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Crucial Role of Conserved Cysteine Residues in the Assembly of Two Iron−Sulfur Clusters on the CIA Protein Nar1[†]

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ABSTRACT: Iron−sulfur (Fe/S) protein maturation in the eukaryotic cytosol and nucleus requires conserved components of the essential CIA machinery. The CIA protein Nar1 performs a specific function in transferring an Fe/S cluster that is assembled de novo on the Cfd1−Nbp35 scaffold to apoproteins. Here, we used systematic site-directed mutagenesis and a combination of in vitro and in vivo studies to show that Nar1 holds two Fe/S clusters at conserved N- and C-terminal cysteine motifs. A wealth of biochemical studies suggests that the assembly of these Fe/S clusters on Nar1 cannot be studied in *Escherichia coli*, as the recombinant protein does not contain the native Fe/S clusters. We therefore followed Fe/S cluster incorporation directly in yeast by a ^{55}Fe radiolabeling method in vivo, and we measured the functional consequences of Nar1 mutations in the assembly of cytosolic Fe/S proteins. We find that both Fe/S clusters are essential for Nar1 function and cell viability. Molecular modeling using a structurally but not functionally related bacterial iron-only hydrogenase as a template provided compelling structural explanations for our mutational data. The C-terminal Fe/S cluster is stably buried within Nar1, whereas the N-terminal one is exposed at the protein surface and hence may be more easily lost. Insertion of an Fe/S cluster into the C-terminal location depends on the N-terminal motif, suggesting the participation of the latter motif in the assembly process of the C-terminal cluster. The vicinity of the two Fe/S centers suggests a close functional cooperation during cytosolic Fe/S protein maturation.

Iron−sulfur (Fe/S)[1] clusters are inorganic cofactors of many proteins found in nearly all prokaryotic and eukaryotic organisms. Fe/S proteins play important roles in many cellular processes, including electron transport, enzyme catalysis, and regulation of gene expression. Cells have developed dedicated systems for synthesizing Fe/S clusters and insert them into apoproteins. In *Saccharomyces cerevisiae*, three different machineries encompassing more than 20 proteins were identified with mature Fe/S proteins located in mitochondria, cytosol, and nucleus (*1*, *2*). The mitochondrial ISC (Fe/S cluster) assembly machinery is needed for the biosynthesis of all cellular Fe/S proteins, whereas the ISC export and CIA machineries are specifically required for the biogenesis of cytosolic and nuclear Fe/S proteins. A central component of the ISC assembly system is the cysteine desulfurase complex Nfs1-Isd11 (*3*−*5*) which converts cysteine to alanine and transfers the sulfur to Isu1 and Isu2 which serve as scaffold proteins for Fe/S cluster assembly (*6*). Thereafter, a dedicated chaperone system is needed for the transfer of the Fe/S cluster from Isu1 and Isu2 to apoproteins. This system includes a mitochondrial Hsp70 protein (Ssq1), its cochaperone Jac1, and nucleotide exchange factor Mge1 (*7*). The mitochondrial ISC assembly machinery is also required for biogenesis of extra-mitochondrial Fe/S proteins. According to a working model, the ISC assembly machinery produces a (still unknown) compound that is exported into the cytosol and used for the maturation of the cytosolic and nuclear Fe/S apoproteins. The central component of the ISC export machinery is the mitochondrial ABC transporter Atm1 (*8*). It is assisted by the other two components of the ISC export system, the sulfhydryl oxidase Erv1 and the tripeptide glutathione (*9*, *10*).

The CIA machinery consists of five known components which are essential for cell viability and have no relation to the components of mitochondrial ISC machineries. Depletion of these proteins specifically affects Fe/S protein maturation in the cytosol and nucleus, while assembly of mitochondrial Fe/S proteins is unaffected. The P-loop NTPases Cfd1 and Nbp35 (*11*, *12*) exhibit sequence similarity to each other in the nucleotide binding domain and in the C-terminal region. In addition, Nbp35 contains at its N-terminus four conserved cysteine residues which coordinate an Fe/S cluster. Recent in vitro and in vivo experiments show that Cfd1 and Nbp35 form a stable heterotetrameric complex and associate [4Fe-4S] clusters at their C-termini (*13*). Assembly of these clusters in vivo depends on the function of the mitochondrial ISC components Nfs1

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[1]Abbreviations: ISC, iron−sulfur cluster; CIA, cytosolic iron−sulfur protein assembly; Fe/S, iron−sulfur; EPR, electron paramagnetic resonance; ABC, ATP binding cassette.

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and Atm1. The C-terminal Fe/S clusters are bound in a labile fashion and can be rapidly and efficiently transferred to apoproteins in vitro which, in the case of yeast Leu1, leads to enzymatic function as an isopropylmalate isomerase. The transient character of binding of an Fe/S cluster to the Cfd1−Nbp35 scaffold suggests that these proteins serve as scaffolds for the assembly of an Fe/S cluster before its transfer to apoproteins. In vivo, the latter reaction depends on the two CIA components Nar1 and Cia1 (*14*, *15*). Cia1 is a member of the large family of WD40 repeat proteins containing a seven-blade β-propeller (*16*). Since WD40 proteins are known to act as protein interaction platforms, Cia1 may mediate the contact between different CIA proteins or between CIA and target Fe/S proteins to facilitate the transfer and incorporation of the Fe/S clusters. The function of the recently discovered Fe/S protein Dre2 is not known yet (*17*).

Nar1 is conserved in eukaryotes. Its essential role for cell growth and in Fe/S protein biogenesis has been established in yeast and human cells (*14*, *18*). The related plant Nar1 protein is crucial for growth under normoxic but not under hypoxic conditions (*19*). Nar1 proteins also exhibit similarity in sequence to bacterial and algal iron-only hydrogenases (Figure 1) (*20*, *21*) but are not known to produce or utilize hydrogen (*14*). According to in vitro studies, recombinant Nar1 from yeast or plant cells binds two interacting Fe/S clusters (*19*, *22*). Assembly of the Fe/S clusters in vivo depends on the mitochondrial ISC machineries and the Cfd1−Nbp35 scaffold, but not on Cia1 (*13*). The coordination of the clusters is a mystery but may involve eight conserved cysteine residues (Figure 1). Four of these residues are concentrated at the N-terminus and resemble a ferredoxin-like domain ($CX_{38}CX_2CX_2C$) that is predicted to bind a [4Fe-4S] cluster. In bacterial iron-only hydrogenases, the corresponding cysteine residues coordinate the medial [4Fe-4S] cluster (FS4A domain) (*23*, *24*). The four other conserved cysteine residues are scattered in the middle and C-terminal parts of Nar1 and correspond to residues which in iron-only hydrogenases coordinate the so-called "H-cluster". This moiety is comprised of the proximal [4Fe-4S] cluster and a connected dithiolate-, CO-, and CN-containing 2Fe center which serves as the active site (*21*). In bacteria, trichomonads, and possibly algae, synthesis of the H-cluster depends on the specialized biogenesis factors (termed HydEFG) which are not conserved in other eukaryotes (*25*−*28*). Hence, it seems unlikely that eukaryotic Nar1 proteins contain an H-type catalytic center, rendering the function of the C-terminal cysteine residues unclear.

For a molecular understanding of Nar1 function it is essential to unravel the identity, mode of coordination, and functional importance of the bound Fe/S clusters. Hence, the purpose of this study was to analyze the potential significance of the eight conserved cysteine residues for Fe/S cluster coordination and for the functionality of Nar1 in the maturation of Fe/S target proteins. To this end, a series of point mutant proteins were generated and analyzed for Fe/S cluster binding in vitro after synthesis and purification from *Escherichia coli*. Various biochemical properties of the recombinant proteins and a comparison of the behavior of the mutant proteins in yeast revealed striking differences which indicate that *E. coli* could not produce a protein with native Fe/S clusters. This finding necessitated the in vivo analysis of Nar1 in its native environment, the yeast cytosol. These studies revealed the importance of all eight cysteine residues for cell growth and for the efficiency of assembly of Fe/S clusters into Nar1 itself and into target proteins. Moreover, the sequence of incorporation of the two Fe/S clusters into Nar1

