Modifications of the Lipoamide-containing Mitochondrial Subproteome in a Yeast Mutant Defective in Cysteine Desulfurase*§

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Comparison and identification of mitochondrial matrix proteins from wild-type and cysteine desulfurase-defective (nfs1-14, carrying a hypomorphic allele of NFS1) yeast strains, using two-dimensional gel electrophoresis coupled to mass spectrometry analyses, revealed large changes in the amounts of various proteins. Protein spots that were specifically increased in the nfs1-14 mutant included subunits of lipoamide-containing enzyme complexes: Kgd2, Lat1, and Gcv3, subunits of the mitochondrial α-ketoglutarate dehydrogenase, pyruvate dehydrogenase, and glycine cleavage system complexes, respectively. Moreover the increased protein spots corresponded to lipoamide-deficient forms in the nfs1-14 mutant. The increased proteins migrated as separate, cathode-shifted spots, consistent with gain of a lysine charge due to lack of lipoamide addition. Lack of lipoylation of these proteins was further validated using an antibody specific for lipoamide-containing proteins. In addition, this antibody revealed a fourth lipoamide-containing protein, probably corresponding to the E2 component of the branched-chain keto acid dehydrogenase complex. Like the lipoamide-containing forms of Kgd2, Lat1, and Gcv3, this protein also showed decreased lipoic acid reactivity in the nfs1-14 mutant. Cysteine desulfurases, such as yeast NFS1, are required for sulfur addition to iron-sulfur clusters and other sulfur-requiring processes. The results demonstrate that Nfs1 protein is required for the proper post-translational modification of the lipoamide-containing mitochondrial subproteome in yeast and pave the road toward a thorough understanding of its precise role in lipoic acid synthesis.


Nfs1 is a protein with cysteine desulfurase activity (1) that is essential in eukaryotes (2, 3). The most abundant localization for Nfs1 is in mitochondria, although extramitochondrial localizations and functions also have been described (2, 4, 5). This pyridoxal phosphate-dependent dimeric enzyme removes sulfur from its substrate cysteine, generating a persulfide intermediate initially bound to the enzyme active site and subsequently donated to recipient proteins (1). The Nfs1 persulfide can also be used for other sulfur-requiring processes in cells. Nfs1 has been implicated in contributing sulfur for thiolation of tRNAs (6), and an allele of Nfs1 was originally identified by its association with tRNA splicing defects (7).

Lipoamide and biotin are important sulfur-containing enzyme cofactors, and in eukaryotes, all known lipoamide-containing proteins are located in the mitochondrial matrix (8–10). Lipoamide cofactor is required for activity of the E2 component of several multisubunit complexes mediating decarboxylation of α-keto organic acids (11) as it forms a tether able to transfer intermediates from one reactive site to another on the enzyme complex. The lipoamide cofactor consists of lipoic acid (a dithiol derivative of octanoic acid) bound by an amide linkage to the ε-amino group of a conserved lysine residue in the peptide backbone of an enzyme subunit. Although a role for Nfs1 in synthesis of biotin in plant mitochondria has been demonstrated recently (12), a similar role in lipoamide biosynthesis has not been shown in any species to date.

The mutant allele nfs1-14 of the essential gene NFS1 of yeast contains an I191S missense mutation associated with decreased Nfs1 protein levels, increased high affinity cellular iron uptake, misregulated mitochondrial iron accumulation, and deficient Fe-S cluster protein activities (3). These varied and pleiotropic phenotypes may be directly or indirectly linked with Nfs1 enzymatic activity. Alternatively they also may be derived from regulatory or toxic effects of iron accumulation. To better characterize the diverse effects of Nfs1 and to evaluate in an unbiased manner the global consequences resulting from loss of function or decreased function of this essential enzyme, we undertook a proteomic approach. The use of the nfs1-14 mutant was attractive because the mutant

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1 The abbreviations used are: E2, specific subunit that contains the lipoic acid in the lipoamide-containing multienzyme complexes; PDC, pyruvate dehydrogenase complex; KGDC, α-ketoglutarate dehydrogenase complex; BCDC, branched-chain keto acid dehydrogenase complex; GCS, glycine cleavage system; nLC, nano-LC; ACTH, adrenocorticotropin hormone; 2D, two-dimensional; 1D, one-dimensional; GE, gel electrophoresis; colloidal blue, colloidal Coomassie Brilliant Blue.
strain grows similarly to wild-type in rich medium, and yet it exhibits mutant phenotypes, such as mitochondrial iron accumulation and partial Fe-S cluster deficiencies. Thus, we surmised that comparing the proteomes of wild-type and nfs1-14 mutant mitochondria might shed light on the involvement of Nfs1 in generating these diverse phenotypes. As the major localization of Nfs1 is in the mitochondrial matrix, we initiated these comparisons using matrix subproteomes, and a number of interesting differences were discovered during the course of these analyses. Of particular interest are changes in the lipoamide-containing subproteome. The results indicate that nfs1-14 mutant mitochondria contain lower amounts of lipoamide-containing proteins concurrent with the accumulation of their lipoamide-deficient immature forms. We therefore conclude that impaired cysteine desulphurase activity severely perturbs the lipoamide-containing mitochondrial subproteome presumably by restricting lipoylation of cognate apoproteins.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Culture Conditions**—The parental yeast strain, a modified version of YPH499, strain 53-75 (MATa ura3-52 lys2-801 amber ade2-101 ochre trpl1ΔΔ3 his3Δ1200 leu2-Δ3 cyh2), was derived from strain YPH499 by growth in the presence of cycloheximide, 100 mg/liter adenine).

