

YajL, Prokaryotic Homolog of Parkinsonism-associated Protein DJ-1, Functions as a Covalent Chaperone for Thiol Proteome*

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Hai-Tuong Le^{‡1}, Valérie Gautier^{‡1}, Fatoum Kthiri^{‡§}, Abderrahim Malki^{‡¶1}, Nadia Messaoudi^{‡§}, Mouadh Mihoub^{‡§}, Ahmed Landoulsi[§], Young Jun An^{||}, Sun-Shin Cha^{||}, and Gilbert Richarme^{‡2}

From the [‡]Stress Molecules Group, Institut Jacques Monod, Université Paris 7/CNRS, 15 rue Hélène Brion, 75013 Paris, France, the ^{||}Marine Biotechnology Research Center, Korea Ocean Research and Development Institute, Ansan 426744, Korea, the [¶]Faculté des Sciences Ben M'Sik, Université Hassan II-Mohammedia, Casablanca, Morocco, and the [§]Laboratoire de Biochimie et Biologie Moléculaire, 03/UR/0902, Faculté des Sciences de Bizerte, Bizerta, Tunisia

Background: A novel function for YajL, the prokaryotic homolog of the Parkinsonism-associated protein DJ-1.

Results: YajL and DJ-1 form mixed disulfides with members of the thiol proteome.

Conclusion: This covalent chaperone function supports their role in oxidative stress protection.

Significance: There is an exciting encounter between the crucial cysteine 106 of these covalent chaperones and the oxidized cysteines of their substrates.

YajL is the closest *Escherichia coli* homolog of the Parkinsonism-associated protein DJ-1, a multifunctional oxidative stress response protein whose biochemical function remains unclear. We recently reported the aggregation of proteins in a *yajL* mutant in an oxidative stress-dependent manner and that YajL exhibits chaperone activity. Here, we show that YajL displays covalent chaperone and weak protein oxidoreductase activities that are dependent on its exposed cysteine 106. It catalyzes reduced RNase oxidation and scrambled RNase isomerization and insulin reduction and forms mixed disulfides with many cellular proteins upon oxidative stress. The formation of mixed disulfides was detected by immunoblotting bacterial extracts with anti-YajL antibodies under nonreducing conditions. Disulfides were purified from bacterial extracts on a YajL affinity column, separated by nonreducing-reducing SDS-PAGE, and identified by mass spectrometry. Covalent YajL substrates included ribosomal proteins, aminoacyl-tRNA synthetases, chaperones, catalases, peroxidases, and other proteins containing cysteines essential for catalysis or FeS cluster binding, such as glyceraldehyde-3-phosphate dehydrogenase, aldehyde dehydrogenase, aconitase, and FeS cluster-containing subunits of respiratory chains. In addition, we show that DJ-1 also forms mixed disulfides with cytoplasmic proteins upon oxidative stress. These results shed light on the oxidative stress-dependent chaperone function of YajL and identify YajL substrates involved in translation, stress protection, protein solubilization, and metabolism. They reveal a crucial role for cysteine 106 and suggest that DJ-1 also functions as a covalent chaperone. These findings are consistent with several defects observed in *yajL* or DJ-1 mutants,

including translational defects, protein aggregation, oxidative stress sensitivity, and metabolic deficiencies.

The gene that encodes YajL has close homologs in many prokaryotes and eukaryotes. YajL is a member of the DJ-1/Hsp31/PfpI superfamily that includes peptidases (1), chaperones (2), and the Parkinson disease protein DJ-1 (3, 4). All members of this superfamily contain a similar domain with a nucleophilic elbow displaying an important cysteine (Cys-106 in DJ-1 and YajL) that is part of a Cys, His, Glu/Asp catalytic triad in the peptidases PfpI and Hsp31³ (5, 6). In other members of the family, such as DJ-1 and YajL, this Cys residue plays an important role in oxidative stress resistance (3, 4, 7).

The crystal structures of YajL and DJ-1 have been solved (8, 9) and are remarkably similar, with essentially identical backbone structures (0.9 Å C_α root mean square deviation). Both YajL and DJ-1 lack the Cys, His, Asp/Glu putative catalytic triad, and their nucleophilic elbow cysteine is oxidized in crystals to cysteine sulfenic or sulfinic acid (6, 8, 9). This conserved cysteine is crucial for the protective functions of DJ-1 and YajL against oxidative stress (3, 4, 7). Many biochemical functions have been proposed for DJ-1. It has been suggested to function as a weak protease (4), an oxidative stress-activated chaperone (6, 10, 11), an atypical peroxiredoxin-like peroxidase that scavenges H₂O₂ (12), a stabilizer of the antioxidant transcriptional regulator Nrf2 (13), an apoptosis inhibitor via its interaction with Daxx (14), a transcriptional or translational (4, 15) regulator of gene expression, and a regulator of uncoupling protein expression affecting mitochondrial potential and production of reactive oxygen species (16). YajL protects bacteria against oxidative stress and oxidative stress-induced protein aggregation, possibly through its chaperone function and control of gene

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¹ Both authors contributed equally to this work.