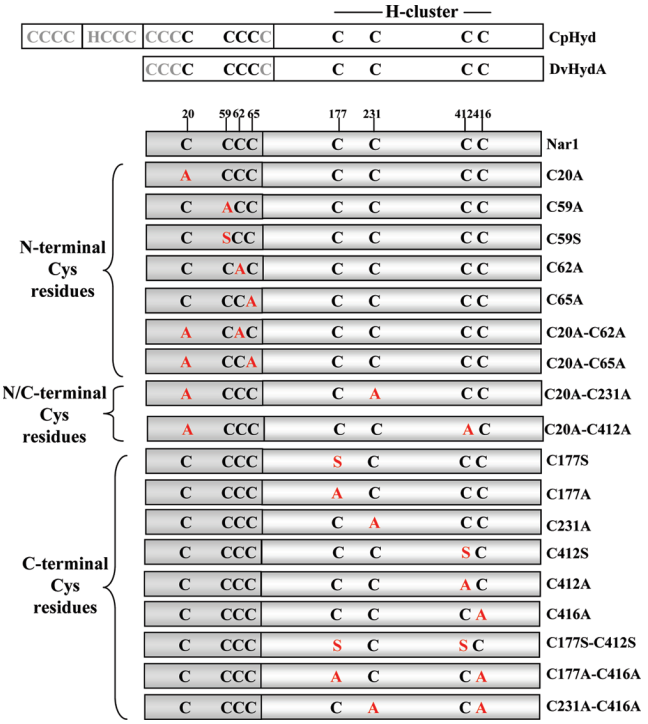

FIGURE 1: Schematic representation of point mutations introduced into yeast Nar1. The figure shows a schematic representation of *S. cerevisiae* Nar1 and the position of the eight conserved cysteine residues. Mutations generated in this work are colored red. In comparison, CpHyd and DvHydA indicate two iron-only hydrogenases from *Clostridium pasteurianum* and *Desulfovibrio vulgaris*, respectively. These proteins show considerable similarities in sequence to Nar1. Residues colored light gray are involved in coordination of Fe/S clusters in hydrogenases yet are not conserved in Nar1.

could be addressed. By combining our functional studies with molecular modeling, we show that Nar1 contains a stably bound C-terminal Fe/S cluster which is deeply embedded in the protein. Its assembly depends on the N-terminal cysteine-rich domain which holds a surface-exposed and thus more labile Fe/S cluster.

EXPERIMENTAL PROCEDURES

Yeast Strains, Cell Growth, and Plasmids. *S. cerevisiae* strain W303-1A (*MATa, ura3-1, ade2-1, trp1-1, his3-11,15, leu2-3,112*) served as the wild type. Mutant strain Gal-NAR1 was derived from W303-1A by exchange of the endogenous *NAR1* promoter for the galactose-inducible and glucose-repressible *GAL1-10* promoter (*14*). Cells were grown in minimal (SC) medium or in minimal medium lacking added iron chloride ("iron-poor"), each containing 2% (w/v) glucose as the carbon source (*29*). The following yeast plasmids were used: p416 containing the *MET25* or *NAR1* promoters and p426 with the *TDH3* promoter (*30*). All constructs were verified by DNA sequencing.

Site-Directed Mutagenesis. Single mutations of the N- and C-terminal cysteine residues of Nar1 to alanine residues were introduced by PCR-mediated mutagenesis of the *NAR1* gene using appropriate mutated primers. The PCR products were then digested with *Bam*HI or *Xho*I and *Xba*I or *Bam*HI, respectively, and cloned into plasmid p416MET25. The double mutations were created by restriction digestion of the single mutants followed by the ligation of a fragment containing one of the desired mutations and the linearized plasmid carrying the other mutation. DNA sequencing was used to verify the desired

sequences of all mutant *NAR1* genes. These plasmids and the plasmids lacking any DNA insert were transformed into the Gal-NAR1 cells.

Structural Modeling. The sequence alignment of *S. cerevisiae* Nar1 and *C. pasteurianum* Fe-only hydrogenase was performed using Multalin (*31*). This alignment was used to calculate the structural model of yeast Nar1 using the CPH 2.0 server (http://www.cbs.dtu.dk/services/CPHmodels/). All structural presentations were prepared with PyMOL (DeLano Scientific, Palo Alto, CA).

Protein Overproduction in E. coli and Purification. The pET15b plasmids encoding the wild-type *NAR1* gene or the *NAR1* cysteine mutant genes were transformed into *E. coli* C41-DE3 competent cells that already contained the plasmid pISC [encoding the *E. coli isc* operon (*32*)]. A 50 mL overnight culture was used to inoculate 2 L of LB medium. Growth was continued 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 incubated at 30 °C for 4 h. The *E. coli* cells were harvested by centrifugation (10 min at 4000*g* and 4 °C) and washed once in lysis buffer [50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole (pH 8.0)]. Cells were lysed at 4 °C in 30−50 mL of lysis buffer by applying a pressure of 10^8 N/m^2 using a high-pressure homogenizer (EmulsiFlex-C3). Intact cells and cell debris were removed by centrifugation (30 min at 100000*g* and 4 °C). The overproduced recombinant His-tagged Nar1 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 of washing buffer [50 mM NaH_2PO_4, 300 mM NaCl, and 25 mM imidazole (pH 8.0)] to remove unspecifically bound proteins. His-tagged Nar1 proteins bound to the Ni-NTA agarose were eluted with 10 mL of elution buffer [50 mM NaH_2PO_4, 300 mM NaCl, and 150 mM imidazole (pH 8.0)]. Immediately after elution, the purified proteins were desalted on a PD-10 column [equilibrated in 25 mM Tris-HCl (pH 7.0) and 150 mM NaCl], frozen in liquid nitrogen, and stored at −80 °C. Similar results were obtained when the procedures were performed anaerobically.

Electron Paramagnetic Resonance. Purified, desalted hexahistidinyl-tagged wild-type Nar1 or mutant Nar1 proteins were used for EPR spectroscopy. Low-temperature X-band EPR spectra were recorded after anaerobic reduction of these proteins using sodium dithionite or 5′-deazaflavin and light with a Bruker ESP 300E cw spectrometer, equipped with an ESR910 helium flow cryostat (Oxford Instruments) (*22*, *33*).

Miscellaneous Methods. The following published methods were used: manipulation of DNA and PCR (*34*); transformation of yeast cells (*35*); enzyme activities of isopropylmalate isomerase and alcohol dehydrogenase (*8*, *36*); quantitative analysis of iron by the colorimetric chelator ferene (*37*) and of sulfur in purified Nar1 proteins (*33*); and radiolabeling and cell lysis of the Fe/S reporter proteins of interest (*38*, *39*). All experiments were repeated at least three times. Error bars represent the standard error of the mean (SEM).

RESULTS

Purification and Analysis of Recombinant Nar1 Cysteine Mutant Proteins. To analyze the role of the eight conserved cysteine residues in Nar1 for function and Fe/S cluster coordination, we used site-directed mutagenesis to create a set of Nar1 mutant proteins in which each of the eight cysteine residues was exchanged with alanine (and in some cases also serine) either alone or in duplicate. On the basis of the Nar1 homology to iron-only hydrogenases, these mutations were grouped into N- and C-terminal residues and are schematically depicted in Figure 1.

To characterize the effects of the cysteine mutations, we first investigated recombinant Nar1 versions purified from *E. coli*. Wild-type Nar1 and the cysteine mutant proteins C20A, C59S, C62A, C177S, C412S, C416A, and C177S/C412S were overproduced in *E. coli* strain C41(DE3) from plasmid pET-15b in the presence of the bacterial *isc* operon (*40*). An N-terminal hexahistidinyl (His) tag was used for purification of Nar1 by Ni-NTA affinity chromatography. While most of the Nar1 mutant proteins were more than 95% homogeneous, the C177S/C412S variant exhibited numerous proteolysis products (Figure 2A). Analytical gel filtration of wild-type Nar1 revealed a major peak with a molecular mass of ≈56 kDa indicating that Nar1 is a monomer (Figure S1 of the Supporting Information).

Iron and Sulfide Content of Wild-Type Nar1 and Cysteine Mutant Proteins. The iron and acid-labile sulfur content of the recombinant "as isolated" Nar1 proteins was investigated by chemical analysis. The amounts of iron and sulfide were in the range of 3−3.5 Fe and 2.5−3 S atoms per wild-type Nar1 protein molecule. Similar results were obtained when Nar1 was purified anaerobically. The amounts of Fe and S did not differ from that of the wild type in the C20A, C59S, C62A, and C412S versions, while C177S and C416A contained significantly less (Table 1). The Nar1 mutant protein C177S/C412S had only 1.5 Fe and 1.5 S atoms bound per protein molecule, i.e., roughly 50% of that of wild-type Nar1. Since previous spectroscopic studies of purified recombinant wild-type Nar1 provided evidence of two Fe/S clusters, which were not of the [2Fe-2S] type, the expected stoichiometry of Fe and S per protein was much higher. Therefore, Nar1 was subjected to in vitro chemical reconstitution under anaerobic conditions (*13*). This procedure did not lead to any increase in iron and sulfide content. Therefore, the spectroscopic analyses described below were conducted 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 that of the wild-type protein with a broad, unstructured shoulder around 420 nm, typical for $[4Fe\text{-}4S]^{2+}$ clusters (Figure S2 of the Supporting Information) (*14*). Similar results were obtained for Nar1 proteins isolated anaerobically. Except for the double mutant C177S/C412S which exhibited a weak signal, the C-terminal cysteine mutant proteins exhibited an absorption peak at 420 nm similar to wild-type Nar1. However, we noted rather large differences in the absorption intensities between the individual Nar1 mutant proteins which do not truly reflect their Fe and S content.