**Preparation and Purification of Mitochondria Using Nycodenz Gradients**—Crude yeast mitochondria were prepared as described previously (13). Briefly the equivalent of 6 g wet weight of cells was incubated for 20 min in 30 ml of DTT buffer (0.1 M Tris-SO4, 10 mM dithiothreitol), treated with Zymolase (3 mg/g/ml of cells) for 30 min at 30 °C, and homogenized using a Dounce tissue grinder (Wheaton Science Products, Millville, NJ). Supernatants were collected after a 10-min centrifugation at 1,600 × g to remove nuclei and unbroken cells, and mitochondria were pelletted by subsequent centrifugation at 12,000 × g for 10 min. Crude mitochondria were resuspended in “mito” buffer with osmotic protection (0.6 M sorbitol, 20 mM Tris-Cl, pH 7.4), and the concentrations of mitochondrial proteins were determined by measuring absorbance at 280 nm and comparing with standard proteins. Crude mitochondria were purified using Nycodenz step gradients prepared by sequentially layering 2 ml each of 25, 20, 15, 10, and 5% Nycodenz solution into a 14 × 89-mm thin wall polycarbonate centrifuge tube (Sorvall, Newtown, CT), loading up to 20 ml of crude mitochondrial proteins in 500 μl of mito buffer on top of the gradient, and centrifuging for 30 min at 40,000 rpm (~200,000 × g). Bands at each layer interface were collected, diluted in the mito buffer, and pelleted by centrifugation for 10 min at 12,000 × g, and pellets were resuspended in the mito buffer at desired concentrations (14).

**Fractionation of Mitochondrial Matrix Proteins**—To release the soluble contents of the intermembrane space, intact mitochondria were resuspended in mito buffer were incubated in a hypotonic solution (20 mM Tris, pH 7.4, 0.15 M sorbitol) for 10 min in an ice-water bath. The mitoplasts thus formed were pelleted by centrifugation for 10 min at 12,000 × g, resuspended in the hypotonic buffer, and sonicated three times, for 20 s each, at 4 °C. Supernatants containing the matrix proteins were collected after a 30-min centrifugation at 20,000 × g to pellet the membrane fraction (15).

**Sample Preparation and Two-dimensional Gel Electrophoresis (2D-GE)**—Mitochondrial matrix protein extracts were precipitated on ice at 4 °C by mixing in a 1:1 (v/v) ratio with a precooled (~20 °C) solution of 20% TCA in acetone (w/v). Precipitated proteins were collected by centrifugation at 25,000 × g, and the pellet was washed twice with ice-cold acetone to remove residual TCA and dried under vacuum using a SpeedVac. Pellets were resuspended in 2D-GE sample solubilization buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% SB 3–10, 40 mM Tris, 0.2% Bio-Lyte 3/10, 65 mM DTT) and vortexed gently at room temperature until completely solubilized. Insoluble materials were removed by centrifugation at 25,000 × g at room temperature, and protein concentrations of the supernatants were determined by using the Bradford method (Bio-Rad).

For 2D-GE, samples containing 150 μg of solubilized mitochondrial matrix proteins were applied to 18-cm, pH 3–10 non-linear IPG strips (Bio-Rad), and following a 12-h passive rehydration, IEF was carried out by using a Protein IEF cell (Bio-Rad) at 20 °C at a maximum of 7000 V for 18 h, and thus prepared strips were kept frozen at −20 °C until use. For the second dimension SDS-PAGE, the IPG strips were thawed, reduced for 15 min with 1% (w/v) DTT, and alkylated for 10 min with 2.5% (v/v) iodoacetamide at room temperature, both prepared in equilibration buffer consisting of 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% SDS, 0.02% bromphenol blue. After equilibration, the IPG strips were layered on top of the second dimension resolving gel slabs and overlaid with a solution of molten 0.5% agarose in SDS electrophoresis buffer. The second dimension Laemmli-type SDS-PAGE was carried out using either 11% homogenous or 10–20% gradient separating gels without any stacking gels. Electrophoresis was conducted at 40 mA/gel in a Protein II XL cell (Bio-Rad), and gels were stained with colloidal Coomassie Brilliant Blue (colloidal blue) (16).

