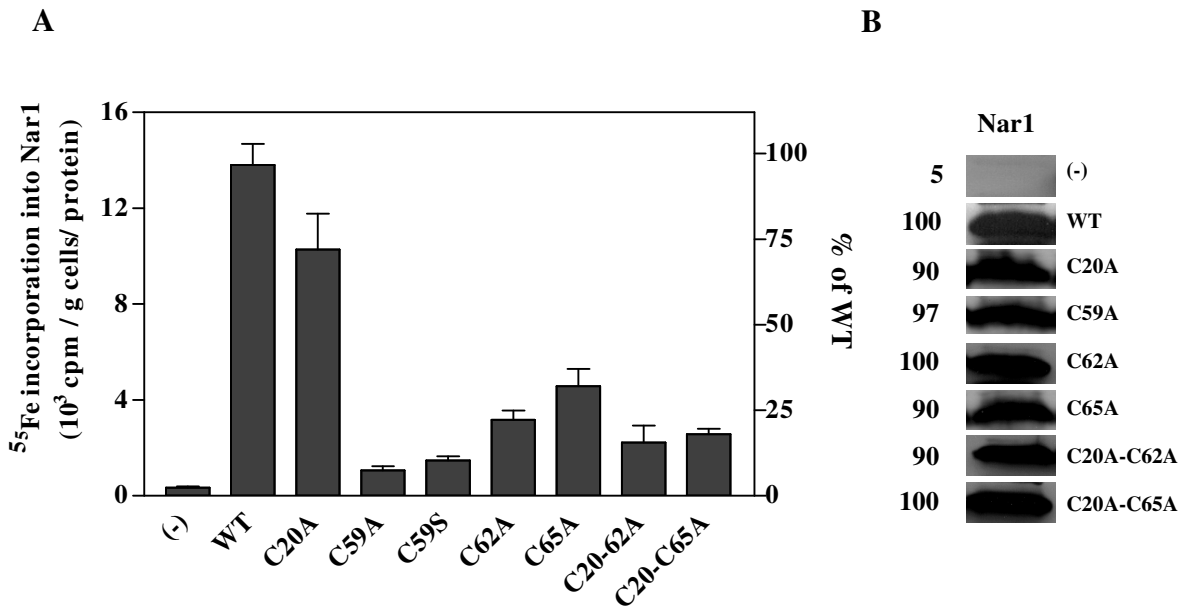


Figure 3.27 Three N-terminal Nar1 cysteine mutants are essential for the incorporation of Fe/S clusters into Nar1. A) Galactose-regulatable Gal-NAR1 cells were transformed with plasmid p416-MET25 coding for wild-type Nar1 (WT), the indicated Nar1 N-terminal cysteine mutants or no gene (-). Cells were grown in SC-medium for 24 h and in iron-poor medium supplemented with glucose for 16 h. Nar1 was immunoprecipitated from cell extracts and the amount of ^{55}Fe was quantified by scintillation counting and corrected for the quantified Nar1 protein levels. **B)** The protein levels of Nar1 were visualised by immunoblot analysis and quantified by densitometry. The levels for wild-type Nar1 were set to 100%.

When the incorporation of ^{55}Fe was followed in the C-terminal single-point C-A (or C-S) Nar1 mutant proteins, the amounts of incorporated ^{55}Fe were comparable to those incorporated into the wild-type protein. Simultaneous mutation of two C-terminal cysteine residues (C177A-C416A, C231A-C416A and C177S-C412S) led to a decrease in ^{55}Fe incorporation to ≈40–60 % of wild-type levels (Figure 3.28 A) which may suggest that the C-terminal Fe/S cluster is lost, yet the Fe/S cluster located in the N-terminal part of Nar1 can still be inserted. The levels of Nar1 (Figure 3.28 B) were diminished in case of some cysteine mutants. They were quantified by densitometry and used to correct the ^{55}Fe incorporation data.

In summary, these data suggest that at least three of the N-terminal cysteines (C59, C62 and C65) are needed for Fe/S clusters assembly on Nar1. The double C-terminal cysteine mutants were able to incorporate only the N-terminal Fe/S cluster, which may indicate that all four C-terminal cysteines (C177, C231, C412 and C416A) coordinate an Fe/S cluster and at least three conserved cysteines at the N-terminus (C59, C62 and C65) are the ligands for another Fe/S cluster. The C-terminal double cysteine mutants (C177A-C416A, C231A-

C416A and C177S-C412S) cannot functionally replace the wild-type Nar1 indicating that the bound Fe/S cluster is essential for Nar1 function.

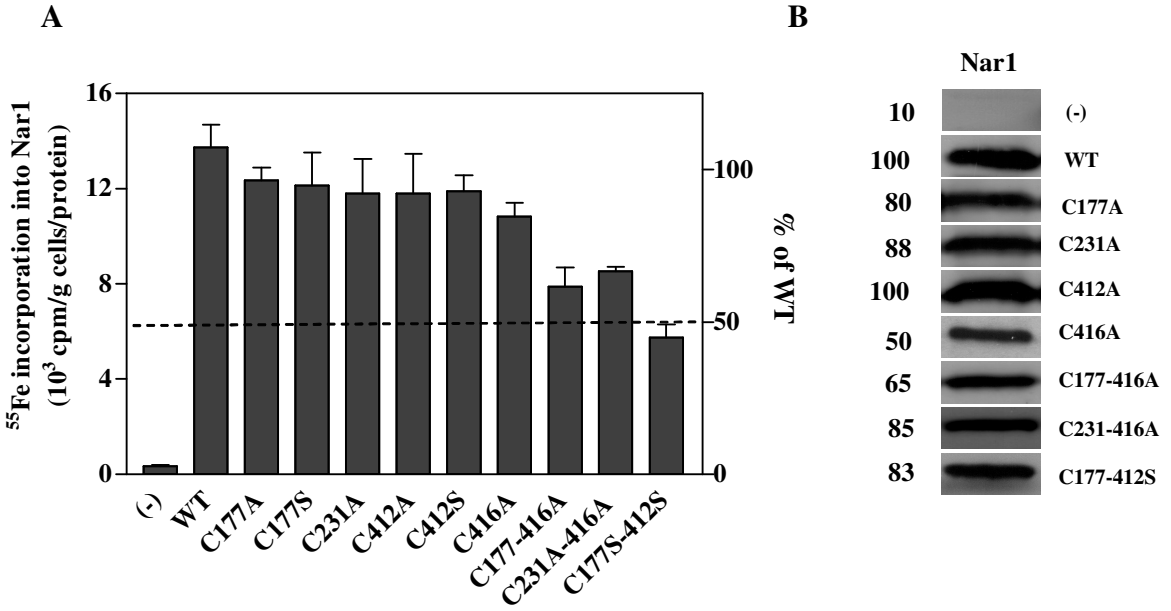


Figure 3.28 Single C-terminal Nar1 cysteine mutants are not essential for incorporation of the Fe/S cluster into Nar1. A) Galactose-regulatable Gal-NAR1 cells were transformed with plasmid p416-MET25 coding for wild-type Nar1, the indicated Nar1 C-terminal cysteine mutants or no gene (-). Cells were grown as described in Figure 3.27A. Nar1 was immunoprecipitated from cell extracts and the amount of ^{55}Fe associated with Nar1 beads was quantified by scintillation counting and corrected for the Nar1 protein levels. The dashed lines indicate the 50% incorporation of ^{55}Fe into wild-type (WT) Nar1. **B)** The protein levels of Nar1 visualized by immunostaining with anti-Nar1 antibodies and quantified by densitometry. The wild-type Nar1 protein levels were set to 100%.

The structural modeling of Nar1 performed above (see part 3.2.2.) showed that the cluster coordinated by the Nar1 C-terminal cysteines (C177, C231, C412 and C416) is buried in the Nar1 protein, whereas the Fe/S cluster at the N-terminus is more exposed to the surface (Figure 3.19 C). This general topology of the N-terminal Fe/S cluster may explain the labile character of this cluster in the biochemical data described above. The surface exposure explains the severe phenotypes shown by the single N-terminal cysteine mutants. Likewise, the shielding of the C-terminal cluster by the polypeptide chain in a cavity might be the reason why mutation of a single C-terminal cysteine residue does not affect the Nar1 function and Fe/S cluster insertion. Only the exchange of two residues eventually leads to a functional impairment.

3.2.6. Residues R144, Q356 and W491 are not essential for Saccharomyces cerevisiae cell viability and for maturation of cytosolic Fe/S proteins

First, two residues (R144A and Q356E) that are conserved only in Nar1-like protein family and correspond to amino acid residues that in Fe-only hydrogenases are surrounding the active site were exchanged by site-directed mutagenesis. In addition, the conserved C-terminal tryptophane residue at position 491 was deleted by PCR to analyse its importance (Figure 3.17, see highlighted residues in grey). To address whether these Nar1 mutant proteins (R144A, Q356E and ΔW491) can functionally replace the wild-type protein, the Nar1 mutants were produced from plasmid p416-MET25 in Nar1-depleted Gal-NAR1 cells (Figure 3.28). All three Nar1 mutants showed growth rates similar to cells expressing the wild-type Nar1 and rescued the lethal phenotype of Gal-NAR1 cells expressing no gene (-). These results indicate that residues R144, Q356 and W491 are not essential for yeast cell viability.

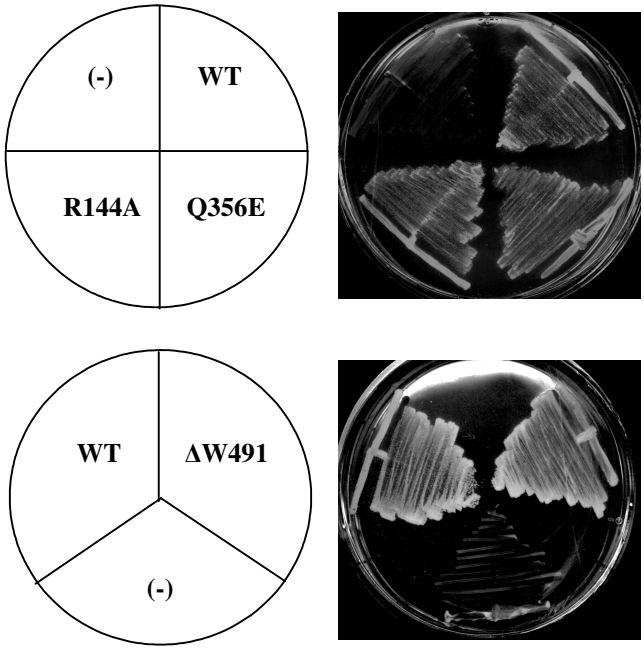


Figure 3.29 Residues R144, Q356 and W491 of Nar1 are not essential for yeast cell viability. Gal-NAR1 cells were transformed with plasmids (p416-MET25) encoding the wild-type Nar1 (WT), the indicated Nar1 mutants or no gene (-). The cells were grown on SD plates for 2x2 days at 30°C.

Further, it was investigated whether these residues might be important for Fe/S cluster insertion into Nar1 or for the *in vivo* function of Nar1 in Fe/S protein maturation. To determine the Fe/S cluster binding capacity of these Nar1 mutants, Gal-NAR1 cells were

transformed with plasmids coding for the wild-type protein, the Nar1 mutants (R144A,Q356E and ΔW491) or no gene (-). Cells were grown in iron-poor minimal medium and radiolabelled with radioactive ^{55}Fe for 2 h. Subsequently, a cell lysate was prepared and Nar1 was immunoprecipitated with specific anti-Nar1 antiserum and the radioactivity coimmunoprecipitated with the immunobeads was quantified by scintillation counting. The amount of ^{55}Fe incorporated into all Nar1 mutants analysed was similar to the amount incorporated into the wild-type Nar1 (Figure 3.30 A), indicating that these residues are not essential for the binding of Fe/S clusters in Nar1.

To test whether these three Nar1 mutants affect the maturation of cytosolic Fe/S proteins, a similar radiolabelling experiment was performed and the ^{55}Fe incorporation into the cytosolic Fe/S protein isopropylmalate dehydrogenase (Leu1) was determined (Figure 3.30 B). The Nar1 mutant proteins showed no significant differences in the amounts of ^{55}Fe incorporated into Leu1 compared to the wild-type Nar1. The protein levels of Nar1 (Fig.3.30 A) and Leu1 (Fig.3.30 B) were not affected in these mutants. Taken together, these data indicate that the insertion of Fe/S clusters into Nar1 or Leu1 does not depend on residues arginine 144, glutamine 356 or tryptophane 491.