Electron Paramagnetic Resonance (EPR) Analyses of Recombinant Nar1 Variants. Previous EPR studies of recombinant wild-type Nar1 indicated the presence of two signals, a sharp rhombic signal, probably from a single cluster, and a broad rhombic signal that was assigned to two magnetically coupled Fe/S clusters (*14*, *19*, *22*). To better understand the chemical nature of the two Fe/S clusters and to possibly identify their ligands, we performed EPR spectroscopy on the recombinant wild-type Nar1 and its cysteine mutant proteins. Nar1 is EPR-silent in the oxidized form and requires reduction to generate EPR signals. As isolated wild-type Nar1 and its cysteine variants

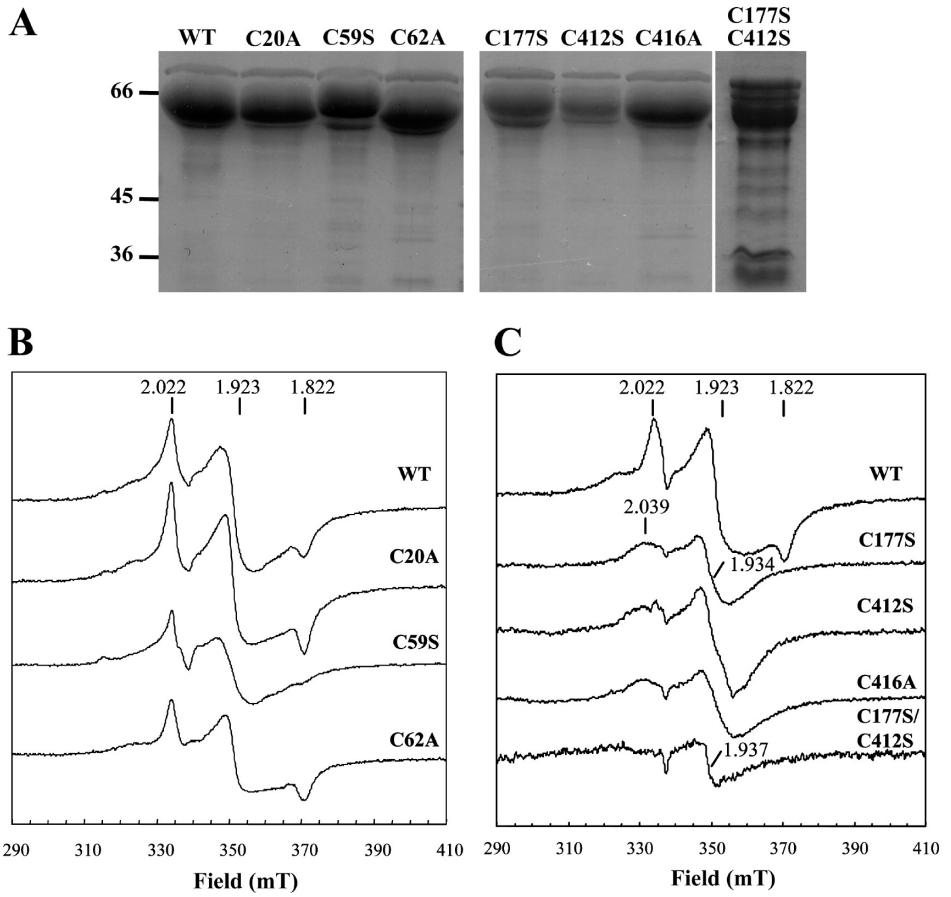

FIGURE 2: Analysis of purified recombinant Nar1 mutant proteins. (A) Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) of His-tagged Nar1 and its cysteine mutants. Protein samples were applied to an SDS−polyacrylamide gel (10%) and stained with Coomassie blue. (B and C) Electron paramagnetic resonance (EPR) spectra of reduced wild-type and cysteine mutant Nar1 proteins. EPR spectra (scaled for protein content) 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. The g values are given. (B) Wild-type and N-terminal cysteine mutant proteins were reduced with 50 μM 5′-deazaflavin and light (4−5 min illumination by a slide projector) at 4 °C and shock-frozen in liquid nitrogen. (C) Nar1 C-terminal cysteine mutant proteins were reduced with 2 mM sodium dithionite in a buffer containing 25 mM Tris-HCl (pH 8.0) and 150 mM NaCl.

Table 1: Analysis of the Content of Iron and Acid-Labile Sulfide in Wild-Type Nar1 and Its Cysteine Variants[a]

Nar1	iron (mol/mol of protein)	sulfide (mol/mol of protein)
wild type	3.2	3.0
C20A	3.6	2.8
C59S	3.2	2.2
C62A	3.6	2.5
C177S	2.4	2.2
C412S	3.0	2.7
C416A	2.4	1.4
C177S/C412S	1.5	1.5

[a] The mean values of 3-5 experiments are given with a typical standard deviation of ± 0.2 for Fe and ± 0.2 for sulfide.

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 exhibited a broad rhombic EPR signal with the following g values: g_z = 2.022, g_y = 1.923, and g_x = 1.822 (Figure 2B). This rhombic EPR signal was similar to the previously described Nar1 signal (*14*, *22*). The cysteine mutations at the N-terminus (C20A and C62A) resulted in a rhombic EPR spectrum similar to that of wild-type Nar1. In the case of the C59S mutant, the negative absorption-shaped g_x = 1.822 feature was almost absent when compared to the wild-type spectrum, but the g_z = 2.022 feature remained (Figure 2B). The altered spectrum was at variance to the almost unchanged amounts of iron and sulfide bound to C59S relative to that in wild-type Nar1 (Table 1).

The broad rhombic EPR signal disappeared when the C-terminal cysteine (C177S, C412S, and C416A) mutants were analyzed. These mutant proteins exhibited a signal typical for $[4Fe\text{-}4S]^{+}$ clusters, but with a lower integrated intensity compared to that of wild-type Nar1 (Figure 2C). Again these data are not consistent with the almost unchanged iron and sulfide contents of these preparations (Table 1). The low-intensity EPR signal of the C177S/C412S variant correlated with the low iron and sulfide values, and most likely, the low content of Fe/S clusters is explained by proteolysis of this mutant protein. These data indicate that mutation of the C-terminal residues C177, C412, and C416 results in changes in the rhombic EPR signal observed in wild-type Nar1. Conspicuously, similar or only slightly smaller amounts of bound iron and sulfide were observed in these cysteine mutant proteins compared to those in wild-type Nar1 (Table 1). Apparently, the spectral differences evident by EPR spectroscopy were not reflected by differences in the iron and sulfide content of the wild-type and mutant Nar1 proteins. These inconsistencies show that *E. coli* produces a non-native Nar1 protein. This conclusion made it necessary to investigate the

physiological relevance of binding of the Fe/S cluster to the mutant Nar1 proteins by functional analyses in vivo, i.e., in their native environment, the yeast cytosol.

Importance of the Conserved Cysteine Residues of Nar1 for Cell Viability. We investigated whether the eight conserved cysteine residues are essential for cell viability. The regulatable yeast strain Gal-NAR1 (*14*) was transformed with centromeric plasmids p416-MET25 encoding wild-type Nar1, the Nar1 mutant proteins, or no protein. The functionality of the mutant Nar1 proteins was tested by depleting the nuclear-encoded (endogenous) Nar1 by growing the cells on minimal medium containing glucose (Figure 3). Plasmid-encoded, wild-type Nar1 restored the growth defect of Gal-NAR1 cells under these conditions. When the N-terminal Nar1 cysteine variants were analyzed, two single-point mutant proteins (C59A and C62A) were not able to restore growth. The C65A mutant protein showed weak complementation, whereas the C20A mutation did not cause any growth defect (Figure 3A). Conspicuously, the double mutant C20A/C65A did not grow at all, indicating the importance of the C20 residue in conjunction with alteration of C65 (Figure 3B). Thus, the three N-terminal cysteine residues (C59, C62, and C65) are crucial for cell growth, while residue C20 also appears to be of functional importance due to the synergistic effect with the C65 mutation.

Nar1 proteins with single-point mutations of the C-terminal cysteine residues (C177A, C231A, C412A, and C416A) supported wild-type cell growth (Figure 3C). In contrast, double-mutant proteins were not able to rescue the growth defect of Nar1-depleted Gal-NAR1 cells (Figure 3D). The cysteine to serine single mutants C177S and C412S (not shown) and the double mutant C177S/C412S (Figure 3D) exhibited growth rates similar to those of the corresponding cysteine to alanine mutants. These results indicate an important function of the C-terminal cysteine residues for Nar1 function. However, it needs two simultaneous mutations to impair its function, demonstrating the synergistic behavior of the C-terminal cysteine residues. In addition, the simultaneous exchange of one N-terminal cysteine, C20, and one of the C-terminal cysteine residues, C231 and C412 (mutants C20A/C231A and C20A/C412A), had no effect on cell growth (Figure 3B), indicating that these residues are not essential for yeast cell viability and likely are not coordinating partners of the same Fe/S cluster. Virtually identical growth complementation effects were observed for C-terminal cysteine to serine mutant proteins produced from the endogenous *NAR1* rather than the *MET25* promoter which supports moderate overproduction (Figure S3 of the Supporting Information), indicating that the growth complementation was not due to high-copy suppression. Taken together, all eight conserved cysteine residues play an important role for Nar1 function, but especially the functional deficits arising from the C-terminal residues are revealed only after double mutations.