**2D Gel Images Analyses**—Following 2D-GE and protein staining, gels were digitized with SilverFast scan software (Epson, Long Beach, CA) on an Epson Expression 1680 flatbed scanner, and the PDQuest 2D gel analysis software (Version 7.1.0, Bio-Rad) was used to process and analyze gel images. For each data analysis set, at least three independently run gels for both wild-type and nfs1-14 mutant strain mitochondrial matrix protein extracts were used. Following automatic detection mode, spots were manually edited so that those that were not present on all replica gels were excluded from analysis. For quasiquantitative comparisons, protein spots observed with the wild-type and nfs1-14 mutant strains were normalized for the total density of each gel after calibration by manual indication of the lowest and highest density spots. Normalized density values were used for comparisons, and spots exhibiting at least a 3-fold increase or decrease were identified by mass spectrometry.

**Sample Preparation and Mass Spectrometry Analyses**—Chosen protein spots were manually excised from the gels and subjected to in-gel trypsin digestion. Briefly gel pieces were destained with 50% CH3CN for 15 min and washed repeatedly for 15 min at room temperature with 100 mM NH4HCO3/CH3CN (50:50, v/v) until completely destained. They were then shrunk for another 5 min by addition of 100% CH3CN, dried in a SpeedVac, and then resuspended in a solution (25 μl/spot) containing 6 ng/μl trypsin (Promega Inc., Madison, WI) in 50 mM NH4HCO3 and incubated at 37 °C for overnight. After incubation, 30 μl of a solution containing 1% HCOOH and 2% CH3CN together was added to each digested sample and vortexed for 30 min at room temperature to complete extraction of digested peptides. After a brief centrifugation at 25,000 × g, the supernatant was collected and stored at −20 °C for mass spectrometric analysis.
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Tryptic peptide extracts were analyzed either by MALDI-TOF-MS using a Micromass M@LDI Reflectron mass spectrometer (Waters/Micromass, Milford, MA) or nano-LC-MS/MS using a Thermo Finnigan LCQ Deca XP Plus mass spectrometer coupled to an Ultimate nano-liquid chromatography system (Thermo Finnigan, San Jose, CA). For the MALDI-TOF-MS, the sandwich method of sample preparation was used. A sample droplet (1.2 µl) was applied on top of a fast evaporated matrix-only bed (0.5 µl of 2.5 mg/ml α-cyano-4-hydroxycinnamic acid in 0.1% (v/v) trifluoroacetic acid, 50% (v/v) acetonitrile) followed by the deposition of a second layer of matrix onto a 96-well stainless steel target plate, sandwiching the sample between two layers of matrix. As needed, prior to MALDI-TOF-MS, reversed-phase Zip-Tips (Millipore, Bedford, MA) were used according to the manufacturer’s instructions for enhanced sample clean-up. For mass calibration, a mixture of angiotensin II (1046.54 Da), angiotensin I (1296.52 Da), substance P (1347.74 Da), Glu-fibrinopeptide B (1383.40 Da), and bradykinin (1084.48 Da) were repeated at least twice both automatically and manually. Calibration was achieved using a Micromass M@LDI Reflectron mass spectrometer (Waters/Micromass). Mass spectra were measured using internal calibration with the ACTH clip-(18–39) peak at m/z 2465.1989 in the lock mass solution, and the calibrated spectra were measured by using a Micromass M@LDI Reflectron mass spectrometer (Waters/Micromass). Protein identifications were assigned by comparing peak lists, generated from peptide mass fingerprinting, to a database containing theoretical peptide sequences for the E2 subunit of α-ketoglutarate dehydrogenase (anti-Kgd2) were gifts from Dr. Alex Tzagoloff (Columbia University, NY) (18), and rabbit polyclonal antibodies against the E2 subunit of α-ketoglutarate dehydrogenase (anti-Kgd2) were gifts from Dr. Alex Tzagoloff (Columbia University, NY) (18), and those against Mir1 (phosphatase carrier protein of mitochondria), cytochrome c, and Ccp1 (cytochrome-c peroxidase) were gifts from Dr. Debkumar Pain (University of Medicine and Dentistry of New Jersey-New Jersey Medical School). Antibodies to Nfs1 (3) and Aco1 (19) were described previously. The rabbit polyclonal antibody raised against lipolipid acid was purchased from Calbiochem (8). Anti-porin antibody was a mouse monoclonal obtained from Molecular Probes/Invitrogen.