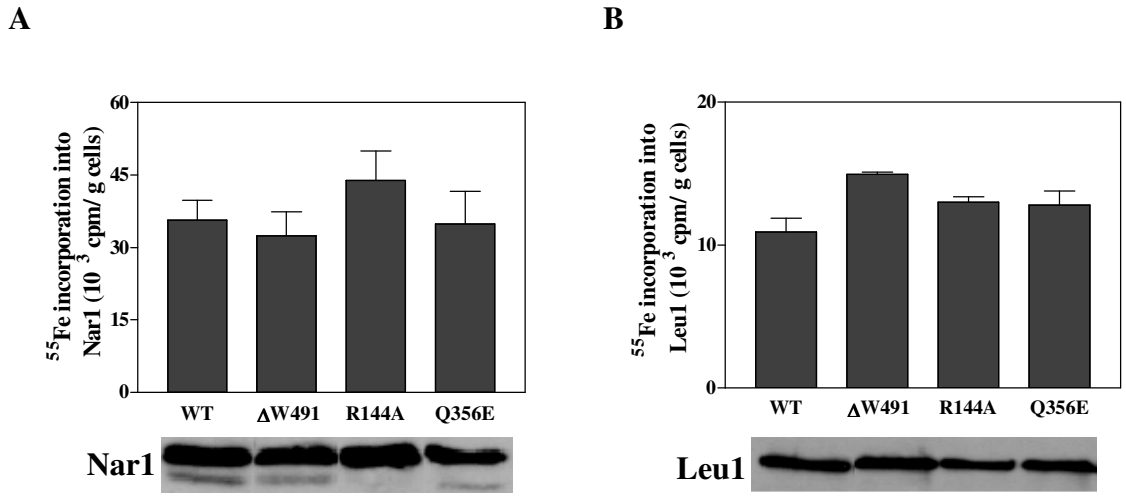


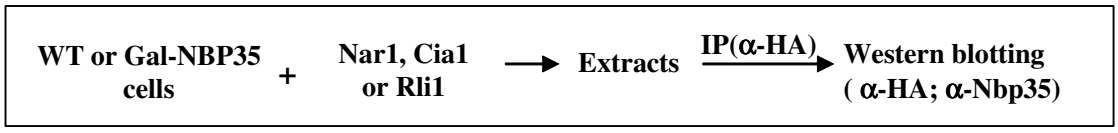
Figure 3.30 Amino acids R144, Q356 and W491 are not involved Fe/S cluster assembly into Nar1 itself and Leu1. Gal-NAR1 cells were transformed with plasmids (p416-MET25) encoding the wild-type Nar1 (WT), the indicated Nar1 mutants or no gene (-). Cells were grown in iron-poor, glucose-containing minimal medium, radiolabelled with $^{55}FeCl_3$ for 2 h and glass beads extracts were prepared. Nar1 **(A)** and Leu1 **(B)** were immunoprecipitated with anti-Nar1 or anti-Leu1 antibodies and the coimmunoprecipitated ^{55}Fe was estimated by liquid scintillation counting. Protein levels of Nar1 and Leu1 were visualized by immunoblot analysis using specific antibodies.

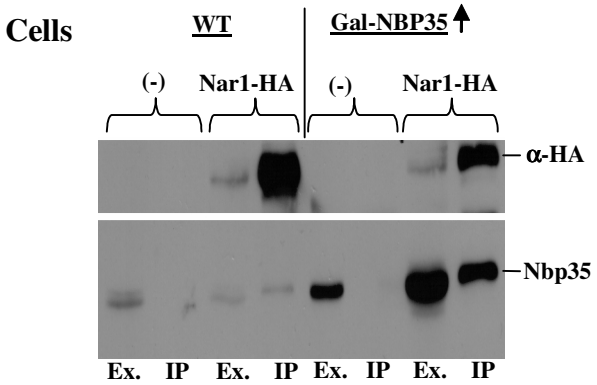
In summary, these results indicate that residues R144, Q356 and W491 are not essential for yeast cell viability and for Nar1 function in the maturation of cytosolic Fe/S proteins. These data clearly shows that the residues R144, Q356 and W491 are not involved in the coordination of Fe/S clusters in Nar1.

3.2.7. Nar1 interacts with Nbp35

Previous studies demonstrated that Nar1 specifically interacts with Cia1 and depletion of Cia1 protein had no effect on Fe/S cluster formation on Nar1 (Balk *et al.*, 2005a). On the other hand, Netz *et al*. (2007) showed that other two components of the CIA machinery, Nbp35 and Cfd1 can form an oligomeric complex *in vivo*. Depletion of Nbp35 lead to impaired Fe/S cluster incorporation into Nar1, indicating that Nar1 must perform its essential function in the cytosol somewhere downstream of Cfd1-Nbp35 complex and upstream of Cia1. Thus, it might be possible that Nar1 also interacts with the upstream CIA proteins. To test whether Nar1 interacts with Nbp35, coimmunoprecipitation assays were performed using yeast cell extracts followed by immunoblotting to estimate the amount of bound protein partner. Because of low endogenous levels of Nar1 and Nbp35 which makes the native proteins almost undetectable by immunoblotting, these proteins were overproduced.

A





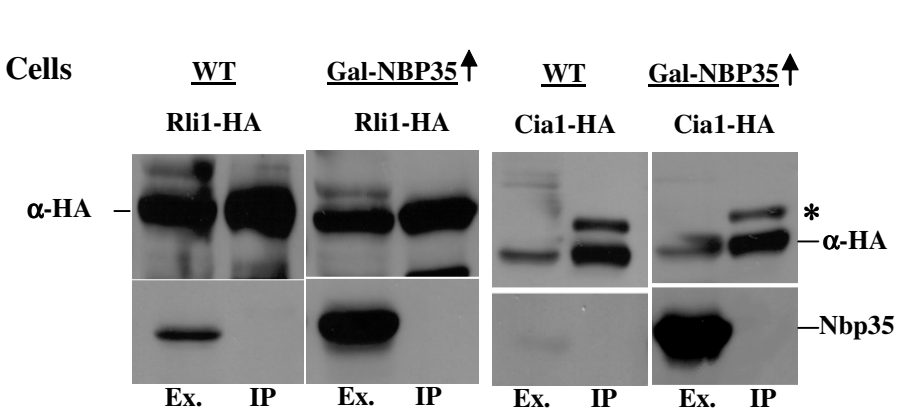


Figure 3.31 Nar1 interacts with Nbp35. Wild-type and Gal-NBP35 cells were transformed with plasmids (p416-MET25) coding for **(A)** Nar1-HA or no gene (-) and **(B)** Cia1-HA or Rli1-HA and grown for 24 h on minimal medium containing galactose. Cell extracts were prepared and the separated cytosol fractions were incubated with HA-agarose for 3 h at 4°C followed by several washing steps in lysis buffer. The cell extracts (Ex.) and the HA-agarose (IP) were subjected to SDS-PAGE and immunoblotting to detect proteins of interest. The asterisk indicates signals detected by the antibodies as a crossreactivity.

HA-tagged Nar1 was overproduced in wild-type and Gal-NBP35 cells grown on galactose-containing minimal medium to induce the Nbp35 expression in Gal-NBP35 cells. Under these conditions a significant amount of Nbp35 was coimmunoprecipitated with Nar1-HA using anti-HA agarose from Gal-NBP35 cell extracts (Figure 3.31 A). This Nar1-Nbp35 interaction was specific because in cells that do not overproduce Nar1 (-) and Nbp35 (WT) or in cells overexpressing other cytosolic HA-tagged proteins (Cia1-HA or Rli1-HA) no Nbp35 could be coimmunoprecipitated (Figure 3.31 A, B).

As a further control, it was tested whether the Nar1-Nbp35 interaction can still occur when Nar1 can no longer perform its essential function in the maturation of cytosolic Fe/S proteins. To test this, Gal-NBP35 cells were transformed with plasmids coding for wild-type Nar1, for some of the Nar1 cysteine mutants or no gene (-). Cells were grown on galactose-containing minimal medium to induce Nbp35 expression. Coimmunoprecipitation was performed as described above using specific anti-Nar1 antibodies. Because in Gal-NBP35 cells there is an endogenous expression of Nar1, in cells containing the empty plasmid we could still coimmunoprecipitate a low, but significant amount of Nbp35 with anti-Nar1 antibodies (Figure 3.32 A). In Gal-NBP35 cells overproducing Nar1, the signal for coimmunoprecipitated Nbp35 was much stronger, confirming the above described Nar1-Nbp35 interaction. When several non-functional Nar1 cysteine mutants (C59A; C62A; C65A; C177A-C416A; C231A-C416A; C177S-C412S) were expressed in Gal-NBP35 cells, the

amount of coprecipitated Nbp35 was diminished compared to the situation when only endogenous wild-type Nar1 was expressed . In case of the C-terminal double cysteine mutants the Nbp35 protein levels were comparable to those found, when Nar1 was only endogenously expressed (Figure 3.32 A). In contrast, Gal-NBP35 cells expressing two Nar1 cysteine mutants that can functionally replace the wild-type Nar1 (C20A and C412A), the immunostaining signal for Nbp35 was similar to that observed for Nar1 wild-type (Figure 3.32 A). Because the Nar1 expression levels were not similar between the wild-type and the cysteine mutants, the Nar1 and Nbp35 immunostaining signals were densitometrically quantified and the ratio of Nbp35 signal to Nar1 signal was calculated (Figure 3.32 B). Except for the C59A and C62A mutants that showed only a weak defect in the Nar1-Nbp35 interaction, all other mutants analysed were not able to support a protein interaction with Nbp35. In summary, these data demonstrate a specific protein interaction between Nar1 and Nbp35. This interaction is impaired in mutations where Nar1 cannot perform its essential function in the cytosol.

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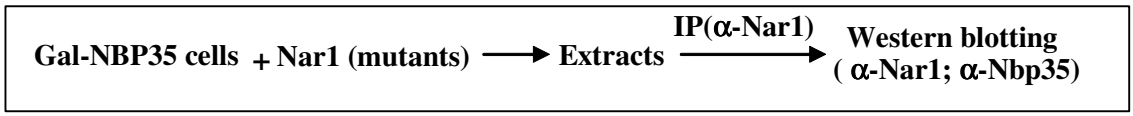