Importance of N- and C-Terminal Cysteine Residues for the Maturation of Cytosolic Fe/S Proteins. The requirement of the N- and C-terminal Nar1 cysteine residues for cytosolic Fe/S protein maturation was analyzed by determination of the enzyme activity of isopropylmalate isomerase (Leu1) as an Fe/S marker protein (*8*). As described above, Nar1-depleted Gal-NAR1 cells producing the various Nar1 (mutant) proteins from plasmids were employed. As a control, the activity of the cytosolic non-Fe/S protein alcohol dehydrogenase (ADH) was measured. ADH activity did not change significantly in any of the cells containing the cysteine mutant proteins compared to wild-type cells (not shown). The Leu1 activities (relative to ADH) are depicted in panels A and B of Figure 4. Two N-terminal cysteine mutants exhibited only a background signal for Leu1 activity (C59A and C62A). Mutation of C65 diminished the Leu1 activity by half, and even further, when C20 was simultaneously exchanged with alanine (C20A/C65A). In contrast, hardly any decrease in Leu1 activity was observed in cells with the C20A mutation.

The C-terminal cysteine single-point mutations in Nar1 did not affect Leu1 activity, yet the activity was strongly diminished when the double mutants C177A/C416A, C231A/C416A, and C177S/C412S were analyzed (Figure 4B). In all cases, the Leu1 protein levels were unchanged relative to the cytosolic marker protein Pgk1 (Figure 4C). The double mutants C20A/C231A and C20A/C412A exhibited slightly lower Leu1 activities as compared to cells producing the wild-type Nar1 protein (Figure 4A). Together, these functional results of the Nar1 mutants fit nicely to the growth data reported above. The data indicate that the N- and C-terminal cysteine residues either alone or together with another cysteine residue are essential for Nar1 function in Leu1 maturation. The growth defects may therefore be a direct consequence of an impairment in Fe/S protein maturation.

To further support this conclusion, the consequences of the Nar1 N-terminal and C-terminal cysteine mutations on the de novo synthesis of cytosolic Fe/S proteins were investigated by following the incorporation of radioactive iron (^{55}Fe) into the cytosolic Fe/S reporter proteins Leu1 and Rli1 (*9*). Gal-NAR1 cells carrying plasmids encoding the various Nar1 proteins were grown in iron-poor minimal medium containing glucose to deplete Nar1. After radiolabeling with ^{55}Fe, endogenous Leu1 and overproduced, HA-tagged Rli1 were immunoprecipitated from a cell extract and the associated radioactivity was quantified by scintillation counting. The radioactivity associated with the immunobeads is a direct measure of the degree of incorporation of Fe/S clusters into Leu1 and Rli1 (*8*, *41*). In most N-terminal cysteine mutants analyzed (C59A, C62A, C65A, C20A/C62A, and C20A/C65A), the amounts of ^{55}Fe incorporated into endogenous Leu1 were decreased to background levels (Figure 5A). Similar results were observed for Rli1 (Figure 5B). The only exception was the C20A mutant which still exhibited ≈30% of the ^{55}Fe associated with both Leu1 and Rli1 compared to wild-type levels. Apparently, the incorporated ^{55}Fe amounts were above the threshold level sufficient for formation of enough functional Leu1 and Rli1 for sustaining growth. Importantly, the amounts of ^{55}Fe associated with Leu1 and Rli1 in the C65A mutant were further diminished in the C20A/C65A double mutant, thus confirming the results obtained for the Leu1 enzyme activity. In all cases, the protein levels of Leu1 and Rli1 were comparable to those present in wild-type cells (Figure 5C).

Nar1 carrying single mutations in the C-terminal cysteine residues supported wild-type incorporation of ^{55}Fe into Leu1 and Rli1 (Figure 5D,E). In cells carrying double mutations of the C-terminal cysteine residues, the level of incorporation of ^{55}Fe into Leu1 and Rli1 was more than 6-fold lower. As observed for the N-terminal cysteine mutant proteins, the Leu1 and Rli1 protein levels were not significantly altered compared to wild-type conditions (Figure 5F), indicating that the assembly of the Fe/S cluster rather than protein synthesis or stability was affected. We also analyzed Nar1 mutant proteins in which one N-terminal and one C-terminal cysteine exchange were combined (C20A/C231A and C20A/C412A) (Figure 5A,B). ^{55}Fe radiolabeling indicated a decrease in the level of maturation of Leu1 and

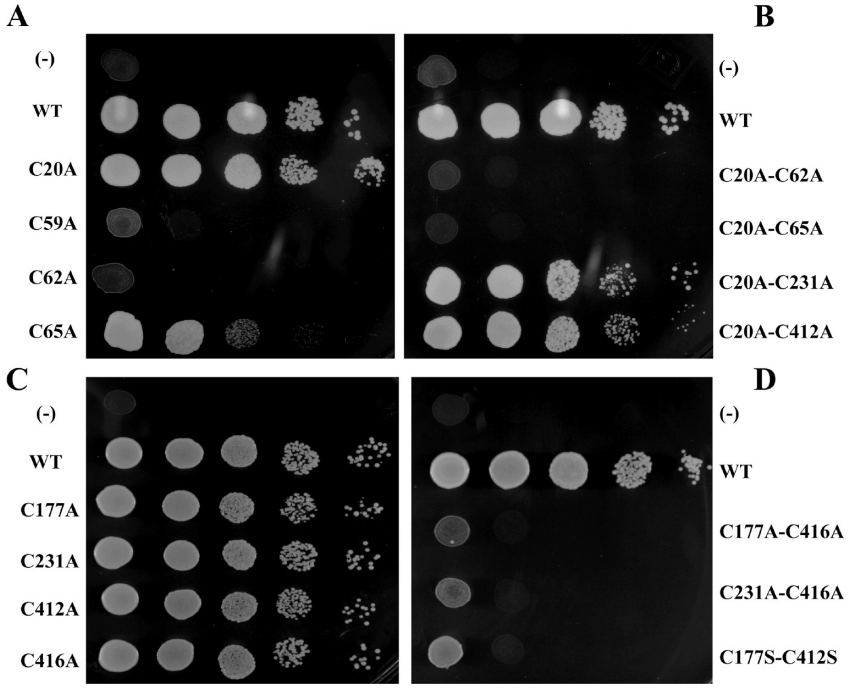

FIGURE 3: Importance of conserved cysteine residues of Nar1 for cell viability. (A−D) Gal-NAR1 cells were transformed with plasmid p416MET25 containing either no insert (−), the wild-type (WT) *NAR1* gene, or the indicated N- and C-terminal cysteine mutant genes. The cells were grown on SD medium for 2 days at 30 °C. Then, 10-fold serial dilutions were grown under the same conditions.

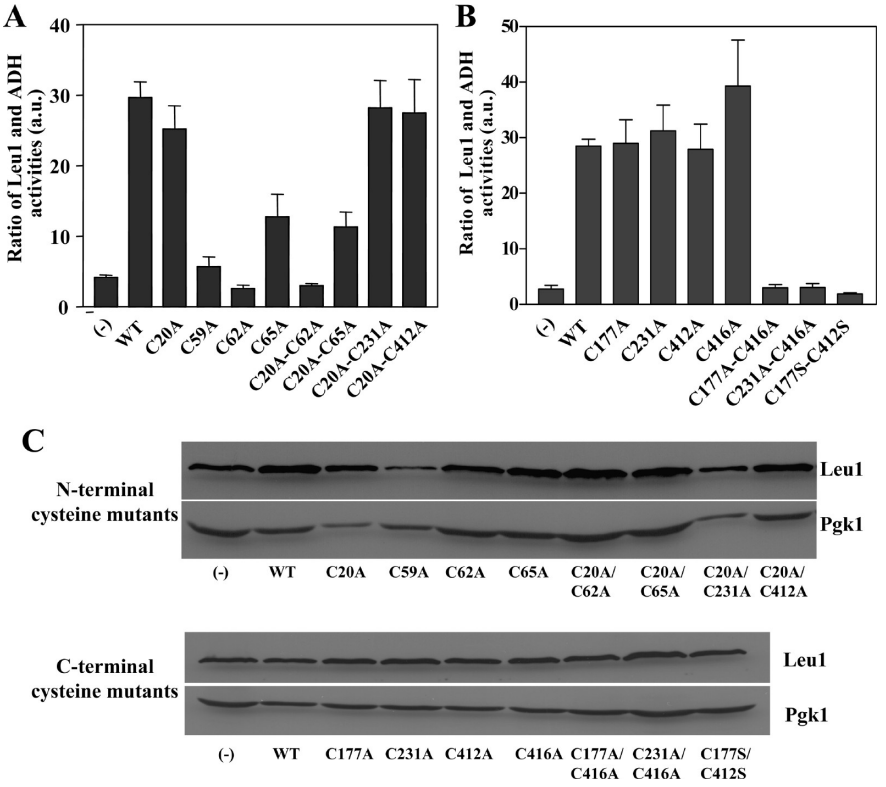

FIGURE 4: Conserved cysteine residues of Nar1 are crucial for maintenance of cytosolic Leu1 Fe/S protein activities. Gal-NAR1 cells were transformed with plasmid p416-MET25 encoding wild-type (WT) Nar1, the indicated N- and C-terminal cysteine mutant Nar1 proteins, or no protein (−). Cells were grown in SC medium for 40 h, and the isopropylmalate isomerase (Leu1) and alcohol dehydrogenase (ADH) activities were measured in cell extracts. (A and B) Ratio of the Leu1 and ADH enzyme activities (a.u., arbitrary units) in the indicated cells. The error bars indicate the standard error of the mean ($n \geq 3$). (C) Protein extracts from panels A and B were subjected to immunostaining for Leu1 and Pgk1 protein levels using specific anti-Leu1 and anti-Pgk1 antibodies, respectively.