RESULTS

Purified Mitochondria and Matrix Fractions from Wild-type and nfs1-14 Mutant—The highly pleiotropic phenotypes of nfs1-14 mutant, including increased cellular iron uptake, iron accumulation in mitochondria, and decreases of mitochondrial Fe-S cluster-containing proteins (3, 20), induced us to compare the mitochondrial matrix proteomes of wild-type and mutant yeast strains. First crude mitochondria were purified by separation on a Nycodenz step gradient (Fig. 1A), and then fractions at the interfaces between 10 and 15% (band 2), 15 and 20% (band 3), and 20 and 25% (band 4) Nycodenz were analyzed by Western blotting to assess their purity. The mitochondrial outer membrane protein porin was present in all fractions from both strains (Fig. 1B), whereas Pgk1, an abundant cytoplasmic protein, or Cpy1, a vacuolar protein marker, were not found in any fraction (not shown). In wild-type preparations, Nfs1 was present in all three mitochondrial fractions although somewhat enriched in bands 3 and 4. As expected, Nfs1 protein was present in much greater abundance in wild-type than in the nfs1-14 mutant strain because this mutant allele destabilizes Nfs1 (3). Aco1 (aconitase), an abundant mitochondrial matrix protein with a 4Fe-4S cluster was preferentially enriched in band 3.
Ccp1 (cytochrome-c peroxidase), which is ordinarily located in the mitochondrial intermembrane space (21), was found primarily in band 3 both in wild-type and nfs1-14 mutant, and much less was found in the other fractions (Fig. 1B). As Ccp1 is released into the supernatant if the mitochondrial outer membrane is ruptured, its copious presence in band 3 reflected the integrity of the mitochondria in this fraction, which was used for preparation of intermembrane space, membrane, and matrix fractions (15). We also noted that the amount of Ccp1 appeared increased in the mutant nfs1-14, possibly indicating response to oxidative stress.

Global Comparison of Wild-type and nfs1-14 Mitochondrial Subfractions—Matrix fractions from wild-type and mutant mitochondria were separated by 2D-GE with the first dimension consisting of isoelectric focusing over a 3–10 non-linear pH gradient and the second dimension consisting of standard SDS-PAGE. The protein spots were identified by colloidal blue staining and compared between the wild-type and mutant using the PDQuest 2D gel analysis program as described under “Experimental Procedures.” This analysis distinguished a minimum of 340 individual spots that were common to both wild-type and nfs1-14 mutant, and one such gel image is presented in Fig. 2. Of these spots, about 310 showed less than 3-fold changes in abundance between the wild-type and the nfs1-14 mutants, and about 60 of them were identified either by MALDI-TOF-MS or by nLC-MS/MS as an identification validity check (Supplemental Table 1S). Of the proteins identified here all but one were previously annotated as being of mitochondrial origin, indicating that the samples used were highly pure and that our identifications were reliable. On the
other hand, several mitochondrial membrane proteins such as Acr2, Atp1, Cyb2, Mas6, and Por1, were detected, indicating that matrix fraction preparations were less pure despite the detergent-free conditions used. A possible source of contamination might be small membrane vesicles formed during the sonication process used for matrix isolation and not eliminated after the centrifugation step. In any event, overall comparisons indicated that at least 15 protein spots showed decreased abundance and at least 11 protein spots showed increased abundance by at least 3-fold in the nfs1-14 mutant in respect to the wild-type strain (Table I).

**Mitochondrial Matrix Proteins Decreased in the nfs1-14 Mutant**

We expected to find decreases in proteins or processes relying on Fe-S clusters in the nfs1-14 mutant because of the established role of Nfs1 in Fe-S cluster synthesis (3, 22, 23).
Mitochondrial Matrix Proteins Increased in the nfs1-14 Mutant—Mitochondrial proteins that were increased in the nfs1-14 mutant included Ssc1, Nar1, Ald4, and Spd1 (Table I). Ssc1 is the major Hsp70 chaperone of mitochondria involved in nuclearly encoded precursor import and subsequent ATP-dependent protein folding (33, 34). Ssq1, a highly homologous Hsp70 protein implicated in Fe-S cluster assembly, interacts with Nfs1 (and Isu1) during Fe-S cluster biosynthesis. Small decreases in Ssc1 abundance have been shown to suppress absence of Ssq1 protein in terms of iron regulatory and Fe-S cluster biosynthetic functions (35). Thus, the increase in Ssc1 abundance seen in the nfs1-14 mutant could represent a copy number plasmid was able to prevent mtDNA damage in an Atm1 temperature-sensitive mutant (24). Atm1 is an ATP-binding cassette half-transporter of the mitochondrial inner membrane implicated in compartmental Fe-S cluster formation and mitochondrial iron homeostasis (25). In other studies, full expression of BAT1 and also ILV5 (encoding another protein involved in branched-chain amino acid biosynthesis) was found to depend on Tpk1, one of three adenylate kinase subunits thought to have a role in controlling mitochondrial iron homeostasis (26). Ilv5 overexpression was able to suppress mtDNA loss due to abf2 mutations (27) similar to effects of Ggc1 overexpression (28). Ggc1 is a mitochondrial carrier protein with exchange activity for GTP and GDP across the mitochondrial inner membrane (29). The Ggc1 deletion mutant accumulates iron in mitochondria and exhibits decreased mitochondrial matrix GAP levels (29). We observed that Abf2 protein was decreased in the nfs1-14 mutant mitochondria (decrease ratio of 0.34) (Table I), although the significance if any of the decrease of Abf2 levels is unclear.