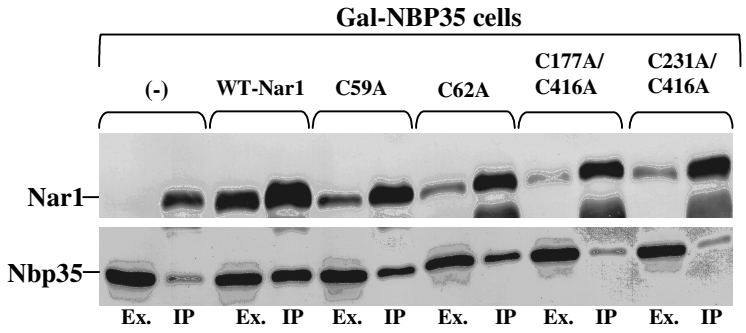

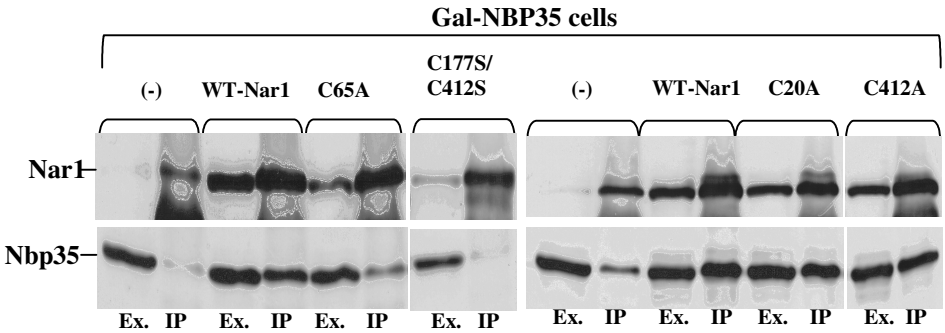

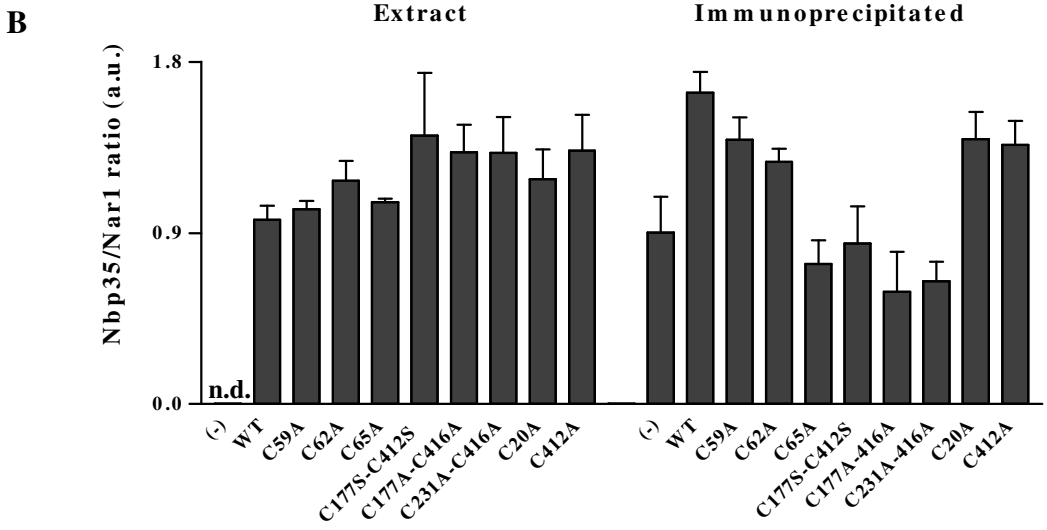

Figure 3.32 Nbp35 interacts with the functional Nar1. Gal-NBP35 cells were transformed with plasmids (p416-MET25) coding for wild-type (WT) Nar1, the indicated Nar1 cysteine mutants or no gene (-) and grown for 24 h on minimal medium containing galactose. Cell extracts were prepared and analysed as described in Figure 3.19 using anti-Nar1 antibodies. **A)** Coimmunoprecipitation employing Nar1 cysteine mutants, **B)** Nar1 and Nbp35 protein levels shown in A were quantified by densitometry and Nbp35/Nar1 ratio of extracts (left panel) and immunoprecipitated proteins (right panel) was calculated (a.u.= arbitrary units; n.d. = not determined).

3.2.8. Purification and Analysis of Recombinant Nar1 Cysteine Mutants

Overproduction and purification of wild-type Nar1 and cysteine mutant proteins

To further characterize the Nar1 cysteine mutant proteins the recombinant *E. coli* proteins were analysed. Wild-type and Nar1 cysteine mutants (C20A, C59S, C62A, C65A, C177S, C231A, C412S, C416A and C177S-C412S) were overproduced in *E. coli* strain C41 (DE3) from pET-15b plasmid in the presence of the bacterial *isc* operon (Nakamura *et al.*, 1999). The *isc* operon was used to overproduce the proteins required for the Fe/S cluster biosynthesis in *E. coli*. The corresponding hexahistidinyl (His)-tagged Nar1 proteins were purified under aerobic conditions by Ni-NTA affinity chromatography in the presence of protease inhibitors. The recombinant wild-type Nar1 protein was brownish on the Ni-NTA column and remained colored during purification. Similar brownish color was observed for some of the Nar1 cysteine mutants (C20A, C59S, C62A, C177S, C412S), whereas the C416A mutants had a yellowish-brown color. In contrast, the other three mutants purified (C65A, C231A and C177S-C412S) were colorless. The purity of the recombinant Nar1 proteins was verified by SDS-PAGE and Coomassie staining (Figure 3.33 A). While most of the Nar1 mutants were more than 95% pure, some mutants (C65A and C231A) were almost completely

degraded and C177S-C412S showed extensive proteolysis products. The former two mutant proteins were not useful and served as "no protein" controls only.

Analytical gel filtration of wild-type Nar1 showed a major peak with a molecular mass of ≈ 56 kDa indicating that Nar1 is a monomer (Figure 3.33 B).

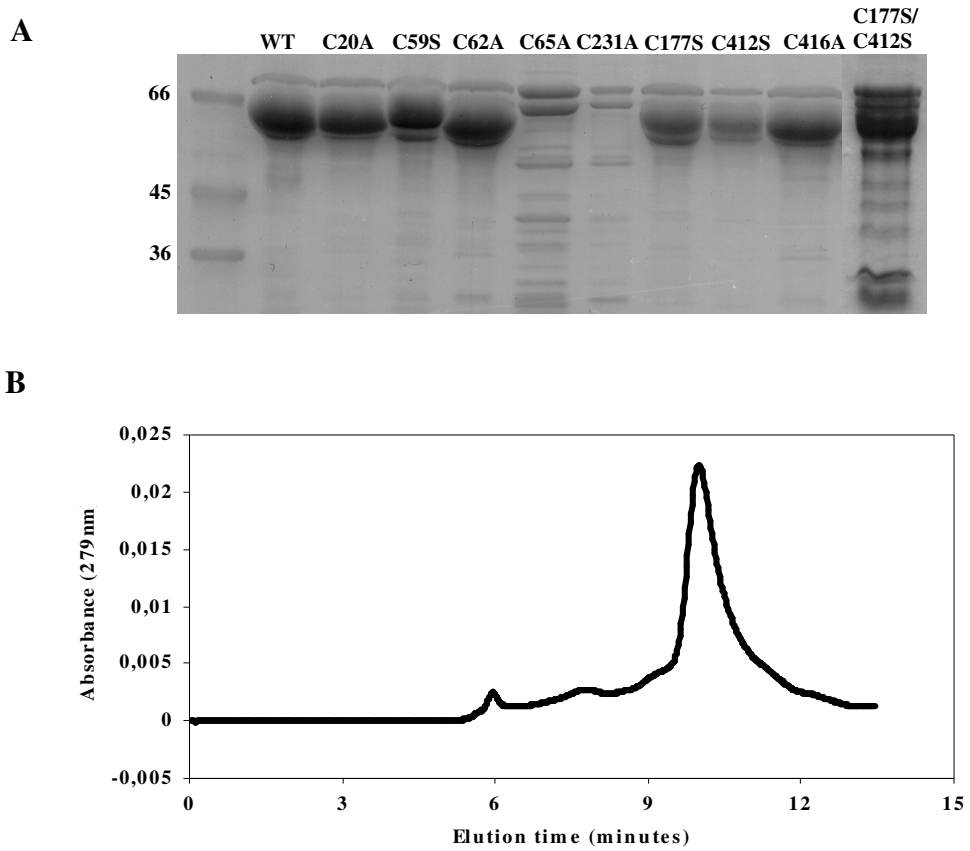

Figure 3.33 Analysis of purified Nar1 mutant proteins. A) SDS-polyacrylamide gel electrophoresis of His-tagged Nar1 and its cysteine mutants. Protein samples were applied to a polyacrylamide gel (10%) in the presence of SDS (0.1%) and stained with Coomassie blue; **B)** Gel filtration of recombinant wild-type Nar1. Buffer conditions: 25 mM Tris-HCl, pH 8.0, 150 mM NaCl. The major peak corresponds to a molecular mass of 56 kDa.

Iron and sulfide content of wild-type Nar1 and cysteine mutant proteins

Next, it was investigated by chemical analysis the non-heme iron and acid-labile sulfur content of the recombinant “as isolated” Nar1 proteins. The amounts of iron and sulfide were in the range of 3-3.5 Fe atoms and 2.5-3 S atoms per wild-type Nar1 protein molecule. Similar amounts (with minor variations) of Fe and S were detected in the C20A, C59S, C62A, C177S and C412S. The C416A Nar1 mutant had only 2.5 Fe atoms and 1.5 S atoms per polypeptide chain (Table 3.2). As expected from the absence of the brownish color and from the SDS-PAGE-Coomassie analysis, the C65A and C231A mutant proteins contained very

low amounts of iron and sulfur, whereas the C177S-C412S had only 1.5 Fe and 1.5 S atoms (Table 3.2).

	Iron (nmol/nmol of protein)	Sulfide (nmol/nmol of protein)
WT	3,19	3,05
C20A	3,6	2,76
C59S	3,18	2,15
C62A	3,61	2,54
C65A	(1,01)	(0,3)
C177S	2,4	2,17
C231A	(0,62)	(0,1)
C412S	2,96	2,68
C416A	2,35	1,42
C177-412S	1,47	1,51

Table 3.2 Analysis of iron and acid-labile sulfide contents for wild-type Nar1 and its cysteine variants. The standard deviation was ± 0.19 for Fe and ± 0.16 for sulfide.

Chemical *in vitro* reconstitution of Fe/S clusters in wild-type and Nar1 cysteine mutants did not lead to an increase in the amount of iron and sulfur content. The chemical reconstitution was performed under similar conditions to those used for the reconstitution of Cfd1 and Nbp35 (Netz *et al.*, 2007). Therefore the following spectroscopic analyses were carried out on the "as isolated" recombinant proteins.

The UV-VIS spectra of three of the N-terminal cysteine mutants (C20A, C59S, and C62A) were similar to the wild-type protein with a broad, unstructured "shoulder" around 420 nm, typical for $[4Fe\text{-}4S]^{2+}$ clusters (Balk *et al.*, 2004). It was observed that the UV-VIS spectrum of the C-terminal cysteine mutant C412S was similar to the wild-type Nar1 spectrum (Figure 3.34 B). The other Nar1 mutants (C177S, C416A and C177S-C412S) gave UV-VIS spectra similar to the wild-type Nar1 protein, but the shoulder visible at 420 nm was much less intense.

In summary, UV-VIS analysis of the N- and C-terminal Nar1 cysteine mutants indicated the presence of signals typical for Fe/S-containing proteins, except for the C65A and C231A samples (not shown) which did not contain mutant proteins.

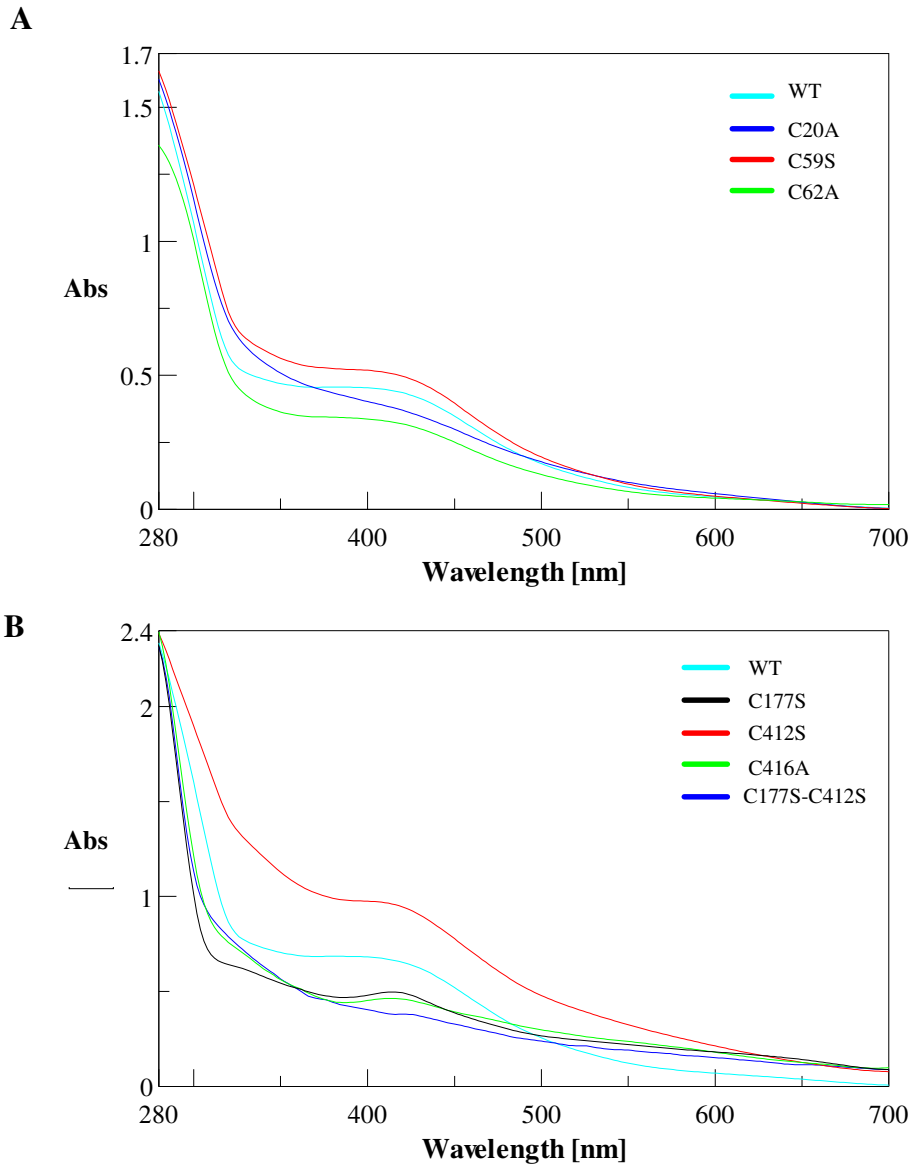

Figure 3.34 UV-VIS spectra of isolated His-tagged wild-type (WT) Nar1 and the cysteine mutant proteins in 25 mM Tris-HCl, pH 8.0, 150 mM NaCl. **A)** N-terminal cysteine mutants and **B)** C-terminal cysteine mutants as indicated.