Rli1 in these mutants relative to wild-type cells. The amount of incorporated ^{55}Fe was similar to that obtained for the C20A single-mutant protein, indicating that there is no cooperative effect with combination of the two mutations. Together, the direct measurement of Fe/S cluster assembly on Leu1 and Rli1 by ^{55}Fe radiolabeling strongly supports the conclusion that all eight

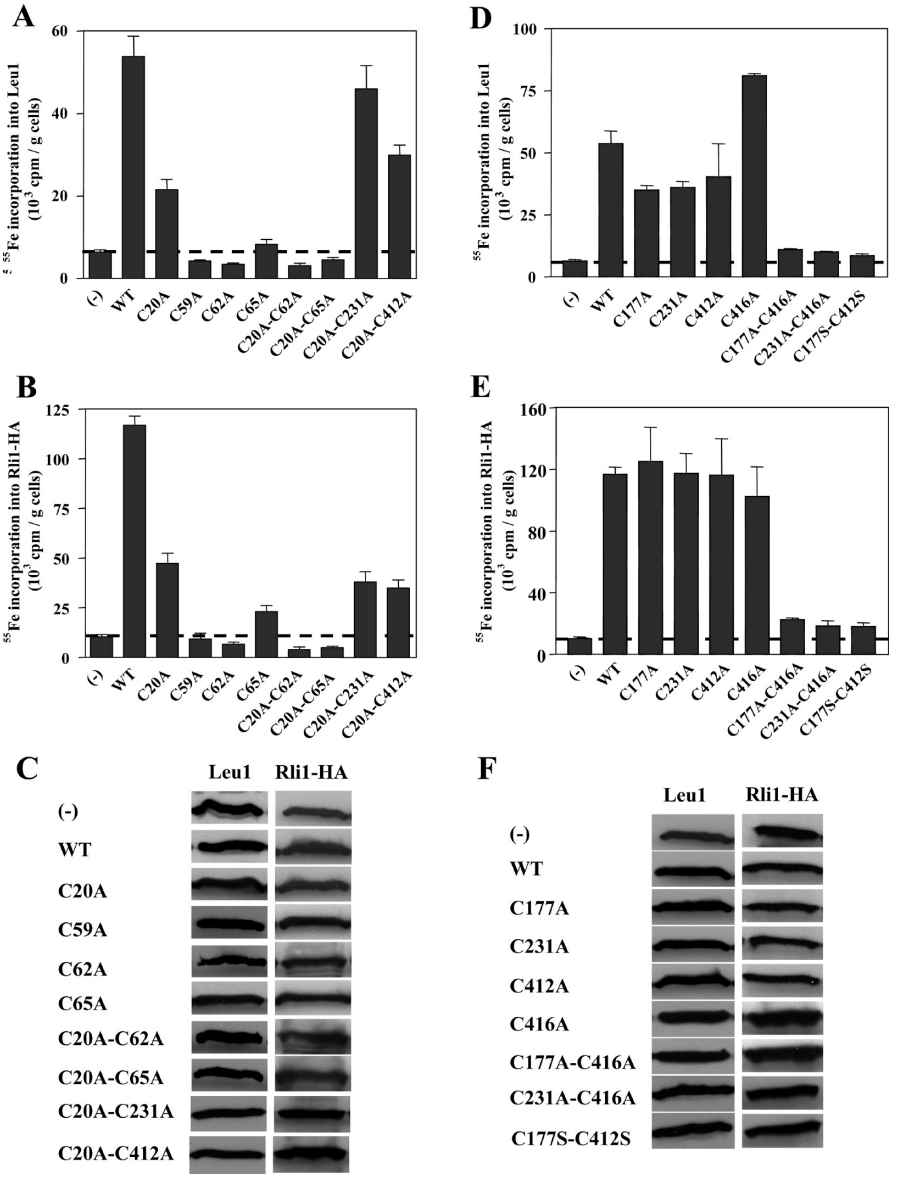

FIGURE 5: Important role of Nar1 cysteine residues for de novo maturation of cytosolic Fe/S proteins. Cells like those described in the legend of Figure 4 were grown in SC medium for 24 h and in iron-poor medium supplemented with glucose for 16 h. Radiolabeling with ^{55}Fe was conducted for 4 h. Endogenous Leu1 (A and D) and Rli1-HA (B and E) synthesized from a high-copy number vector (p424-TDH3) were immunoprecipitated from cell extracts using Leu1-specific and anti-HA antibodies, respectively. The radioactivity associated with Leu1 and Rli1 was quantified by scintillation counting and corrected for the slight variations in Leu1 and Rli1-HA protein levels which were determined by immunostaining and quantitative densitometry (see panels C and F). The dashed lines indicate the background levels of incorporation of ^{55}Fe into Leu1 and Rli1.

conserved cysteine residues of Nar1 are important for the assembly of cytosolic Fe/S proteins. Even though the single mutant C20A and all four C-terminal single mutants showed no severe defects in ^{55}Fe/S cluster assembly, the combined mutation of C-terminal cysteine residues led to strongly impaired incorporation of Fe/S clusters into both Leu1 and Rli1, showing the functional importance of these Nar1 residues for Fe/S cluster assembly.

N- and C-Terminal Cysteine Residues Are Essential for Fe/S Cluster Assembly on Nar1. The results presented above made it likely that at least those cysteine residues of Nar1 that are essential for viability and Nar1 function in Fe/S protein maturation are involved in the coordination of the two Fe/S clusters on Nar1. To directly investigate the consequences of the Nar1 cysteine mutations on binding of Fe/S clusters to Nar1 itself, the ^{55}Fe radiolabeling assay described above was employed, and Nar1 was immunoprecipitated with specific antibodies. When the N-terminal cysteine mutant Nar1 proteins were analyzed for their ability to bind Fe/S clusters, the ^{55}Fe associated with the C20A mutant protein was only slightly diminished (to ≈75−80% of the wild-type levels), whereas the amount of ^{55}Fe incorporated into the C59A and C62A mutant proteins was substantially decreased to 10−20% of wild-type levels (Figure 6A, left panel). The C65A mutant protein could still incorporate ≈35% of ^{55}Fe compared to wild-type amounts, but the double mutation of C20 and C65 led to a further decrease to ≈20%. The Nar1 protein levels were almost similar in all mutant cells (Figure 6A, right panel). These results clearly indicate that each individual N-terminal cysteine residue is crucial for binding of Fe/S clusters to Nar1. Residue C20 is not essential for incorporation of Fe/S clusters on Nar1, but the substantially lower efficiency of binding of Fe/S clusters to the C20A/C65A mutant demonstrates the requirement of both residues for binding of Fe/S clusters to Nar1. Notably, the C59A, C62A, C20A/C62A, and C20A/C65A mutant proteins exhibited