Another protein that was decreased in the nfs1-14 mutant was Mrp21, a nuclearly encoded protein component of the mitochondrial ribosome. The nfs1-14 mutation confers increased frequency of mtDNA damage and mtDNA loss, a condition characterized by complete absence of mitochondrial ribosomal RNA (30). Thus, the effect on mitochondrial ribosomal subunits proteins such as Mrp21 might be secondary to mtDNA damage in the mutant. However, the extent of mtDNA damage in the mutant is unclear as it was able to grow on non-fermentable carbon sources and was rho− by 4′,6-diamidine-2-phenylindole staining. The specific decrease in Mrp21 might reflect sensitivity of this particular ribosomal component to Fe-S cluster deficiency or to iron-mediated damage and turnover. Additional proteins that were decreased in the nfs1-14 mutant included Lap3 and Din7. Lap3 is a leucine aminopeptidase with dual (cytoplasmic and mitochondrial) localizations recently shown to be part of a large protein complex of about 250 kDa (31). Din7 is a DNA damage-inducible protein (32). Whether these decreases reflect secondary effects of impaired Fe-S cluster protein activities, increases in iron accumulation, oxidative stress, or some other effect of the nfs1-14 mutation is unknown and beyond the scope of this work.

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Fig. 3. Western blot analysis of lipoamide-containing proteins. 20 μg of mitochondrial matrix proteins from wild-type and nfs1-14 strains were separated on a 13% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blotted separately with antibodies raised against lipoic acid (anti-LA, upper panel), E2 component of α-ketoglutarate dehydrogenase complex (anti-Kgd2, middle panel), or Nfs1 (anti-Nfs1, lower panel). A short exposure for the anti-lipoic acid antibody blot is shown to the right. To the left of the anti-lipoic acid blot, assignments for reactive bands were made on the basis of predicted sizes from databases (www.yeastgenome.org): Lat1, E2 component of PDC; Kgd2, E2 component of KGDC; Gcv3, H component of GCS. For BCDC E2 component, the corresponding protein and thus its size have not been ascertained and is thus only tentatively labeled as BCDC (38).

For example, the tricarboxylic acid cycle contains critical Fe-S cluster proteins, including aconitase and succinate dehydrogenase. Thus, it was reassuring to observe decreases in several components of the tricarboxylic acid cycle in nfs1-14 mutant in particular in Cit1 (decrease ratio of 0.37) and Idh1 (decrease ratio of 0.17) (Table I). Aconitase (Aco1) was minimally changed at the level of protein abundance, although aconitase activity in the mutant was decreased to 30–50% of the wild-type level (not shown). The data suggested that limitation in availability of the Fe-S clusters was associated with negative regulatory effects on enzyme protein levels perhaps as a result of decreased substrate flux through the tricarboxylic acid cycle. Mitochondrial proteins implicated in branched-chain amino acid metabolism were also decreased in nfs1-14 mutant, including Ilv3 (decrease ratio of 0.27) and Bat1 (decrease ratio of 0.34) (Table I). Ilv3 is the dihydroyx-acid dehydratase producing 2-ketoisovalerate for the branched-chain amino acid biosynthetic superpathway, and Bat1 acts downstream in the same pathway, transferring amino groups from glutamate for each of the three branched-chain amino acids. The connections of branched-chain amino acid metabolism with iron metabolism are not well understood but are highlighted by multiple observations. Overexpression of Bat1 from a high
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Fig. 4. Western blots of 2D gels with anti-lipoic acid antibody (cells grown in rich medium). Mitochondrial matrix proteins from wild-type and nfs1-14 purified mitochondria (equivalent to 150 μg of mitochondrial protein) were separated by IEF in one dimension followed by SDS-PAGE in a second dimension. The gels were stained with colloidal blue, and colloidal blue-stained proteins were electrophoretically transferred to nitrocellulose membranes. Membranes were then incubated with anti-lipoic acid antibody followed by peroxidase-conjugated donkey anti-rabbit antibody, and signals were developed with ECL. Lipoic acid signals of four different sizes were observed as darker staining spots on lighter staining background of colloidal blue-stained spots. The lipoic acid-reactive proteins were labeled as a, b, c, and d, and spots a, b, and d were identified by mass spectrometry to represent Lat1 (E2 subunit of PDC), Kgd2 (E2 subunit of KGDC), and Gcv3 (H subunit of GCS), respectively. Spot c is thought to represent the E2 subunit of BCDC. Arrow a indicates anti-lipoic acid-non-reactive spots for Lat1, arrowhead b indicates a non-reactive spot for Kgd2, open arrow d indicates a non-reactive spot for Gcv3. These are seen only in the nfs1-14 mutant (right panel).

specific compensatory effect, overcoming the loss of activity of Nfs1. Nar1 has been implicated in Fe-S cluster formation in the cytoplasm and nucleus (36, 37) but has not been localized to mitochondria. A dual (mitochondrial and extramitochondrial) localization of Nar1 or its mislocation to mitochondria in nfs1-14 if confirmed could be relevant to Fe-S cluster assembly in different cellular compartments. Finally we observed that Ald4 (increase ratio of 3.2), an aldehyde dehydrogenase and a component of the mitochondrial nucleoid, and Spd1, a stress-inducible tyrosine phosphatase that has not been previously found in mitochondria, were also increased in nfs1-14 mitochondria.