Electron-Paramagnetic-Resonance (EPR)

Previous electron-paramagnetic-resonance studies on recombinant wild-type Nar1 protein indicated the presence of a broad rhombic EPR signal that might be assigned to two magnetically coupled Fe/S clusters (Balk *et al.*, 2005c; Balk *et al.*, 2004). To further investigate the chemical nature of the two Fe/S clusters and to possibly assign their ligands, electron-paramagnetic-resonance (EPR) spectroscopy on the recombinant wild-type Nar1 and its cysteine mutant proteins was performed. Nar1 is EPR-silent in the oxidized form and requires reduction to generate EPR signals. Oxidized, "as isolated" wild-type Nar1 and its

cysteine mutants were either chemically reduced with sodium-dithionite or photoreduced with 5´-deazaflavin and light at 4°C under anaerobic conditions. The reduced recombinant wild-type Nar1 showed a broad rhombic EPR signal with g values of g_z = 2.022, g_y = 1.923 and g_x = 1.822 which was more intense when the wild-type Nar1 was reduced by 5´-deazaflavin and light (Figure 3.35). This rhombic EPR signal is similar to the previously described Nar1 signal (Balk *et al.*, 2005c; Balk *et al.*, 2004).

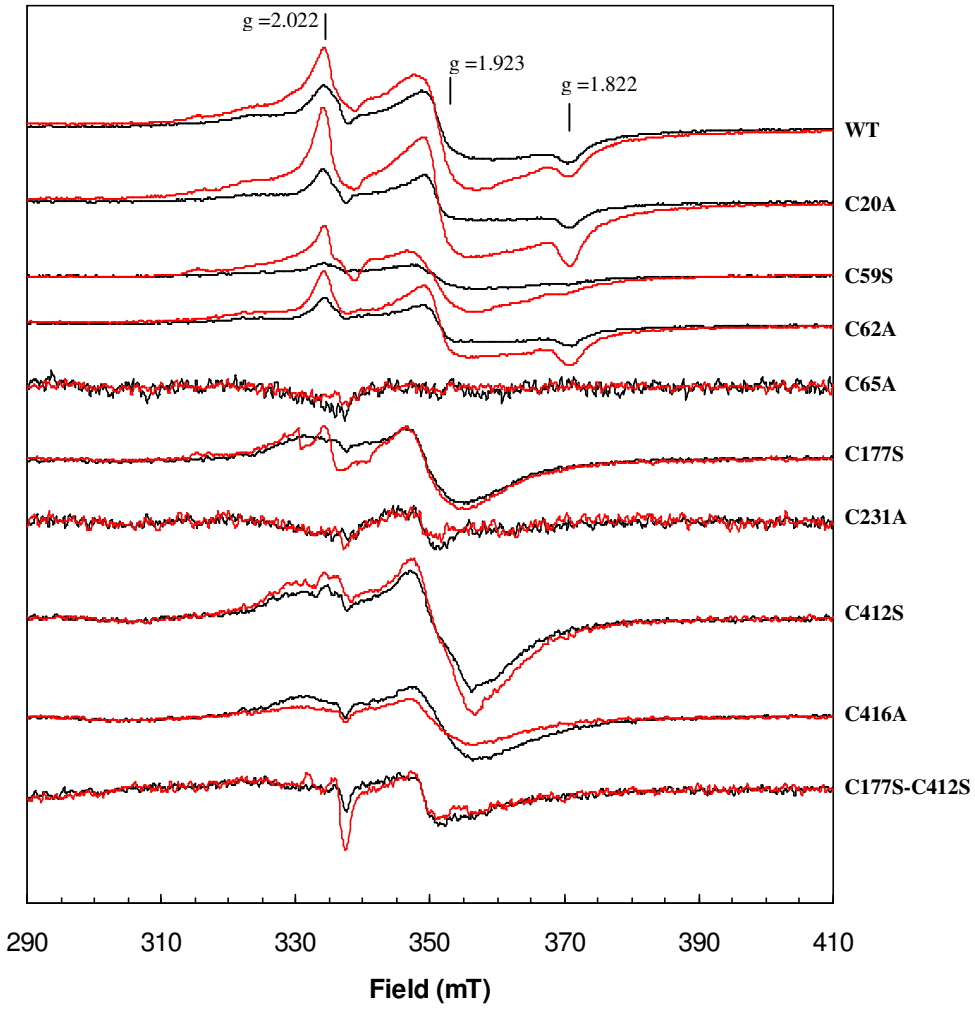

Figure 3.35 Electron paramagnetic resonance spectra of reduced wild-type and cysteine mutant Nar1 proteins. EPR spectra were recorded under the following conditions: microwave power, 0.80 mW; temperature, 10 K; microwave frequency, 9.460±0.001 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.25 mT. Black lines, proteins were reduced with 2 mM Na-dithionite in a buffer containing 25 mM Tris/Cl (pH 8.0) and 150 mM NaCl; red lines, proteins were reduced with 50 µM 5´-deazaflavin and light (4-5 min illumination by a slide projector) at 4°C. The C65A and C231A mutants served as "non-protein" controls.

The cysteine mutant proteins of the N-terminus (C20A and C62A) gave a rhombic EPR spectrum similar to that of wild-type. In case of the C59S mutant the negative

absorption-shaped feature at g_x = 1.822 was almost absent when compared to the wild-type spectrum (Figure 3.35 and 3.36 A).

The broad rhombic EPR signal disappeared when the C-terminal cysteine (C177S, C412S and C416A) mutants were analysed. These mutants showed a rather typical $[4Fe\text{-}4S]^{1+}$ signal, whereas the C177S-C412S mutant gave a very weak EPR signal. (Figure 3.35 and Figure 3.36 B). The low intensity EPR signal in the C177S-C412S mutant correlates with the low iron and sulfide values. The EPR signal observed in the C59S mutant shows similar features to those found for the C-terminal cysteine mutants (C177S, C412S and C416A).

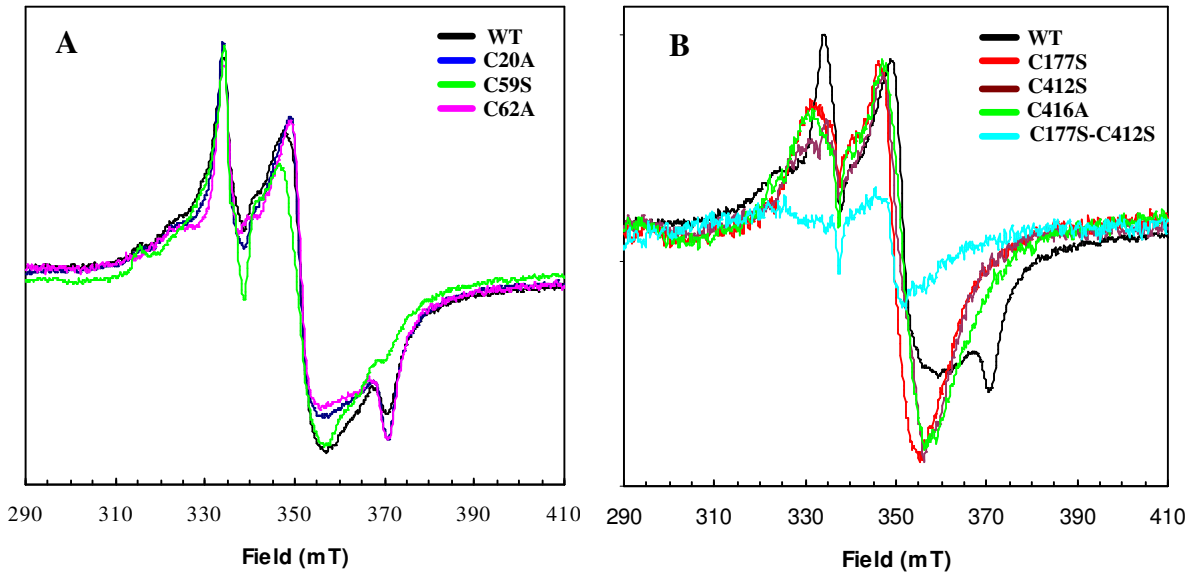

Figure 3.36 Superimposed Electron Paramagnetic Resonance spectra of wild-type and cysteine Nar1 mutant proteins. (A) EPR signals from 5´-deazaflavin/light-reduced wild-type and N-terminal cysteine mutants. **(B)** EPR signals from dithionite-reduced Nar1 C-terminal cysteine mutant proteins.

These data indicate that mutations of the N-terminal cysteine C59 and of the C-terminal cysteines C177, C412 and C416 results in changes in the rhombic EPR signal when compared to that observed for the wild-type Nar1. The other mutations have no influence (C20A and C62A) or gave weak EPR signals (C177S-C412S).

It is surprising that mutations in cysteine residues 59 and 62 did not lead to a complete loss of the EPR signal, as these mutations caused cluster loss in *vivo* (see Chapter 3.2.5). Likewise, the EPR signal for at least one Fe/S cluster was expected for the C177S-C412S mutant, based on the yeast *in vivo* data (see Figure 3.28). Apparently, there are major differences between the results obtained *in vivo* in yeast and for purified recombinant Nar1. These differences will be further addressed in Discussion.

4. Discussion

Fe/S protein biogenesis in eukaryotes is a complex process involving more than 20 components inside and outside mitochondria. In this work an additional component of the ISC-assembly system, designated Isd11 was identified and functionally characterized (see Fig. 4.1). Further I have analysed the role of Nar1, a member of the CIA-machinery in the cytosol. In this part of the work, the requirement of conserved residues for Nar1 function was addressed. In particular, the potential role of eight conserved cysteines for Fe/S cluster coordination in Nar1 was analysed.

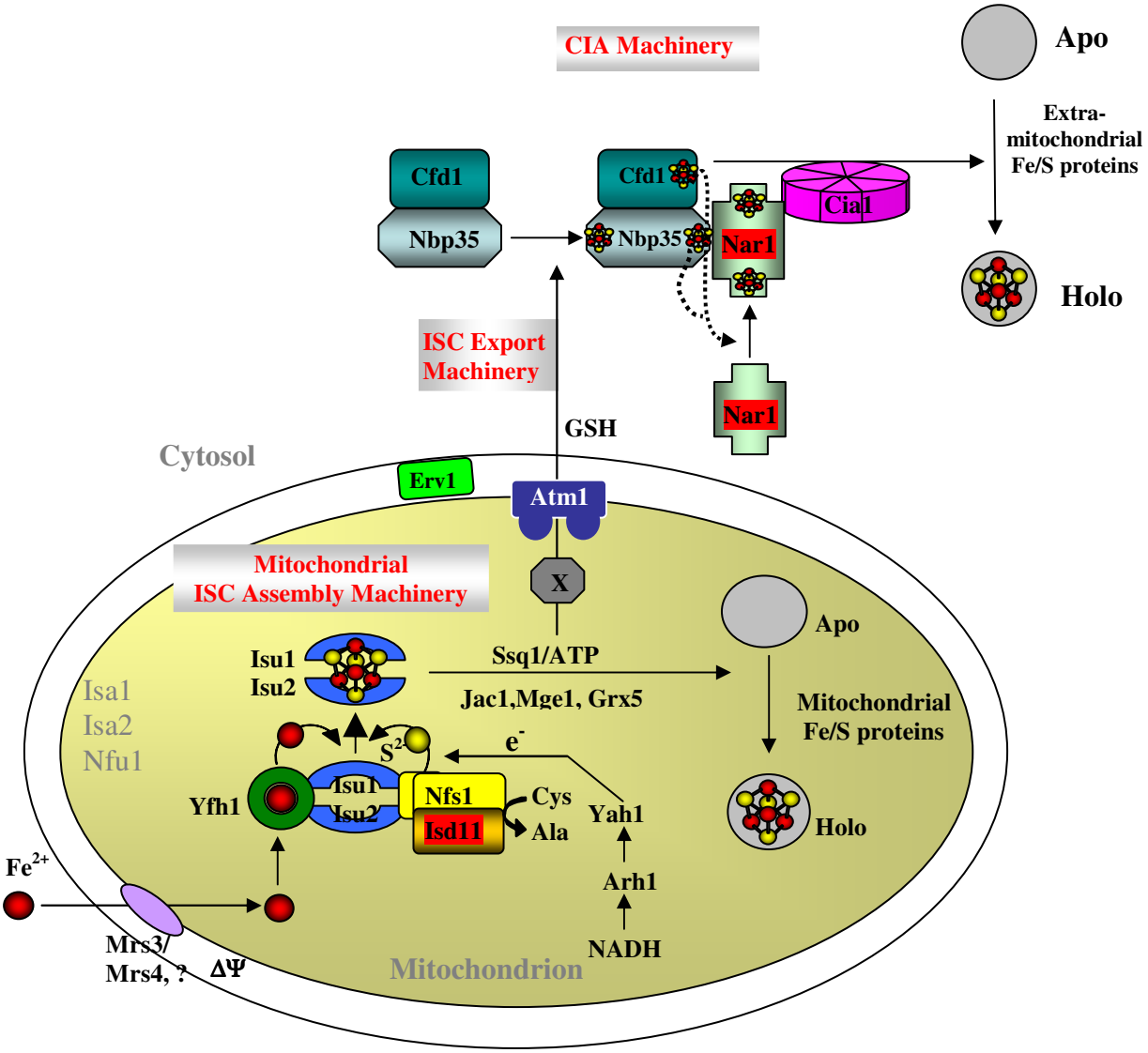

Figure 4.1 An updated model for the function of the mitochondrial ISC-assembly and export machineries and of the CIA-machinery in the maturation of cellular Fe/S proteins in *S. cerevisiae*. The novel component of the mitochondrial ISC-assembly machinery, Isd11, found in this work was shown to form a complex with the cysteine desulfurase Nfs1. The Nfs1-Isd11 complex, rather than Nfs1 alone is the physiological active