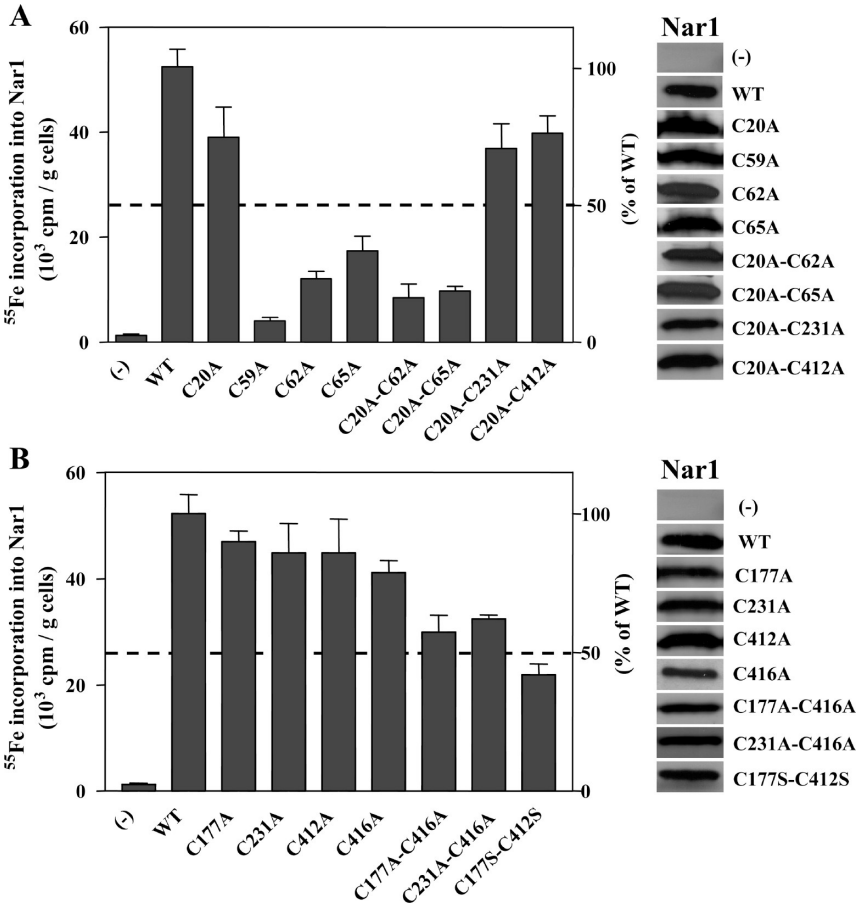

FIGURE 6: Importance of conserved Nar1 cysteine residues for the assembly of Fe/S clusters on Nar1. Gal-NAR1 cells expressing the indicated N-terminal (A) or C-terminal (B) cysteine mutant Nar1 proteins were grown as described in the legend of Figure 5. After ^{55}Fe radiolabeling had been conducted, Nar1 was immunoprecipitated from cell extracts and the amounts of ^{55}Fe bound to Nar1 were quantified by scintillation counting (left panels). These levels were corrected for differences in Nar1 protein levels determined by immunoblot analysis and densitometry (right panels).

an almost complete loss of Fe/S cluster incorporation to background levels. This result clearly indicates that mutations of the N-terminal cysteine residues severely affect incorporation of both Fe/S clusters into Nar1, suggesting a critical role of the N-terminal cysteine residues in the assembly of the C-terminal Fe/S cluster.

A different result was obtained for the C-terminal cysteine residues of Nar1. Simultaneous mutation of two residues (C177A/C416A, C231A/C416A, and C177S/C412S mutants) led to a decrease in the level of ^{55}Fe incorporation to ~50% of wild-type levels (Figure 6B, left panel), while incorporation of Fe/S clusters into the C-terminal single-cysteine Nar1 mutant proteins was comparable to that for the wild-type protein. These data suggest that in the double-mutant proteins the C-terminal Fe/S cluster is specifically lost while the N-terminal one can still be inserted. The levels of Nar1 (Figure 6B, right panel) were slightly diminished in some cases and used to normalize the ^{55}Fe incorporation data. The double-mutant proteins C20A/C231A and C20A/C412A incorporated amounts of ^{55}Fe similar to that of the C20A single-mutant protein (Figure 6A). This unaltered Fe/S cluster insertion efficiency strongly suggests that the C20 and C231 or C412 residues do not perform cooperative roles in the assembly of the Fe/S cluster in Nar1. In all other cases, the simultaneous mutation of two cysteine residues led to severe effects on Fe/S cluster association, i.e., either the loss of the C-terminal cluster (in C177A/C416A, C231A/C416A, and C177S/C412S mutant proteins) or the loss of both Fe/S clusters (in the N-terminal C20A/C65A mutant). This indicates that these residues cooperate in binding of an Fe/S cluster to Nar1. Collectively, the results demonstrate a crucial role for binding of Fe/S clusters to the C-terminal cysteine motif, even though in its absence the N-terminal one can still be incorporated.

In summary, our mutational data suggest that all eight conserved cysteine residues are important for Fe/S cluster assembly on Nar1. The data are compatible with the idea that both the N- and C-terminal cysteine residues each bind one Fe/S cluster. These clusters perform an essential role for the functionality of Nar1, as seen from their importance for Nar1 function in both cytosolic Fe/S protein maturation and cell growth. The propensity of the single C-terminal mutant proteins to hold almost wild-type levels of the Fe/S cluster indicates that the Fe/S center coordinated by these residues is more stably bound than the N-terminal cluster, for which mutation of only one cysteine residue results in complete loss of the coordinated Fe/S cluster. The concomitant defect of binding of Fe/S clusters to the C-terminal residues suggests that the N-terminal Fe/S cluster may play a direct role in the assembly of the C-terminal Fe/S center.

Structural Modeling of Nar1. To determine where the eight conserved cysteine residues of Nar1 might be located in the three-dimensional structure, a theoretical model structure was calculated on the basis of the published X-ray structure for the Fe-only hydrogenase from *C. pasteurianum* (Protein Data Bank entry 1feh) (*24*). The sequence alignment of the yeast Nar1 and bacterial Fe-only hydrogenase showed an overall level of amino acid identity of 28% and was used to derive a structural model of Nar1 by means of the CPH 2.0 server (Figure 7A, left).

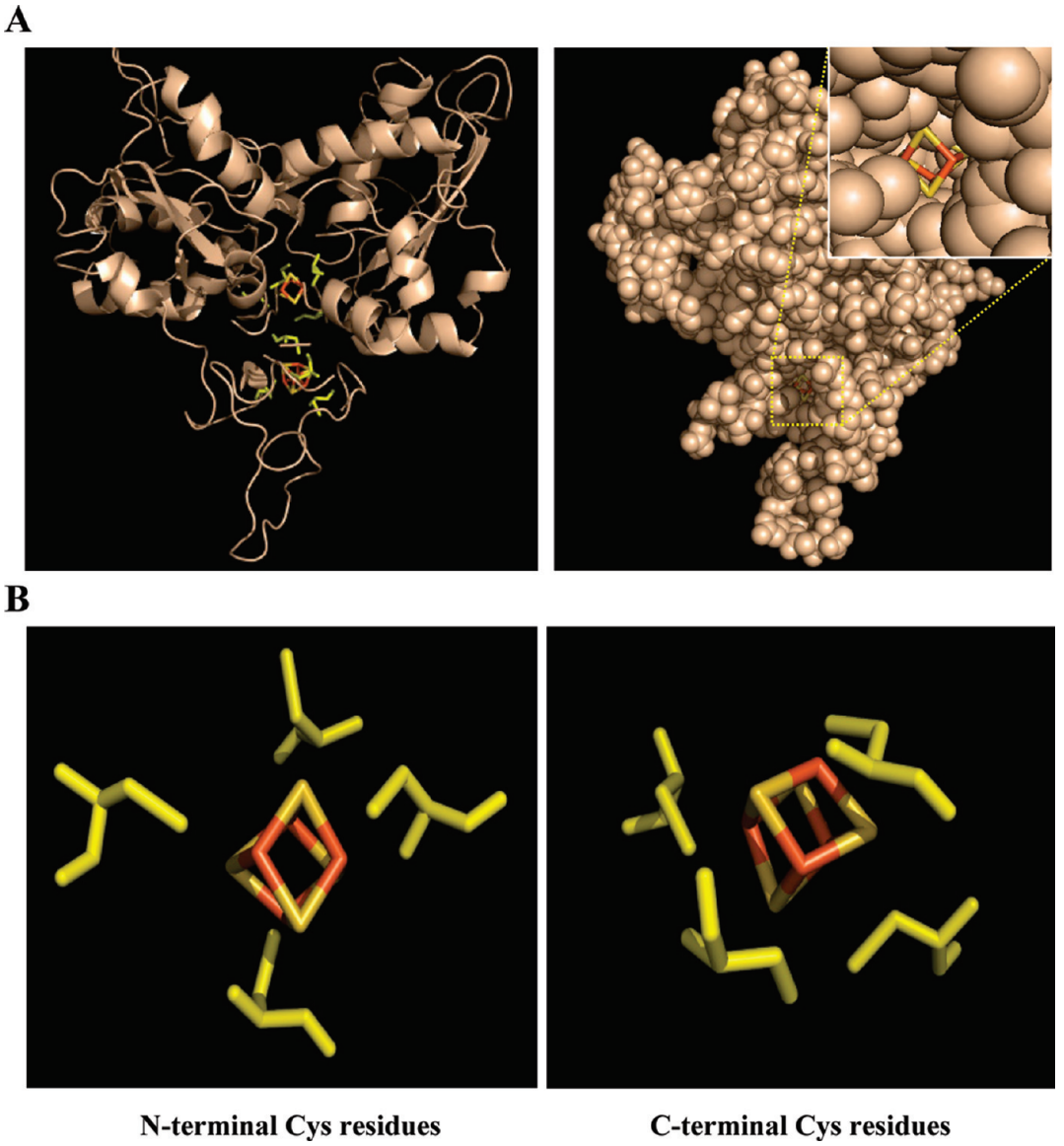

FIGURE 7: Structural model of the *S. cerevisiae* Nar1 protein. A structural model for Nar1 was calculated using the Protein Data Bank coordinates of *C. pasteurianum* iron-only hydrogenase (Protein Data Bank entry 1FEH) with CPHmodels 2.0 (http://www.cbs.dtu.dk/services/CPHmodels/). (A) The left panel shows a ribbon diagram of the Nar1 structural model with the two Fe/S clusters (red and yellow sticks) inserted according to the X-ray structure of Fe-only hydrogenase from *C. pasteurianum*. The right panel highlights the surface exposure of the N-terminal Fe/S cluster in a space-filling representation. The region of the N-terminal Fe/S cluster is enlarged in the inset. Compared to the left panel, the structure was rotated by 90° from left to front. (B) Close-up views of the modeled N- and C-terminal Fe/S clusters of Nar1. This figure was prepared with PyMOL (DeLano Scientific).