Among the matrix proteins identified here, the largest -fold increases were seen for Gcv3 (increase ratio of 52), Lat1 (increase ratio of 10), and Kgd2 (increase ratio of 6.4) (Table I). Gcv3 is part of the glycine cleavage system (GCS), mediating the cleavage of the amino acid glycine to produce CO₂ and NH₃. The concomitant transfer of a methylene carbon unit to tetrahydrofolate generates the C-1 donor N⁵,N¹⁰-methylene-tetrahydrofolate. Lat1 is part of the pyruvate dehydrogenase complex (PDC) that regulates the entry of carbon into the tricarboxylic acid cycle, mediating the provision of acetyl-CoA. Kgd2 is part of the a-ketoglutarate dehydrogenase complex (KGDC) that acts within the tricarboxylic acid cycle to synthesize succinyl-CoA. We noted that all of these enzyme complexes contain a lipoamide moiety covalently bound to a lysine residue in one of their protein subunits, and in all three cases, the lipoamide-requiring components of the multisubunit enzyme complexes were increased. This striking observation led us to further examine the lipoamide-containing subproteome of yeast.

Lipoamide-containing Subproteome—The availability of a commercial antibody against lipoic acid, capable of specifically recognizing lipoamide-containing proteins, allowed us to examine the lipoamide-containing yeast subproteome. First the same samples previously used for 2D-GE were submitted to 1D-GE and immunoblot analyses with various antibodies. Nfs1 antibodies indicated that, as expected, the abundance of Nfs1 protein was markedly decreased in nfs1-14 mutant compared with wild type (Fig. 3). In agreement with previous data showing that this mutation destabilizes the protein (3), longer exposure of the blot detected trace amounts of Nfs1 protein in the mutant (not shown). Next the antibody against lipoic acid recognized several bands both in the wild-type and the mutant matrix protein extracts, but the reactivity was noticeably diminished in the latter case, reflecting decreased overall lipoylation. Finally polyclonal antibodies against the E2 subunit (Kgd2) of the KGDC (a generous gift from Dr. A. Tzagoloff) allowed us to directly monitor this protein. Although the lipoic acid antibodies were invaluable for detecting the lipoamide-containing subproteome of the mitochondrial matrix proteins, the anti-Kgd2 antibodies made possible the identification and comparison of the Kgd2 protein independently of its lipoic acid content and its gel migration pattern. Based on the molecular weight estimations and comparison with 2D-GE data (Fig. 2), the upper band migrating at about 57 kDa corresponds to Lat1 protein, or the E2 component of PDC. Moving downward in the gel, the apparent doublet (~56 and 54 kDa) of which only the top band was recognized by anti-Kgd2 antibodies corresponds to Kgd2 protein, which is the lipoamide-containing E2 subunit of KGDC. The bottom band of the doublet is likely to correspond to the lipoamide-containing subunit of BCDC (38, 39) (see below). The lipoic acid antibody-reactive bands migrating at 28 and 25 kDa
dicted molecular mass of 19 kDa. Although the lipoic acid retardation in SDS gels as compared with the predicted molecular mass of 19 kDa. Although the lipoic acid reactivity of Kgd2 was slightly lesser in the nfs1-14 mutant, the lipoic acid reactivity was most severely decreased for bands tentatively assigned to Gcv3.

Variants of Lipoamide Proteins with Altered Migration in the nfs1-14 Mutant—Consistent with the 1D gel data, lipoamide-containing proteins of four distinct molecular weights were detected when immunoblots of 2D gels were probed with anti-lipoic acid antibody (Fig. 4, spots a, b, c, and d), and all spots in both wild-type and nfs1-14 mutant extracts were identified by mass spectrometry (Supplemental Table 1S). First, the protein with the largest molecular weight represented Lat1, the E2 subunit of PDC (Fig. 4, spots a). Both wild-type (left panel) and nfs1-14 mutant (right panel) exhibited two anti-lipoic acid reactive spots (molecular weight estimated at 56.7, pl 6.12 and 6.32; Fig. 4, spots a) at this \( M_r \). An additional spot of the same apparent \( M_r \) was only overproduced in the nfs1-14 mutant (upward arrow). This latter cathode-shifted spot was clearly detected in the nfs1-14 mutant sample as a colloidal blue signal on the membrane used for immunoblotting (Fig. 4, upward arrow; molecular weight 56.7, pl 6.50). Second, two anti-lipoic acid-reactive spots (Fig. 4, spots b; molecular weight 55.6; pl 5.75, 5.88, and 6.02) and an additional spot that was non-reactive with anti-lipoic acid antibody, but again only overproduced in the nfs1-14 mutant, were identified by mass spectrometry as Kgd2 protein, the E2 subunit of the PDC (Supplemental Table 1S). Again the latter cathode-shifted spot was clearly discerned in the nfs1-14 mutant sample (Fig. 4, upward arrowhead; molecular weight 55.6, pl 6.16).