cysteine desulfurase which provides the sulfur for the synthesis of transiently bound Fe/S cluster on Isu1/2 scaffold proteins. A critical step for Fe/S cluster synthesis in the cytosol is their assembly on the scaffold proteins Cfd1-Nbp35. This step is dependent on the mitochondrial ISC-assembly and export machineries. Next, two Fe/S clusters are incorporated into Nar1 to convert it into an active component of the cytosolic CIA-machinery. Then, Nar1 interacts with Nbp35 and Cia1 and assists the transfer of Fe/S clusters from Cfd1/Nbp35 to the recipient cytosolic Fe/S apoproteins.

4.1. Isd11 plays an essential role in Fe/S protein biogenesis

4.1.1. Isd11 - a novel component of the mitochondrial ISC-assembly machinery

Isd11 is an essential protein of the mitochondrial matrix and is a well conserved protein in eukaryotes, but no prokaryotic homologs have been found (Wiedemann *et al.*, 2006). The eukaryotic homologs of the Isd11 were grouped into the LYR (or LYK) family named after the conserved LYR (or LYK) tripeptide found at the N-terminus of these proteins. Among the members of this family are the eukaryotic accessory subunits B14 and B22 of the mitochondrial complex I of the respiratory chain. A recent study showing that Isd11 is conserved in organisms containing primitive forms of mitochondria (the so-called mitosomes and hydrogenosomes) reported that Isd11 is an exclusively eukaryotic addition in evolution that originated during or after endosymbiosis that gave rise to mitochondria, mitosomes and hydrogenosomes (Richards and van der Giezen, 2006).

Biogenesis of Fe/S proteins is an essential process since many components of the mitochondrial ISC-assembly machinery are encoded by essential genes (Lill and Muhlenhoff, 2006b). As *ISD11* is essential and the encoded protein was found in mitochondria (Adam *et al.*, 2006; Huh *et al.*, 2003; Wiedemann *et al.*, 2006), it seemed likely that Isd11 is involved in either protein import or ISC assembly. Import experiments with mitochondria isolated from a temperature-sensitive mutant of Isd11 (*isd11-1*) showed no defect in the translocation of several mitochondrial proteins of various sub-mitochondrial locations. Thus, Isd11 has no detectable function in protein import into mitochondria. An interesting observation in these import experiments that pointed towards a role of Isd11 in the biogenesis of Fe/S proteins was that Isd11 is required for the formation of the holo-form of Yah1, an Fe/S protein. Using BN-PAGE it was demonstrated that formation of holo-ferredoxin was strongly diminished in *isd11-1* mitochondria, although the import of the apo-form into mitochondria was not affected. The second indication pointing to a function of Isd11 in Fe/S protein maturation was obtained from *in vivo* experiments. When the *isd11-1* mutant cells were grown at non-permissive temperature the mitochondrial protein levels of the ISC-assembly machinery

component Nfs1 were strongly diminished. Furthermore, after a heat-shock of *isd11-1* mitochondria followed by lysis and separation into pellet and supernatant fractions, the usually soluble Nfs1 was completely found in the pellet suggesting that Isd11 is involved in maintaining the Nfs1 in a soluble form.

The role of Isd11 in mitochondrial Fe/S protein biogenesis was verified by measuring the enzyme activities of several mitochondrial Fe/S proteins and the *de novo* biosynthesis of the cofactors. The activities of aconitase, succinate dehydrogenase (complex II) and cytochrome *c* reductase (complex III) were strongly decreased in *isd11-1* mitochondria. The Isd11 requirement in the *de novo* maturation of Fe/S proteins was investigated by the determination of the ^{55}Fe incorporation into mitochondrial aconitase (Aco1) and biotin synthase (Bio2). The amounts of radioactive ^{55}Fe incorporated into Aco1 and Bio2 were strongly decreased in Isd11-defective cells, indicating that Isd11 is required for the *de novo* biosynthesis of Fe/S clusters and their incorporation into mitochondrial apoproteins.

Several studies employing yeast mutants with defects in the mitochondrial ISC-assembly machinery have documented that the components of this machinery also play an essential role in the maturation of extra-mitochondrial Fe/S proteins (Lill *et al.*, 2006a; Lill and Muhlenhoff, 2005). Therefore it was interesting to know whether Isd11 is also required for the maturation of cytosolic and nuclear Fe/S proteins. Using the same *isd11-1* temperature-sensitive mutant cells or the Isd11-depleted Gal-ISD11 cells it was shown that the activity of the cytosolic Fe/S enzyme isopropylmalate-isomerase (Leu1) was strongly impaired. The cells defective in Isd11 also displayed a strong defect in the incorporation of radioactive ^{55}Fe into the cytosolic Fe/S proteins Leu1 and Rli1 and into the nuclear DNA repair enzyme Ntg2 (Wiedemann *et al.*, 2006). Thus, the experimental data presented in this study clearly demonstrated that Isd11 is required for the maturation of mitochondrial, cytosolic and nuclear Fe/S proteins, identifying Isd11 as a novel component of the mitochondrial ISC-assembly machinery. The essential character of Isd11 in yeast may be well explained by its role in the maturation of the extra-mitochondrial Fe/S protein Rli1 which performs an indispensable function in the biogenesis of ribosomes (Kispal *et al.*, 2005; Yarunin *et al.*, 2005).

As discussed above, Isd11 is present only in eukaryotes. This is an unusual characteristics for an ISC-component, since all members of the mitochondrial ISC-assembly machinery are either derived from the bacterial *isc* operon (Nfs1, Isu1/2, Yah1, Nfu1, Ssq1, Jac1, Isa1/2) or possess close homologs in bacteria (Arh1, Yah1, Grx5) (Johnson *et al.*, 2005; Lill and Muhlenhoff, 2006b; Zheng *et al.*, 1998). However, functional counterparts of Isd11

with no detectable similarity at the protein sequence level are not excluded to be present in bacteria.

4.1.2. S. cerevisiae cells defective in Isd11 show a deregulation of cellular iron homeostasis

In *S. cerevisiae* the cellular iron uptake is regulated via the two transcription factors Aft1 and Aft2. Under iron-replete conditions Aft1 is localised in the cytosol, whereas under iron-deplete conditions Aft1 translocates into the nucleus and activates the transcription of genes involved in iron uptake (Yamaguchi-Iwai *et al.*, 2002). Yeast mutants with defects in mitochondrial ISC-assembly and export machineries display a misregulated cellular iron homeostasis that is manifested by the Aft1-dependent activation of Fe-regulon genes involved in cellular iron uptake. In all these mutants there is also an accumulation of iron within mitochondria (Kispal *et al.*, 1997; Kispal *et al.*, 1999; Muhlenhoff *et al.*, 2002; Sipos *et al.*, 2002). The Aft1 translocation from cytosol into the nucleus to activate the transcription of genes involved in iron uptake does not depend on the CIA-machinery (Rutherford *et al.*, 2005).

The *isd11* mutant cells showed increased cellular iron uptake and the induction of the Aft1-dependent *FET3* gene, encoding a copper-dependent ferroxidase under iron-replete conditions indicating that these cells display a defect in cellular iron homeostasis. The content of non-heme and non-Fe/S iron in mitochondria isolated from *isd11-1* mutant cells was 3-fold higher compared to wild-type mitochondria. Thus, depletion of Isd11 shows specific effects that are similar to those observed for other mutants in the ISC-assembly and export machineries indicating that the cells defective in Isd11 are impaired in the regulation of cellular iron homeostasis.

4.1.3. Isd11 is required for the sulfur transfer to Isu1 scaffold protein

In vivo experiments performed to obtain better insights into the molecular role of Isd11 in *S. cerevisiae* showed that in cells defective in Isd11 the protein levels of Nfs1 were strongly diminished (Adam *et al.*, 2006; Wiedemann *et al.*, 2006). Moreover, Nfs1 was found to aggregate after a heat-shock of mitochondria isolated from *isd11* mutant cells. These data indicated that Isd11 might play a role in the stabilisation of Nfs1, by preventing its aggregation.

The function of Isd11 in maintaining the stability of Nfs1 suggested that these two proteins may interact. The physical interaction between Isd11 and Nfs1 was documented in

the present study using different methods (BN-PAGE, affinity precipitation or coimmunoprecipitation). Isd11 forms a stable complex of about 200 kDa with the cysteine desulfurase Nfs1. The Isd11-Nfs1 complex (designated ISD complex) contains both proteins in similar amounts (Wiedemann *et al.*, 2006). Previous data provided evidence for an interaction of Nfs1 with Isu1 (Gerber *et al.*, 2003) and for the requirement of Nfs1 for the *de novo* Fe/S cluster formation on Isu1 (Mühlenhoff *et al.*, 2003). From these studies it was concluded that Nfs1 provides the S for the transient Fe/S cluster synthesis on Isu1 scaffold. Likewise, the bacterial Nfs1 homologs NifS/IscS form stable complexes with the scaffold proteins NifU/IscU to allow the transfer of the sulfur atom derived from L-cysteine (Smith *et al.*, 2001; Urbina *et al.*, 2001; Yuvaniyama *et al.*, 2000). Here it was shown that Nfs1 interacts with Isd11, but also Isu1 was found in the purification eluate. Isd11 and Nfs1 were found to be present in similar amounts, whereas Isu1 was present in 10-fold lower amounts. Thus, Isd11 interacts with both, Nfs1 and with Isu1. Isd11 and Isu1 may be bound to Nfs1 independently, but the formation of a trimeric complex is not excluded. All these data may indicate a dynamic assembly and disassembly of Nfs1, Isd11 and Isu1 during the sulfur release from cysteine and its transfer to Isu1/2 scaffold proteins.

Nfs1 catalyses the conversion of cysteine to alanine to provide the sulfur for the synthesis of Fe/S clusters on Isu proteins (Mühlenhoff *et al.*, 2004). The yeast Nfs1 and its bacterial counterparts NifS, IscS and SufS facilitate the elimination of sulfane sulfur (S^0) from L-cysteine via the formation of a protein-bound cysteine persulfide intermediate (Kessler, 2006; Mueller, 2006; Mühlenhoff *et al.*, 2004; Zheng *et al.*, 1993). The Isd11-Nfs1 complex could also be labelled with radioactive [^{35}S]-cysteine indicating that it carries the radioactive sulfur released from cysteine. This [^{35}S] sulfur was likely bound as a persulfide intermediate, as indicated by the disappearance of the radioactivity in the Isd11-Nfs1 complex upon incubation with DTT which reacts with persulfides. All these data strongly suggested that the Isd11-Nfs1 complex rather than Nfs1 alone functions as the mitochondrial cysteine desulfurase *in vivo*. On the other hand, Nfs1 is functional as a cysteine desulfurase in the absence of Isd11. Surprisingly, the Nfs1 desulfurase activity was even moderately increased in *isd11* mutant cells (Adam *et al.*, 2006; Wiedemann *et al.*, 2006) indicating that Isd11 is not required for the cysteine desulfurase activity of Nfs1 *in vitro*. How can these results be reconciled? The typical biochemical test for Nfs1 function is the realease of sulfide from cysteine (Mühlenhoff *et al.*, 2004). This likely does not reflect the physiological reaction of Nfs1 which, as detailed above, involves the transient formation of an enzyme-bound persulfide (and not of free sulfide). Hence, the *in vitro* sulfide release reaction might not be

the appropriate physiological test for Nfs1 function. Rather, the activity of Nfs1 and of the Isd11-Nfs1 complex in generating an Fe/S cluster on Isu1 seems the relevant assay system. This assay clearly shows that Isd11, together with Nfs1, is essential for the sulfur transfer to Isu1 for the synthesis of the transient Fe/S cluster and thus represents the physiological cysteine desulfurase activity *in vivo*.