The orientation of the four N-terminal cysteine residues suggests that they could be involved in the coordination of one Fe/S cluster, whereas the other four conserved cysteine residues in the middle and C-terminal parts of Nar1 may bind a second Fe/S cluster. On the basis of this result, it was possible to insert two [4Fe-4S] centers into the calculated Nar1 model structure (Figure 7B). As seen in Figure 7A (right panel), the Fe/S cluster at the C-terminus is deeply buried in the Nar1 structural model, whereas the N-terminal Fe/S cluster is more exposed at the protein surface. These relative topographic arrangements of the N- and C-terminal Fe/S clusters explain several of the experimental observations reported above. The surface exposure of the N-terminal Fe/S cluster may contribute to its looser binding behavior, leading to a loss of cluster association when only a single N-terminal cysteine residue is mutated. On the other hand, the shielding of the C-terminal cluster within the Nar1 polypeptide chain in a cavity might be the reason why mutations of a single C-terminal cysteine residue do not detectably affect Fe/S cluster insertion and Nar1 function. Only the exchange of two C-terminal cysteine residues leads to Fe/S cluster loss and a concomitant functional impairment of Nar1.

DISCUSSION

Our attempts to improve the molecular understanding of the functional mechanisms of Fe/S protein assembly by components of the CIA machinery prompted us to initiate a detailed investigation of the structural basis of Fe/S cluster coordination on Nar1. Previous studies have provided evidence of the presence of two magnetically coupled Fe/S clusters on recombinant forms of yeast and plant Nar1 (*14*, *19*, *22*). The association of Fe/S clusters with Nar1 could be verified by in vivo studies, yet it had remained unclear how the clusters are coordinated and whether they represent the native form. On the basis of the considerable

sequence homology between Nar1 and bacterial iron-only hydrogenases, we predicted two sets of four conserved cysteine residues as potential Fe/S cluster-coordinating residues. The first one at the N-terminus is similar to the medial [4Fe-4S] cluster binding motif of hydrogenases, while the second one in the central and C-terminal regions corresponds to the hydrogenase H-cluster which consists of a [4Fe-4S] cluster linked to a 2Fe center representing the active site (*23*, *42*−*44*). Since yeast is unlikely to generate such a special Fe/S cluster, it was unclear whether these cysteine residues have maintained their Fe/S cluster-coordinating roles. To investigate Fe/S cluster binding to Nar1, we systematically constructed single- and double-cysteine mutant versions of Nar1 and produced them in both *E. coli* and *S. cerevisiae* for biochemical, spectroscopic, and cell biological investigations.

Our biochemical work on recombinant wild-type and mutant Nar1 proteins purified from *E. coli* established that yeast Nar1 is not produced in its native form in this bacterium, even if the Nar1 isolation was performed under strictly anaerobic conditions. First, the iron and sulfur contents of recombinant wild-type Nar1 were more than 2-fold lower that the expected stoichiometry of 8 (for two [4Fe-4S] clusters) and could not be further improved by chemical reconstitution with iron and sulfide under anaerobic conditions. Second, mutations of several cysteine residues of Nar1 did not significantly decrease the iron and sulfur contents relative to those of wild-type Nar1. These observations were in contradiction to the substantial changes in the EPR spectra which indicate alterations in the type and/or coordination of the bound Fe/S clusters (compare Table 1 and Figure 2B,C). Even though these new EPR spectroscopic studies on mutant Nar1 support our earlier conclusion of two types of magnetically coupled Fe/S centers in recombinant wild-type Nar1 (*14*, *15*), it became clear in this investigation that the Fe/S clusters associated with recombinant Nar1 are not identical to the ones present in native Nar1. These conclusions were further substantiated by comparing the data for the recombinant protein with results for mutant forms of Nar1 synthesized in its native environment, the yeast cytosol. The growth behavior of yeast cells expressing mutant versions of *NAR1*, the functionality of Nar1 in cytosolic Fe/S protein maturation, and the amount of radiolabeled ^{55}Fe associated with Nar1 were at variance with the findings for recombinant Nar1. For instance, mutations of cysteine residues 59 and 62 showed no changes in the iron and sulfur contents of recombinant Nar1, whereas these residues are essential for growth and function in yeast with strongly diminished ^{55}Fe association (see below). Further, the mutant protein C59S gave rise to an EPR spectrum indicative of an associated Fe/S cluster, yet no ^{55}Fe was tightly bound to this (and other N-terminal) mutant Nar1 in yeast. Likewise, the EPR spectra and the iron−sulfur contents of recombinant Nar1 with single C-terminal cysteine mutations were significantly different from those of the wild type, yet in yeast no (or only slight) effects were observed for mutant cell growth, Nar1 function, or ^{55}Fe binding. The observation that ectopic synthesis of the Nar1 Fe/S protein in *E. coli* leads to a non-native metal center is not unprecedented. Studies on the *Desulfovibrio vulgaris* iron-only hydrogenase showed that *E. coli* supports the incorporation of the distal and medial [4Fe-4S] clusters, but not the H-cluster (*45*). Further examples are the single-iron-containing *C. pasteurianum* rubredoxin and the [2Fe-2S] cluster-containing *Haemophilus influenzae* IscU, which are isolated as zinc rather than Fe/S proteins upon purification from *E. coli* (*46*, *47*).

The non-native character of recombinant Nar1 made it necessary to study the importance of the two conserved cysteine motifs in the native environment of Nar1, the yeast cytosol. Yeast cells producing the site-directed Nar1 mutant proteins were analyzed for the growth behavior, for the functional consequences on cytosolic and nuclear Fe/S protein biogenesis, and directly for binding of ^{55}Fe/S clusters to mutant Nar1 in vivo. The results obtained in the various assays in yeast were highly similar and are schematically summarized in Figure 8. Generally, any mutation affecting the efficiency of binding of Fe/S clusters to Nar1 (Figure 8C) elicited a physiological defect observed as an impaired enzymatic function and maturation of cytosolic Fe/S proteins (Figure 8B). Since this functional defect affected the assembly of essential Fe/S proteins such as the ABC protein Rli1 (*41*, *48*), a severe growth defect was observed upon Fe/S cluster loss on Nar1 (Figure 8A). Together, these in vivo findings unequivocally demonstrate that association of Fe/S clusters with Nar1 is crucial for its function in cytosolic Fe/S protein maturation.

A set of four cysteine residues (termed the C-terminal motif) is scattered in the central and C-terminal parts of Nar1 and corresponds to residues which hold the H-cluster of iron-only hydrogenases (Figure 1). Single mutations of these cysteine residues caused no growth retardation of yeast cells and no defects in the maturation of cytosolic Fe/S proteins, and Nar1 was able to assemble almost wild-type amounts of Fe/S clusters (Figure 8). Apparently, the four residues individually are not essential for Fe/S cluster coordination and function of Nar1. In contrast, pairwise mutation of these C-terminal cysteine residues resulted in a strong decrease in cell viability. Three different combinations of cysteine mutations elicited similar strong phenotypical consequences. Moreover, the maturation of Leu1 and Rli1 Fe/S proteins was severely hampered. These synergistic effects of the C-terminal Nar1 cysteine residues suggest an important role of this conserved cysteine motif for Nar1 function. Direct measurement of incorporation of ^{55}Fe into doubly mutated Nar1 indicated that the level of Fe/S cluster binding was diminished to ~50% compared to the level in wild-type Nar1 or Nar1 carrying only a single mutation. Since Nar1 function was completely lost in these double mutants, the simplest explanation is that Nar1 binds an essential Fe/S cluster via the C-terminal cysteine motif. Apparently, the N-terminal Fe/S cluster can still remain bound in the absence of the C-terminal one.