Third, two additional anti-lipoic acid antibody-reactive spots, migrating approximately at 53.8 kDa and pl of 5.32 and 5.51 in the wild-type sample (Fig. 4, spots c) but exhibiting markedly reduced reactivity with anti-lipoic acid antibody in the nfs1-14 mutant, were detected. These spots were tentatively attributed to the E2 component of BCDC as this is the only remaining yeast protein predicted to contain lipoamide modification. These spots were poorly resolved in the colloidal blue-stained gels because of more abundant overlapping proteins in this area, rendering unambiguous identification impossible by mass spectrometry. Curiously although genes for this protein have been cloned from other species and the activity has been measured in yeast, the gene corresponding protein for the S. cerevisiae homologue have not been identified (38, 39). Genes and proteins for BCDC from other species, including Arabidopsis (40) and humans, have been identified (41), but apparently they do not have readily identifiable homologues in the yeast genome.

Finally the smallest protein that was reactive with lipoic acid antibody migrated at ~28 kDa and with a very acidic pl between 3 and 4 at the margin of the gel (Fig. 4, spots d). This was identified by mass spectrometry as the Gcv3 protein or the H subunit of the GCS (Supplemental Table 1S). In the nfs1-14 mutant, the reactivity with anti-lipoic acid antibody in this area of the gel was decreased, and a single protein spot identified as Gcv3 protein by mass spectrometry became visible as a colloidal blue-staining spot. This spot was non-reactive with lipoic acid antibody (Fig. 4, open arrow) and migrated more rapidly than its lipoic acid-reactive forms (molecular weight 24.8, pl between 3 and 4) in agreement with the presence of two species of anti-lipoic acid antibody-reactive proteins seen on the 1D gels with only the upper form being present in the nfs1-14 mutant (Fig. 3). In summary, the overall data indicate that, when cysteine desulfurase activity becomes deficient, anti-lipoic acid antibody reactivity decreases, and non-reactive protein forms accumulate concomitantly, reflecting severe perturbations of the mitochondrial lipoamide subproteome.

Closer Examination of Lipoic Acid-reactive and -non-reactive Kgd2 Protein Variants—The availability of both anti-lipoic acid and anti-Kgd2 antibodies made it possible to examine more closely the region of the colloidal blue-stained gel where Kgd2 migrates (molecular mass of 45–70 kDa and pl 5–7). Four anti-Kgd2-reactive spots were identified (Fig. 5, spots 1, 2, 3, and 4 from acidic to basic pl). Indeed mass spectrometry analyses confirmed that all four spots corresponded to Kgd2...
protein, and comparison of the wild-type and nfs1-14 mutant patterns on colloidal blue-stained gels indicated that the spots located at positions 2 and 3 were attenuated in the mutant, whereas the spot at position 4 was increased in intensity. It is noteworthy that it was the spot at this position 4 that was initially identified by 2D-GE analyses as specifically increased in the nfs1-14 mutant.

Probing membranes first with anti-lipoic acid antibody and then, without its removal, reprobing with anti-Kgd2 antibodies (Fig. 5) showed that in the case of the wild type all four spots seen with the colloidal blue staining also reacted with anti-lipoic acid antibody. The intensity of the reactivity correlated with the protein abundance with the most intense signal associated with spot 3, a lesser signal from spot 2, and even lesser signals from spots 1 and 4. The subsequent probing of the same blot with anti-Kgd2 antibodies gave a pattern similar to that observed with anti-lipoic antibody (Fig. 5, wild type, middle and lower panels). By contrast, blots obtained using the nfs1-14 mutant extracts showed overall decreased anti-lipoic acid antibody reactivity as before. In particular, very little reactivity was seen with spot 4, which still reacted strongly with the anti-Kgd2 antibodies, consistent with its intense staining with colloidal blue. Thus, only for spot 4 of nfs1-14 mutant extracts was there a lack of correlation between the reactivity of the anti-lipoic acid and anti-Kgd2 antibodies (Fig. 5, nfs1-14, middle and lower panels). As the former antibody is highly specific for lipoic acid and as all four spots have been identified as Kgd2 protein (Supplemental Table 1S), we therefore concluded that spot 4 in the nfs1-14 mutant corresponded to a Kgd2 protein variant devoid of its natural lipoamide modification. These findings established that, at least in the case of Kgd2 and most likely also Lat1 and Gcv3, lipoamide-containing forms of these proteins were decreased at the expense of the accumulation of their non-lipoylated forms.

**DISCUSSION**

In this study, we aimed to elucidate the multiple functions of the yeast cysteine desulfurase, NFS1, which is encoded by an essential gene. We used a global approach, using 2D-GE and mass spectrometry, to compare the proteomes of yeast mitochondrial matrix fractions from wild type and the congenic nfs1-14 mutant carrying a hypomorphic allele of NFS1 (3). This comparison revealed a number of interesting differences between these proteomes beyond the predictable decrease of several proteins that rely on mitochondrial synthesis of iron-sulfur clusters. Perhaps most intriguing was a set of proteins that became more abundant in the mitochondrial extracts of the nfs1-14 mutant. These spots were identified as lipoamide-lacking variants of matrix proteins that require lipoamide for their enzymatic function.