A previous work (Mühlenhoff *et al.*, 2003) employing regulatable yeast mutants demonstrated that Nfs1, Yah1 and Yfh1 are required for Fe/S cluster synthesis on Isu1 scaffold protein. Depletion of Nfs1, Yah1 or Yfh1 led to dramatic decrease in the incorporation of ^{55}Fe into Isu1. On the other hand, Ssq1, Jac1 or Grx5 are not needed for the formation of transiently-bound Fe/S cluster on Isu1, but are required for the incorporation of Fe/S clusters into recipient apoproteins. Depletion of Ssq1, Jac1 or Grx5 showed up to 5-fold increases in ^{55}Fe incorporation into Isu1, whereas the ^{55}Fe incorporation into Bio2 or Yah1 was strongly diminished (Mühlenhoff *et al.*, 2003). Thus, it is possible to discriminate *in vivo* whether an ISC component is involved in either the *de novo* Fe/S cluster synthesis on the Isu1 scaffold protein or in later steps after the formation of Fe/S clusters on Isu1. These staging experiments were applied to ask at which step of mitochondrial Fe/S protein biogenesis Isd11 is necessary. It was demonstrated that in *isd11* mutant cells (*isd11-1* or Isd11 depleted Gal-ISD11) there was strong reduction of Fe/S cluster formation on Isu1. Thus, Isd11 behaves like Nfs1, Yah1 or Yfh1, and consequently is required for Fe/S cluster synthesis on the Isu scaffold proteins.

Although, in the absence of Isd11, Nfs1 is still active *in vitro* as a cysteine desulfurase, this activity does not seem to represent the physiological sulfur donor *in vivo*, since Isd11 is essential for the formation of the transient Fe/S cluster on the Isu1 scaffold proteins. The results obtained here and in previous studies can be summarised in the model depicted in Fig. 4.2 addressing the mechanism of the Isd11-Nfs1 complex function and the formation of the persulfide intermediate on Nfs1.

The bacterial Nfs1 homologs, NifS and IscS, perform their function in sulfur formation without the help of other proteins. However, the function of Isd11-Nfs1 complex on the first glimpse seems to be similar to another Nfs1 homolog, the bacterial SufS which forms a complex with SufE. Intensive investigations in *E. coli* and *Erwinia chrysanthemi* have demonstrated the involvement of the SUF system in Fe/S cluster biogenesis (Fontecave *et al.*, 2005). The SUF system consists of six proteins termed SufA, SufB, SufC, SufD, SufS and SufE. The SUF system fulfills similar functions as the bacterial ISC system, but is used mainly under stress conditions such as iron limitation or oxidative stress. SufS and SufE form

a stable complex *in vivo* (Loiseau *et al.*, 2003). SufS has a weak cysteine desulfurase activity which is enhanced by SufE. Experimental data from *E. coli* suggested that the release of sulfur from cysteine occurs first via the formation of a persulfide on Cys364 of SufS followed by the sulfur transfer from the SufS persulfide to Cys51 of SufE and the formation of a persulfide on SufE (Loiseau *et al.*, 2003; Ollagnier-de-Choudens *et al.*, 2003; Outten *et al.*, 2003). The mechanism of Isd11 function however, seems to be different from that of SufE because a persulfide cannot be formed on Isd11 due to the lack of conserved cysteine residues.

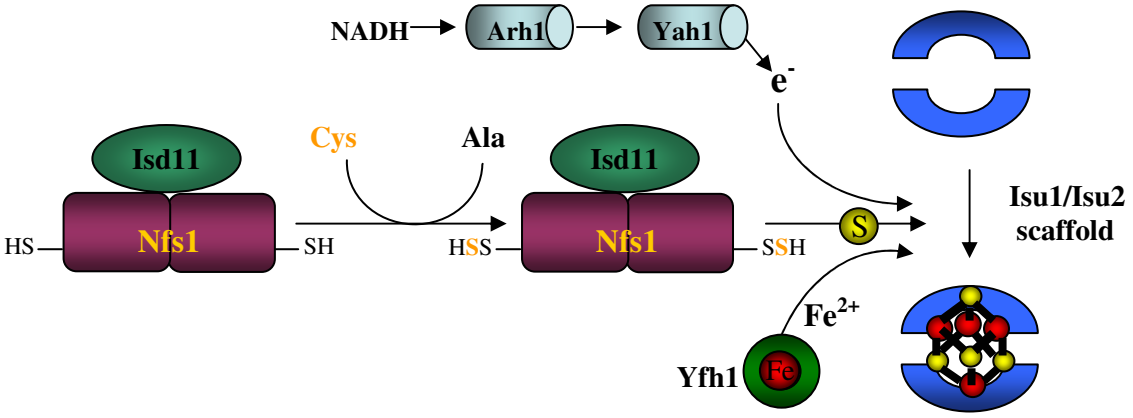

Figure 4.2 Mechanism of sulfur generation via a persulfide intermediate by the Isd11-Nfs1 complex and Fe/S cluster formation on Isu1/2 scaffold proteins. The Isd11-Nfs1 complex catalyses the conversion of L-cysteine to L-alanine and the release of elemental sulfur (S^0) via the formation of a Nfs1-bound persulfide. Then the persulfide is transferred to the Isu1/2 proteins. This reaction possibly involves a direct interaction between Nfs1 and the Isu proteins (see Fig. 3.14). For the synthesis of Fe/S clusters on scaffold Isu1 proteins Fe^{2+} is required which is probably delivered by the yeast frataxin homologue Yfh1. Additionally, the sulfur atom (S^0) has to be reduced. The electrons needed are most likely provided by an electron transfer chain formed by Arh1 and Yah1.

In all organisms the transfer RNA (tRNA) contains modified nucleosides including thionucleosides containing a sulfur atom. Several studies provided evidence that the bacterial cysteine desulfurase IscS is essential not only for Fe/S clusters biogenesis, but also for delivering the sulfur to thionucleoside biosynthesis (Lauhon, 2002; Lauhon and Kambampati, 2000). Studies in *S. cerevisiae* have shown that the yeast cysteine desulfurase Nfs1 plays an essential role to liberate the sulfur needed for thio-modification of both mitochondrial and cytosolic tRNAs (Mühlenhoff *et al.*, 2004; Nakai *et al.*, 2004). In bacteria, thio-modification of all tRNAs requires IscS. This pathway involves proteins that can be grouped into two classes. One pathway is Fe/S protein-dependent and, for example involves the [4Fe-4S]

protein MiaB as an essential component (Pierrel *et al.*, 2002). An Fe/S protein-independent pathway involves proteins such as ThiI which is needed for thiolation of tRNAs in *E. coli* using the sulfur delivered by IscS (Kambampati and Lauhon, 2000; Mueller *et al.*, 2001). The situation might be similar in *S. cerevisiae*, where a recent study (Nakai *et al.*, 2007) has shown that members of the mitochondrial ISC-assembly machinery (Isu1/2) or of the cytosolic CIA-machinery (Nbp35, Cfd1, Nar1 and Cia1) are required for the thio-modification of cytosolic tRNAs, but not for the mitochondrial tRNAs (Nakai *et al.*, 2007), suggesting that the thiolation of cytosolic tRNAs depends on a yet to be identified cytosolic/nuclear Fe/S protein.

Several studies have shown that the eukaryotic Nfs1 has multiple cellular localizations. In eukaryotes Nfs1 is predominantly localised in mitochondria (Kispal *et al.*, 1999; Land *et al.*, 1998; Li *et al.*, 1999). A fraction of Nfs1 is also localised in the nucleus. In both, *S. cerevisiae* and human cells this nuclear Nfs1 is essential for cell viability (Biederbick *et al.*, 2006; Nakai *et al.*, 2001) and it was therefore suspected to be involved in extra-mitochondrial Fe/S protein biogenesis and/or tRNA thiomodification. However, recent findings have excluded a role of the nuclear localised Nfs1 in either Fe/S protein biogenesis or thio-modification of tRNAs (Mühlenhoff *et al.*, 2004; Nakai *et al.*, 2007). Thus, there seems to be yet another essential function of Nfs1 in eukaryotes which remains to be elucidated. In this context, it will be interesting to study the existence of extra-mitochondrial Isd11, its complex formation with nuclear Nfs1 and its function in extra-mitochondrial Fe/S protein maturation and tRNA thiomodification.

4.2. Characterization of Fe/S cluster binding to Nar1

4.2.1. N- and C-terminal conserved cysteine residues play an essential role for Nar1 function

Biogenesis of cytosolic and nuclear Fe/S proteins in *S. cerevisiae* requires the CIA machinery which is comprised of at least four proteins: Cfd1, Nbp35, Nar1 and Cia1 (Lill *et al.*, 2006a). The present study was focused on the CIA component Nar1 and identified several amino acids that are essential for the function of Nar1 in the maturation of extra-mitochondrial Fe/S proteins and for the coordination of its Fe/S clusters.

Nar1 from *S. cerevisiae* is a highly conserved protein in all eukaryotes and exhibits sequence similarity to bacterial and algal Fe-only hydrogenases (Horner *et al.*, 2002). The cysteine residues responsible for the coordination of the proximal (H-cluster) and medial [4Fe-4S] cluster in Fe-only hydrogenases are conserved in the yeast Nar1 protein (Nicolet *et al.*, 2002). Here, it has been demonstrated that the eight conserved cysteine residues of Nar1 are important for the function of Nar1, and that they are involved in the coordination of two Fe/S clusters. Four of the cysteine residues are located at the N-terminus and resemble a ferredoxin-like domain ($CX_{38}CX_2CX_2C$), whereas the other four cysteine residues are distributed in the middle and C-terminal parts of Nar1.

Previous studies showed that Nar1 is an Fe/S protein (Balk *et al.*, 2005c; Balk *et al.*, 2004). The four conserved N-terminal cysteines might coordinate a [4Fe-4S] cluster like the corresponding cysteines in Fe-only hydrogenases. The other four cysteine residues at the C-terminal part of Nar1 may be involved in the coordination of the second Fe/S cluster of, so far unknown nature. The cysteine residues in Fe-only hydrogenases that correspond to the Nar1 C-terminal cysteines coordinate the H-cluster. The H-cluster is an unusual type of Fe/S cluster which consists of a [4Fe-4S] cluster linked by a cysteine residue to a [2Fe-2S] center (Nicolet *et al.*, 2002; Nicolet *et al.*, 1999). The unusual feature is the presence of non-protein ligands (CN^- and CO) at each Fe atom of the [2Fe-2S] center. The sulfur atoms in the [2Fe-2S] center are connected by a di-thiolate compound, probably a di-(thiomethyl)-amine (Nicolet *et al.*, 2002). It is unlikely that the C-terminal Nar1 cysteines are the coordinating ligands for an H-cluster because specialized proteins are needed for the maturation of H-clusters in bacteria. These proteins are not conserved in eukaryotes (Böck *et al.*, 2006; King *et al.*, 2006; Posewitz *et al.*, 2004).