The four N-terminal cysteine residues of Nar1 form a typical Fe/S cluster binding motif and thus were likely to coordinate an Fe/S cluster in vivo. Mutations in the two N-terminal cysteine residues, C59 and C62, affected the viability of yeast cells and the function of Nar1 in Fe/S protein maturation. The resulting complete loss of binding of Fe/S clusters to Nar1 indicates that apparently also the C-terminal cysteine motif can no longer stably assemble or bind an Fe/S cluster without the N-terminal one. This differs from the case discussed above. The C65A mutant protein could only partially replace wild-type Nar1, whereas the C20A mutant protein fully rescued both the growth phenotype of Nar1-depleted cells and the function of Nar1. When residues C20 and C65 were simultaneously exchanged (mutant C20A/C65A), the partial complementation observed for the single C65A mutant was lost, resulting in a nonfunctional Nar1 with hardly any Fe/S cluster bound. These data indicate that also the C20 residue is of functional importance due to this synergistic effect. Thus, C20 appears to be an Fe/S cluster-coordinating residue, even though its contribution to cluster

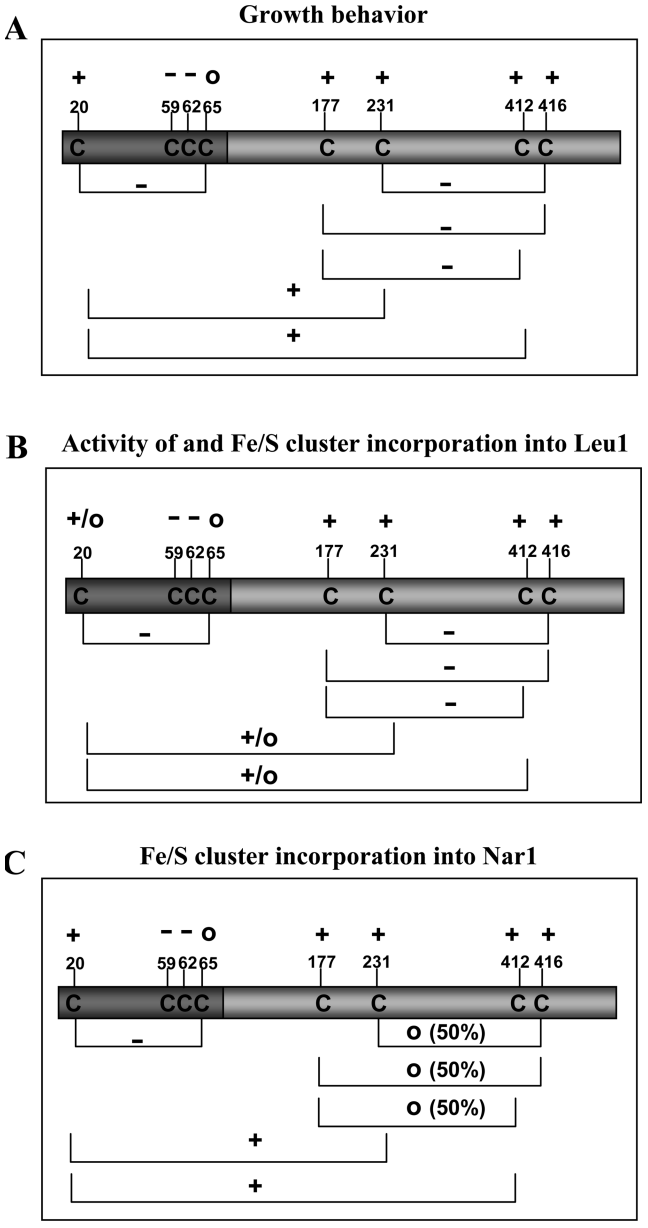

FIGURE 8: Schematic overview of the in vivo data obtained with the Nar1 mutants. The positions of the conserved N- and C-terminal cysteine residues of Nar1 are depicted. The consequences of the various single (above the Nar1 scheme) and double (below) Nar1 cysteine mutations on (A) yeast cell growth, (B) enzyme activity of and incorporation of ^{55}Fe into the cytosolic Fe/S protein Leu1, and (C) binding of ^{55}Fe/S clusters to (mutant) Nar1 are summarized (see Figures 3−6). Results similar to those in panel B were obtained for incorporation of ^{55}Fe into the cytosolic Fe/S protein Rli1. Abbreviations: +, behavior similar to that of the wild type; ○, intermediate effects; −, no growth, function, or Fe/S cluster binding.

stability is not essential, and becomes obvious only in the C65A background. The dispensable character of one or more coordinating residues of Fe/S clusters is a well-known phenomenon. In some cases, another cysteine residue takes over the position of the natural Fe/S cluster ligand (*49*, *50*). Such cysteine ligand swapping seems unlikely in the case of Nar1 as both the primary sequence of Nar1 and the modeled Nar1 structure lack cysteine residues in the vicinity of C20. Alternative candidates for a swap with a non-natural ligand may be residue E7, D9, or D12. In summary, we conclude from our data that the four N-terminal cysteine residues are coordinating ligands for an essential Fe/S cluster in Nar1.

The results of our mutational studies fit nicely to the structural model derived for Nar1 using a bacterial iron-only hydrogenase as a template (Figure 7). The model suggests that all eight conserved cysteine residues at the N- and C-termini are clustered in two patches, showing orientations that could favor the binding of the two Fe/S centers which are located in the immediate vicinity, suggesting a coordinate function. It appears from the model that the N-terminal Fe/S cluster is partially exposed at the surface of the protein (Figure 7A, right panel), while the C-terminal one is completely buried within the protein (Figure 7A, left panel). These observations may provide a logical explanation for why the Fe/S cluster bound to the N-terminal motif is less tightly coordinated than the buried Fe/S cluster at the C-terminus where two cysteine residues have to be exchanged for cluster loss. The surface exposure may explain why the N-terminal cluster is lost more easily, especially upon mutation of residues C59 and C62. With regard to the dependence of the assembly of the C-terminal Fe/S cluster on the N-terminal cysteine motif, we propose that the C-terminal cluster requires transient interaction with the N-terminal binding motif to reach its buried location within the Nar1 protein. Conversely, the N-terminal Fe/S cluster can still bind in the absence of detectable Fe/S cluster occupancy of the C-terminal cysteine motif.

At the molecular level, the function of Nar1 is still unknown. On the basis of the similarity of Nar1 to the iron-only hydrogenases (with regard to the overall amino acid sequence and the binding of two Fe/S clusters to conserved cysteine residues), one can speculate that Nar1 may perform an electron transfer function. Bacterial and algal Fe-only hydrogenases use their bound Fe/S clusters to transfer electrons for the interconversion of protons and hydrogen at the active site, the H-cluster (*21*). At which step of cytosolic Fe/S protein assembly may the putative electron transfer function of Nar1 become effective? Nar1 is not required for the de novo assembly of Fe/S clusters on the cytosolic Cfd1−Nbp35 scaffold yet appears to be essential for transfer of Fe/S clusters to target apoproteins (*13*, *14*). Thus, Nar1 is unlikely to play a role similar to that of the mitochondrial ferredoxin Yah1 which is essential for Fe/S cluster assembly on the Isu1 scaffold, possibly by catalyzing the reduction of sulfur to sulfide (*6*). Instead, Nar1 may supply electrons to facilitate the dislocation of the Fe/S cluster from the Cfd1−Nbp35 scaffold, thus initiating its transfer to apoproteins. The identification of the exact molecular function of yeast Nar1 and the test of its proposed role in electron transfer may require in vitro studies with purified proteins of the cytosolic CIA machinery.

The human genome encodes two relatives of yeast Nar1 termed IOP1 and IOP2 that also show similarities in sequence to Fe-only hydrogenases (*51*). IOP1 was first assumed to modulate the activity of hypoxia-inducible factor 1α that represents the global mediator of the mammalian response to hypoxia. Recently, depletion of the protein was shown to be essential for the formation of cytosolic Fe/S proteins identifying IOP1 as a functional Nar1 orthologue (*18*). Depletion of IOP2 (also termed NARF) was without effects on these Fe/S proteins, suggesting an alternative cellular function. In fact, this protein was reported to bind to prenylated lamin A in the nucleus (*52*) and thus may perform a role in prelamin A processing. At present, it is not known whether the human Nar1-like proteins contain Fe/S clusters, but the conservation of the eight cysteine residues shown here to be critical for binding of Fe/S clusters to yeast Nar1 makes this idea highly likely. It appears from these considerations that, during evolution, Nar1-like proteins have functionally diverged

from their ancestral iron-only hydrogenases to factors involved in different and independent processes. Nevertheless, their function apparently remained dependent on the Fe/S clusters present in both hydrogenases and yeast Nar1.

In summary, our work on *S. cerevisiae* Nar1 has identified two conserved cysteine motifs at the N- and C-termini which serve as essential determinants for the coordination of two Fe/S clusters. Impairment of the association of each Fe/S cluster is detrimental for Nar1 function. Our study indicates that the recombinant Nar1 protein purified from *E. coli* does not contain native Fe/S clusters. Hence, it will be necessary to purify functional Nar1 (and its mutant forms) from its native environment, the eukaryotic cytosol. Such studies are ongoing and will provide insights into the types of Fe/S clusters associated with Nar1, their mode of coordination with the protein, and their interaction. This information will be crucial for further progress on the understanding of the molecular function of Nar1 in the transfer of nascent Fe/S clusters from the scaffold proteins Cfd1 and Nbp35 to target Fe/S apoproteins.

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SUPPORTING INFORMATION AVAILABLE

Characterization of recombinant Nar1 by sizing chromatography and by UV−vis spectroscopy and growth behavior of Nar1 mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

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