Under physiological conditions, lipoamide is covalently attached to the ε-amino group of lysine residues in the subunit proteins of mitochondrial complexes involved in oxidative decarboxylation of various substrates. During isoelectric focusing, loss of a free amino group via cofactor addition would shift the modified protein toward the anode, and failure to do so would generate an apoprotein with relatively greater mobility toward the cathode. The appearance in the colloidal blue-stained gels of mutant mitochondria of a new cathode-shifted protein spot identified as Kgd2, the E2 subunit of KGDC, was a first clue indicating that a Kgd2 variant devoid of lipoamide was appearing in the mutant. The lack of reactivity of this spot with anti-lipoic acid antibody further supported that it contained no lipoamide group. However, as we were so far unable to reproducibly detect specific lipoic acid-containing, or -lacking, peptides of Kgd2 protein by mass spectrometry, whether spot 4 indeed corresponds to an unmodified, or a differently modified, variant of Kdg2 remains unknown.

Further investigation of the lipoamide-containing subproteome indicated that three additional proteins were similarly deficient in lipoic acid content to varying degrees in the leaky nfs1-14 mutant. Lat1 protein, which corresponds to the E2 subunit of the PDC, appeared in the mutant as a new cathode-shifted protein spot that was non-reactive with anti-lipoic acid antibody. Similarly the probable E2 subunit of the BCDC also showed decreased reactivity for anti-lipoic acid antibody in the mutant, although the lipoic acid-non-reactive form was difficult to identify due to proximity of abundant interfering proteins in the 2D gels. Finally Gcv3 corresponding to the H protein of the GCS appeared in the nfs1-14 mutant as a spot that stained intensely with colloidal blue but that showed markedly decreased reactivity with anti-lipoic acid antibody, representing a variant of Gcv3 devoid of its lipoamide adduct.

A role for cysteine desulfurase, including yeast Nfs1, in the lipoic acid addition to cognate proteins has not been demonstrated previously. The biosynthesis of lipoic acid has been characterized mostly in prokaryotes, and work has focused on a critical Fe-S cluster-containing enzyme, LipA (42, 43). The yeast homolog of LipA, Lip5, is a mitochondrial protein sharing 43% amino acid identity with its prokaryotic homologue. Lipoamide contains critical sulfur atoms, and the biosynthetic process is thought to proceed via the Fe-S-containing protein Lip5 (42–44). Thus as Nfs1 is the sole cysteine desulfurase in yeast (3, 6, 12), its involvement in lipoamide formation could be envisioned. By analogy with bacterial LipA, *S. cerevisiae* Lip5 probably has two Fe-S clusters, one for activation of S-adenosylmethionine and the other for donating sulfur to the octanoic acid of the apoprotein, as has recently been worked out for biotin synthase and even more recently for LipA. The biochemical steps of this process have been elucidated using bacterial proteins (45). Accordingly S-adenosylmethionine radicals are produced by reduction of a Fe-S cluster in LipA, and then radical chemistry is used to activate the C-6 and C-8 carbons of octanoic acid. Subsequently sulfurs derived from the second buried cluster in LipA are covalently attached to C-8 and then at C-6. The lipoamide cofactor thus formed then remains attached to specific lysine residues of the lipoamide
enzymes (46). Although these reaction sequences have not yet been established in the case of yeast Lip5 enzyme, accumulation of lipoamide-containing protein subunits that lack reactivity to anti-lipoic acid antibody in the nfs1-14 mutant suggests that the eukaryotic Nfs1 is required for lipoylation of proteins. Although the exact role of Nfs1 in lipoamide synthesis is unknown, this process is likely to proceed via an Nfs1-dependent liberation of sulfur from cysteine, which is required for the formation of the Fe-S clusters of Lip5. By analogy with biotin synthase (47), sulfur derived from one of the Lip5 clusters (the buried 4Fe-4S cluster) might act as the immediate sulfur donor for lipoamide formation as has been suggested for LipA (48).

How homeostasis of lipoic acid is controlled in mitochondria is an intriguing issue. In physiological situations, such as in mitochondria from the wild-type strain grown in rich medium, apoproteins of the lipoamide enzymes are not present. This observation suggests that sufficient lipoic acid is produced and efficiently ligated to all cognate apoproteins or that apoprotein synthesis and degradation are tightly coupled to lipoic acid availability. However, in the nfs1-14 mutant, both the apo and apo forms of the E2 subunits of PDC and KGDC complexes coexist due to inadequate cysteine desulfurase activity. This finding implies that precursor apoprotein levels are not directly coupled to lipoic acid availability and that the nfs1-14 mutant is perhaps the first example of uncoupling the two processes. The homeostatic mechanisms by which lipoic acid availability is kept in line with needs for various lipoamide-requiring proteins remain unknown but are likely to involve one or more Fe-S cluster substrates of Nfs1. As a characteristic feature of the nfs1-14 mutant is its pleiotropic phenotypes, further studies of the mitochondrial proteome or other subproteomes of this mutant might help to elucidate the molecular basis of some of its phenotypes, such as iron accumulation in mitochondria.

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Lipoamide-containing Enzymes in Cysteine Desulfurase Mutant