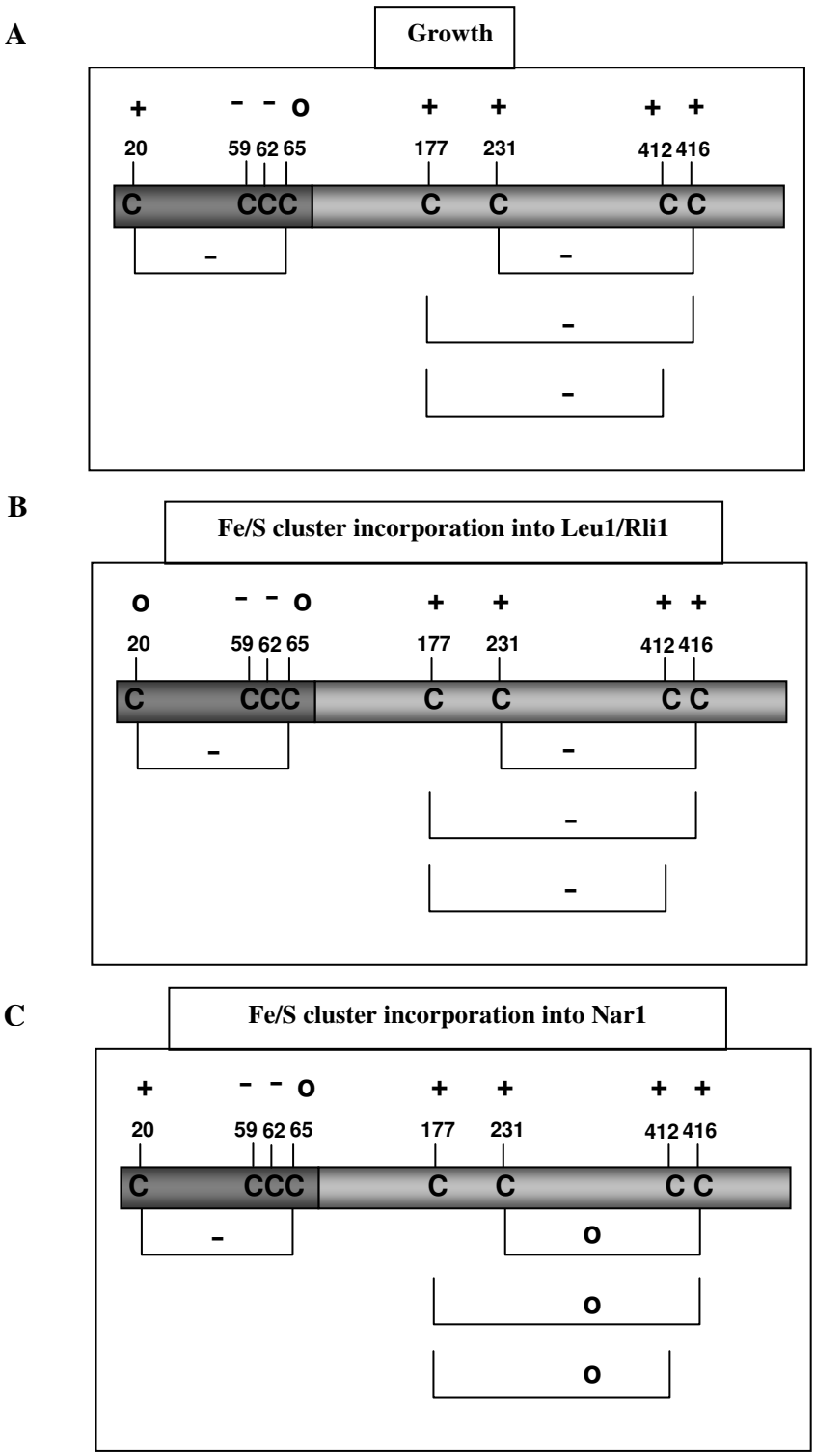

Figure 4.3 Schematic comparison of the *in vivo* data obtained with the Nar1 mutants. Nar1 N- and C-terminal conserved cysteine residues are depicted and their positions are indicated. Effects of **A)** functional complementation by mutant Nar1 proteins of the growth defect of Nar1-depleted Gal-NAR1 cells, **B)** incorporation of ^{55}Fe into Leu1 and Rli1 Fe/S proteins and **C)** Fe/S cluster binding of various Nar1 cysteine mutants. +, similar behaviour to wild-type; o, intermediate effects; -, no growth, function or Fe/S cluster binding, respectively.

Here, it was analysed whether the conserved cysteine residues are essential for yeast cell viability (Figure 4.3 A). When the N-terminal cysteines (C59 and C62) were exchanged to alanine residues by site-directed mutagenesis these Nar1 mutants were not able to rescue the growth defect of Gal-NAR1 cells depleted for the nuclear-encoded Nar1. The C65A mutant only partially replaced the functional wild-type Nar1, whereas the C20A mutant fully rescued the growth phenotype. However, when the C20A and C65A were simultaneously exchanged (C20A-C65A) the partial complementation observed for the single C65A mutant was lost, indicating that also the C20 is important for cell viability due to this cumulative effect. Surprisingly, the single C-terminal cysteine mutants showed no growth defect, but they are important for cell viability because several double mutants were not able to restore the wild-type growth (Figure 4.3 A). Thus, also the C-terminal cysteines are important for Nar1 function.

All eight conserved N- and C-terminal cysteine residues in Nar1 perform a crucial role for the function of Nar1 in the *de novo* maturation of cytosolic Fe/S proteins (Figure 4.3 B). *In vivo* experiments demonstrated that three N-terminal cysteines (C59, C62 and C65) are essential for Nar1 function in the maturation of Leu1 and Rli1. The fourth conserved cysteine at the N-terminus of Nar1 (C20) is also an important residue due to its cumulative effect observed after its mutation in a C65A background. ^{55}Fe incorporation studies indicated that all conserved cysteine residues at the C-terminus of Nar1 are important for Nar1 function. Single mutations of these cysteines showed no defects in the maturation of cytosolic Fe/S proteins, as Leu1 and Rli1 were able to incorporate their Fe/S clusters as in the wild-type Nar1 strain. However, the simultaneous mutation of two of these C-terminal cysteine residues resulted in a strong impairment in the maturation of Leu1 and Rli1. These synergistic features of the C-terminal Nar1 cysteines suggest an important role of these residues for Nar1 function. The important role of all eight conserved cysteine residues was further confirmed by the measurements of the enzyme activities of cytosolic Fe/S protein Leu1. The Leu1 activity found in various N- and C-terminal cysteine mutants showed similar profile to the results obtained by the *in vivo* radiolabelling assay and correlated well with the functional complementation data.

4.2.2. Conserved cysteines at the N- and C-terminus of Nar1 are involved in the coordination of two Fe/S clusters

This study made it likely that the eight N- and C-terminal conserved cysteine residues are the coordinating ligands for the two Fe/S clusters. *In vivo* studies employing ^{55}Fe

radiolabelling have shown that at least three conserved cysteines in the N-terminal part of Nar1 (C59, C62 and C65) are required to bind an Fe/S cluster. Site-directed mutagenesis of these residues resulted in a severe loss of Fe/S cluster association with Nar1. The C20A mutation had no significant effect on Fe/S cluster binding. This observation might indicate that C20 is either not an essential ligand for Fe/S cluster binding, or that a ligand exchange might take place and another residue that is in close proximity may substitute the coordinating function. Nevertheless, the C20 may be directly involved in the Fe/S cluster coordination, as the cluster binding to the C20A-C65A mutant was diminished in comparison to that of the single C65A mutant (Figure 3.27 and Fig. 4.3 C).

The *de novo* incorporation of Fe/S clusters into the Nar1 C-terminal single cysteine mutants was comparable to the cluster incorporation into wild-type Nar1. Nevertheless, these four C-terminal cysteines likely are the coordinating ligands for an Fe/S cluster, because the double cysteine mutants were able to incorporate only the N-terminal Fe/S cluster. Structural modelling of Nar1 (Fig. 3.18 and Fig. 3.19) showed similarities between the calculated Nar1 structure and the experimentally determined structure of Fe-only hydrogenases. Except for the cysteine residue C62 which has a different predicted orientation compared to the corresponding cysteine in Fe-only hydrogenases, all other conserved N- and C-terminal cysteine residues show orientations that could favour the binding of the two Fe/S clusters. The reversed orientation of the C62 residue might well be a calculation artifact.

The single mutations in the N-terminal cysteine residues (C59, C62 and C65) led to severe defects manifested by impaired growth or cell death, loss of Nar1 function or Fe/S cluster binding. In contrast, single C-terminal cysteine mutations had no effects on Nar1 function or in coordination of its Fe/S clusters. These observations may indicate that the Fe/S cluster bound at the N-terminal part of Nar1 is a more labile entity compared to the Fe/S cluster coordinated by the C-terminal cysteines. The structural model of Nar1 suggests that the N-terminal Fe/S cluster is exposed at the protein surface, whereas the second Fe/S cluster is buried in the Nar1 protein. This different spatial orientation of the two Fe/S clusters in Nar1 might explain the distinct phenotypes observed for the N-and C-terminal cysteine mutants. The N-terminal cluster is lost easily, whereas the C-terminal one requires mutation of two coordinating ligands for cluster loss.

Several Nar1 mutant proteins carrying N-and C-terminal cysteine exchanges could not rescue the growth defect of Nar1-depleted Gal-NAR1 cells. Because these cysteine residues are the coordinating ligands for the two Fe/S clusters, it is evident that both Fe/S clusters are required for Nar1 function in cytosolic/nuclear Fe/S protein biosynthesis. At the molecular

level, the function of Nar1 is still unknown. Based on the similarity to the iron-only hydrogenases it can be speculated that Nar1 may perform an electron transfer function. Nar1 may supply electrons to the cytosolic scaffold proteins Cfd1-Nbp35 for Fe/S cluster dissociation. Also the bacterial and algal Fe-only hydrogenases catalyse the electron-dependent reduction of protons for hydrogen production in bacteria (*Clostridium pasteurianum* and, *Desulfovibrio vulgaris*), hydrogenosomes of trichomonads and cythrid fungi and in chloroplasts of green algae (Chlorella, Chlamydomonas) (Horner *et al.*, 2002). The identification of the exact molecular function of yeast Nar1 may require *in vitro* studies with purified proteins of the cytosolic CIA machinery.

The human genome encodes two components of the Nar1-like family, NARF and IOP1 that also show sequence similarities to Fe-only hydrogenases. NARF is a nuclear protein that was reported to bind to prenylated lamin A in the nucleus (Barton and Worman, 1999). A recent work (Huang *et al.*, 2007) showed that the second mammalian Nar1-like member, IOP1 may regulate the expression of HIF1α (hypoxia inducible factor) that represents the global mediator of the mammalian response to hypoxia. Both IOP1 and NARF do not possess hydrogenase activities and IOP1 is not involved in the iron incorporation into the active site of PHD2 (Proline hydroxylase domain-containing protein) (Huang *et al.*, 2007). At present, it is not known whether these human Nar1-like members are Fe/S containing proteins and whether they support cytosolic Fe/S protein biogenesis, but this seems likely. Thus, during evolution, the Nar1-like members have developed from their ancestors (Fe-only hydrogenases) to factors involved in different and independent pathways, including the *S. cerevisiae* Nar1 which is an Fe/S protein supporting cytosolic Fe/S protein assembly.

In previous studies (Balk *et al.*, 2005c; Balk *et al.*, 2004), spectroscopic analyses suggested that Nar1 binds two magnetically interacting Fe/S clusters. This phenomenon can be observed when two Fe/S clusters are in close proximity. In Fe-only hydrogenases and bacterial ferredoxins two [4Fe-4S] clusters are close to each other and have been shown to give rise to broad, magnetically coupled EPR signals (Mathews *et al.*, 1974). Purification of recombinant Nar1 from *E. coli* and analytical gel filtration analysis indicated that Nar1 is in a monomeric form. Spectroscopic analysis of wild-type Nar1 demostrated that Nar1 is an Fe/S protein yielding an unusual rhombic EPR signal (Balk *et al.*, 2004) that was different from that observed for the H-cluster (Adams, 1987) or for typical Fe/S clusters (Hagen *et al.*, 1982; Thamer *et al.*, 2003). EPR analysis of the C-terminal cysteine mutant C177S-C412S indicated the absence of this Fe/S moiety (Fig. 4.4 A). Also the chemical analysis of Fe and S content showed that this mutant protein is predominantly in the apoform (Fig. 4.4 B). The SDS-gel

analysis indicated a high proteolytic sensitivity of this mutant possible due to the loss of the Fe/S cluster (see Fig. 3.29 A). Likewise, the C65A mutation resulted in protein instability and degradation precluding further analysis. The recombinant C20A mutant gave a similar EPR spectrum to that found for the wild-type Nar1. In yeast cells, the C20A mutation had no impact on Fe/S cluster association or Nar1 function (see above). Analysis of the C59S, C177S, C412S and C416A mutants indicated the disappearance of the rhombic EPR signal. These mutant proteins gave a signal typical for $[4Fe\text{-}4S]^{1+}$ clusters. The N-terminal cysteine mutant C62A gave an EPR signal that was indistinguishable from the rhombic EPR features of wild-type Nar1 (Fig. 4.4 A). Analysis of the Fe and S content showed that this mutant contains similar amounts of Fe and S atoms as the wild-type Nar1 protein (Fig. 4.4 B) suggesting that C62 residue is not involved in coordination of Fe/S clusters in recombinant Nar1.

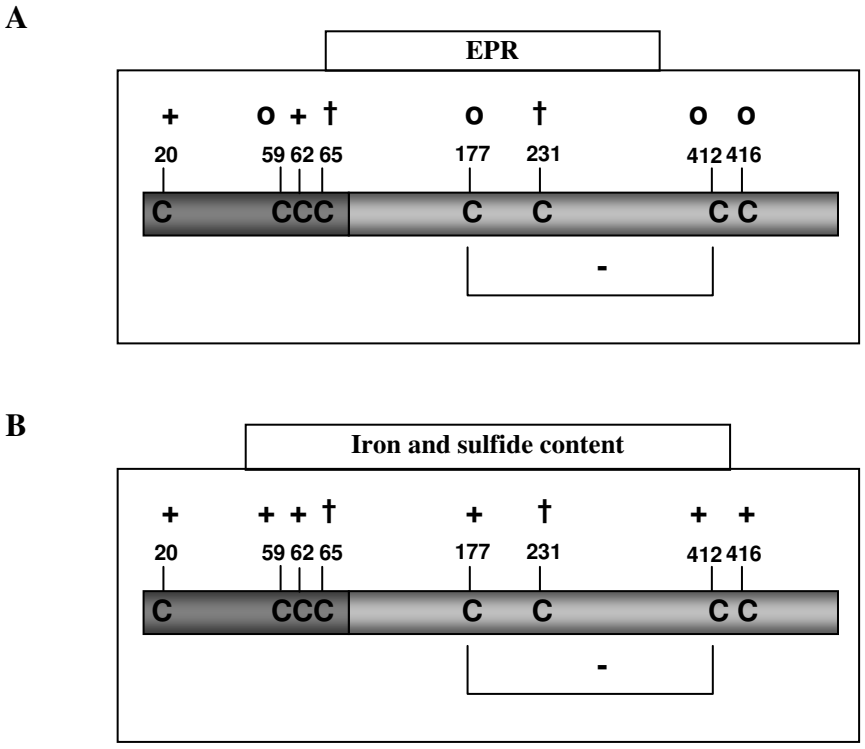

Figure 4.4 Schematic comparison of the *in vitro* data obtained with the Nar1 mutants purified from *E. coli*. Nar1 N- and C-terminal conserved cysteine residues are depicted and their positions are indicated **A)** EPR analysis of mutant NAR1 proteins **B)** analysis of iron and sulfide content. +, similar behaviour to wild-type; o, indicates spectral changes when compared to wild-type recombinant Nar1; -, no EPR signals or low iron and sulfide content, †, no stable protein.

These *in vitro* results are in striking contradiction with the *in vivo* data which indicated that mutations of the C59 and C62 residues led to severe effects on Fe/S cluster association and Nar1 function in the maturation of extra-mitochondrial Fe/S proteins (see Figure 3.27 and

Figure 3.36 A). Further, the single mutations of the C-terminal cysteines C177S, C412S and C416A had no effect on Fe/S cluster binding *in vivo*, whereas *in vitro* the EPR spectra showed characteristic changes (see Figure 3.28 and Figure 3.36 B). These differences clearly show that *E. coli* apparently does not produce a physiologically relevant wild-type Nar1 protein, and hence contains an artificial Fe/S moiety. Other results indicating that the recombinant Nar1 protein is not a functional relevant protein are represented by the determination of the Fe and S content. Chemical analysis indicated the presence of 4 Fe and 4 S atoms per protein and not of, e.g., 8 Fe and 8 S atoms as it would be expected in the situation that Nar1 binds two [4Fe-4S] clusters.

The incomplete association of the Fe/S clusters on Nar1 after expression and purification from *E. coli* is not an unusual observation. Several earlier studies indicated that many recombinant Fe/S proteins do not carry the correct physiologically relevant complement of Fe/S clusters after purification from *E. coli.* These proteins either needed *in vitro* reconstitution to reach the complete association of Fe/S clusters or had to be produced in their native organisms. Examples include *S. cerevisiae*, Nbp35 which after purification from *E. coli* contains only 1 Fe and 1 S atoms per monomer, yet after chemical *in vitro* reconstitution binds up to 8 Fe and 8 S atoms (corresponding two [4Fe-4S] clusters). Another example in yeast is Cfd1 which only after reconstitution can associate a [4Fe-4S] cluster (Netz *et al.*, 2007). Aerobically isolated bacterial biotin synthase (*BioB*) and lipoate synthase (*LipA*) monomers were previously shown to contain a [2Fe-2S] cluster. However, after incubation with dithionite under anaerobic conditions one [4Fe-4S] cluster per dimer was obtained (Ollagnier-De Choudens *et al.*, 2000). This study was not successful in applying *in vitro* chemical reconstitution of Fe/S clusters to improve the Fe and S content of the recombinant Nar1 protein. The apparent incorrect assembly of Nar1 upon synthesis and purification from *E. coli* may require expression in eukaryotic organisms such as *Pichia pastoris* or *Neurospora crassa*. Also purification under anaerobic conditions may be useful. It is clear that Nar1 containing a full complement of two Fe/S clusters will be required for further functional studies.

In summary, the thorough investigation of the recombinant Nar1 protein and its cysteine mutants indicated that this protein contains an artificial Fe/S species that is coordinated differently from that demonstrated *in vivo* in yeast cells. The Fe/S species of recombinant Nar1 seems to be coordinated by the cysteines C59, C177, C412 and C416, since mutation of these residues had a strong impact on the spectroscopic signals. The coordination of the artificial Fe/S cluster on recombinant Nar1 does not appear to be functionally relevant.

This conclusion could previously not be derived from the spectroscopic and biochemical analysis of the wild-type protein. Only the detailed analysis of a number of Nar1 mutants allowed me to convincingly dissect the striking differences between the properties of Nar1 *in vivo* and *in vitro*.

4.2.3. Residues R144, Q356 and W491 are not essential for Nar1 function

Yeast Nar1 contains at the C-terminus a tryptophane residue (W491) which is conserved in all eukaryotic Nar1 homologs, but not in Fe-only hydrogenases. Another residue which is conserved only in the Nar1-like proteins is Q356. This residue is at a position that in Fe-only hydrogenases forms specific hydrophobic patches. In Fe-only hydrogenases these hydrophobic patches form a continous channel that runs from the molecular surface to the buried active site. The channel may be used for either the uptake or the production of molecular hydrogen. Fe-only hydrogenases contain also important amino acids that surround the enzyme active site (H- cluster) (Nicolet *et al.*, 2002). Residue R144 of yeast Nar1 corresponds to such an amino acid. These three residues were exchanged by site-directed mutagenesis (R144A and Q356E) or deleted (ΔW491) to analyse their role for Nar1 function.

In the present study it was shown that these Nar1 residues (R144, Q356 and W491) are not important for Nar1 function in the maturation of extra-mitochondrial Fe/S proteins. Deletion of W491 (ΔW491) or the exchange of R144 and Q356 to alanine and glutamine respectively, had no effect on yeast cell viability, coordination of the Fe/S clusters in Nar1 or its function. As these residues showed to be conserved in the eukaryotic Nar1 homologs, the results are surprising, as we expected that they will play an important role for Nar1 function. Anyway, the mutational analysis of residue R144 and Q356 reassumes that Nar1 performs a different function that the Fe-only hydrogenases.

4.2.4. Functionally active Nar1 interacts with Nbp35

In this study it was demonstrated that Nar1 specifically interacts with Nbp35. A significant amount of Nbp35 was immunoprecipitated with HA-tagged Nar1 using anti-HA agarose from cell extracts of Gal-NBP35 cells. The Nar1-Nbp35 interaction is specific because Nbp35 could not be immunoprecipitated in cells that do not overproduce Nar1 or in cells overproducing other cytosolic proteins such as Cia1 and Rli1. The Nar1-Nbp35 interaction requires a fully functional Nar1 protein, because Nar1 can no longer interact with Nbp35 when Nar1 looses its Fe/S clusters and subsequently, its function. Previous work (Balk *et al.*, 2005a) has shown that Nar1 also interacts with another component of the CIA

machinery, namely Cia1. A recent work demonstrated that Nbp35 and Cfd1 form a stable complex *in vivo* and *in vitro* (Netz *et al.*, 2007). Nbp35 and Cfd1 can assemble three [4Fe-4S] clusters: one at the N-terminus of Nbp35 and one each at the C-termini of Cfd1 and Nbp35. The C-terminal Fe/S clusters can be transferred to recipient Fe/S apoproteins (Leu1) indicating a transient binding of these clusters on the Cfd-Nbp35 complex. This transient binding suggested a scaffold function of Cfd1-Nbp35, similar to what has been demonstrated for the Isu1/Isu2 proteins (Mühlenhoff *et al.*, 2003). These results have been summarized in the model depicted in Fig. 4.1. The insertion of Fe/S clusters into Cfd1, Nbp35 and Nar1 depends on the function of mitochondrial ISC-assembly and export machineries (Balk *et al.*, 2004; Netz *et al.*, 2007). Assembly of Fe/S clusters on Nar1 requires the function of the scaffold proteins Cfd1 and Nbp35 (Netz *et al.*, 2007). It is thus conceivable that the Fe/S cluster transfer from Cfd1/Nbp35 activates Nar1. The Nar1-Nbp35 interaction could be important in two different steps. First, during Fe/S cluster assembly on apo-Nar1 to form functional holo-Nar1. Second, the Nbp35-Nar1 interaction could be essential for directed Fe/S cluster transfer from Cfd1/Nbp35 to target apoproteins. Like Cia1, Nar1 is required only for the maturation of "true" Fe/S protein targets such as Leu1, Rli1 or Ntg2, but not for the assembly of the Fe/S clusters on Cfd1/Nbp35 (Balk *et al.*, 2004; Netz *et al.*, 2007). Thus, Nar1 becomes a member of the CIA-machinery only after the protein receives its Fe/S clusters from Cfd1/Nbp35. This "chicken and egg" situation seems similar to that described for the mitochondrial ferredoxin Yah1, where the assembly of its [2Fe-2S] cluster requires the activity of the mitochondrial ISC-assembly machinery (Lange *et al.*, 2000).

Based on the biochemical data, the CIA components can be grouped into two classes: The first one includes Cfd1 and Nbp35 that serve as scaffolds for the assembly of Fe/S clusters, and the second class comprises of the other two members, Nar1 and Cia1, which are required for later steps in the cytosolic/nuclear Fe/S proteins maturation. The Nar1-Nbp35 interaction may be needed to facilitate the Fe/S cluster transfer from Cfd1/Nbp35 to apoproteins. In this situation Nar1 functions as mediator between the early and late class of CIA proteins. Binding of Nar1 to Nbp35 could also labilize Fe/S cluster binding on Cfd1/Nbp35 complex to facilitate Fe/S cluster transfer.

This study provided biochemical data that help to understand the molecular function of Nar1. Future i*n vitro* studies should now focus on the molecular function of Nar1 in the pathway of Fe/S cluster transfer from Cfd1/Nbp35 to apoproteins. Identification of additional factors needed for the maturation of cytosolic/nuclear Fe/S proteins will help to gain further insights into the molecular mechanism of this process.

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Acknowledgments

Firstly I would like to express my profound gratitude to Prof. Dr. Roland Lill for offering me the opportunity to work in his team as a PhD student on a captivating and intriguing topic, and for his full support through suggestions and active debates concerning the scientific research.

I would like to thank Prof. Dr. Klaus Lingelbach for serving as the external adviser of this work.

Special thanks go to Priv. Doz. Dr. Ulrich Mühlenhoff for being always beside me with small tips and tricks and for helping with correction of my thesis.

I would like to thank Prof. Dr. Klaus Pfanner and Dr. Nils Wiedemann for the nice and productive collaboration on the Isd11 project. I am also deeply appreciative to Dr. Antonio Pierik for its help on the EPR experiments and to Prof. Dr. Wolfgang Buckel and Prof. Dr. Rolf Thauer for supporting the EPR studies.

I would like to say a sincere “Danke schön”, for the nice and friendly atmosphere given to the lab to all my colleagues.

My bottomless gratitude belongs to my family, to my parents, my sister Dr. Anca Gassmann and to Prof. Dr. Ecaterina Merica who always encouraged me in taking new prospects in life and offered me their support, without which I would never have made this reality.

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