² To whom correspondence should be addressed. Tel.: 33-01-57-27-80-57; Fax: 33-01-57-27-81-01; E-mail: richarme@paris7.jussieu.fr.

³ The abbreviations used are: Hsp, heat shock protein; PDI, protein disulfide isomerase.

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expression (7). Moreover, *yajL* mutants display translational accuracy defects (17).

In vitro, DJ-1 exhibits a chaperone activity for citrate synthase and luciferase (6, 11) and for synuclein under oxidizing conditions (10). Contradictory results previously identified either Cys-53 or Cys-106 as the key residue for the chaperone function of DJ-1 (10, 11). *In vivo* studies of the DJ-1 chaperone activity produced mixed results (10, 18) so that the significance of this function in protecting cells against oxidative stress is not yet clear (19). YajL exhibits a chaperone activity toward citrate synthase and the ribosomal proteins S1 and L3, and protein aggregation occurs in the *yajL* mutant under aerobic conditions but not in anaerobiosis (7).

In both DJ-1 and YajL, cysteine 106 is required for protecting cells against oxidative stress (7, 19). It is easily oxidizable to a sulfenic acid form, but it is not clear whether this oxidation is important for the function of these proteins, or whether it is incidental or even detrimental (19). Cysteine 106 of DJ-1 has a low pK_a value of ~ 5 and might function as a potent nucleophile (19, 20). The two other cysteines of DJ-1, Cys-47 and Cys-53, have not been reported to play essential roles (except in Ref. 10). YajL possesses 4 cysteines (Cys-8, Cys-47, Cys-81, and Cys-106), of which only Cys-106 is conserved in all YajL variants.

In the present work, we show that YajL displays a weak protein oxidoreductase activity and functions as a covalent chaperone by forming mixed disulfides with many cellular proteins upon oxidative stress, most of which belong to the cellular thiol proteome (21, 22) and are involved in stress protection. Finally, we show that DJ-1 also displays protein oxidoreductase and covalent chaperone activities.

EXPERIMENTAL PROCEDURES

YajL Expression and Purification—The *yajL*-disrupted strain JW5067 and the YajL expression strain containing plasmid pCA24N-*yajL* (23) were kindly provided by Dr. Mori (Nara Institute of Sciences and Technology, Japan). The YajL C106A and YajL C47A mutants were constructed by *in vitro* site-directed mutagenesis of the appropriate codon in the pCA24N-*yajL* plasmid (7). YajL, YajL_{C106A}, and YajL_{C47A} were purified using DEAE-Sephacel and hydroxyapatite chromatography (7). The multimeric states of YajL, YajL_{C106A}, and YajL_{C47A} were investigated by gel filtration of the purified proteins (1 mg/ml) on a Bio-Gel P200 column (1-ml bed volume, flow rate 50 μ l/min) equilibrated in 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, at 20 °C (molecular weight markers were from Bio-Rad).

DJ-1 Expression and Purification—The DJ-1 gene was amplified by polymerase chain reaction from a human kidney cDNA library (6). The gene was inserted downstream of the T7 promoter of the expression plasmid pET-21a, and the plasmid was introduced in *Escherichia coli* strain BL21 (DE3). For the analysis of mixed disulfides between DJ-1 and *E. coli* proteins, cells were grown at 37 °C in LB medium to an A_{600} of 0.6, and expression of DJ-1 was induced for 40 min with 1 mM isopropyl-D-thiogalactoside. For DJ-1 purification, cells were grown at 37 °C in LB medium to an A_{600} of 0.6, and expression of DJ-1 was induced for 4 h with 0.5 mM isopropyl-D-thiogalactoside. Cells were lysed by sonication, and DJ-1 was purified on Q-Sepharose

and SP-Sepharose fast flow columns (Amersham Biosciences). The DJ-1 C106S mutant was constructed by *in vitro* site-directed mutagenesis of the appropriate codon in the pET-21a-*DJ-1* plasmid (6, 7).

Rescue of *yajL* Mutant by YajL- and DJ-1-overproducing Plasmids—For rescue of aconitase B and NADH dehydrogenase 1 activities in the *yajL* mutant by the *yajL* and *DJ-1* plasmids, we used a *yajL* derivative of the *E. coli* strain BL21 (DE3) (constructed by P1 transduction of the *yajL* mutation into BL21 (DE3)) transformed by plasmids pCA24N-*yajL* or pET-21a-*DJ-1*.

Reactivation of Reduced and Scrambled RNases and Insulin Reduction Assay—Preparation of reduced and scrambled RNases from RNase A (Sigma) and the RNase assay were performed in oxygen-free buffers as described previously (24). Before use, YajL was incubated for 30 min with 30 mM dithiothreitol and isolated by gel filtration through a Bio-Gel P10 column (Bio-Rad) equilibrated with oxygen-free 0.1 M potassium phosphate, pH 6.8, and 1 mM EDTA (24). Reactivation of reduced RNase by air was initiated by diluting the reduced enzyme in air-containing buffer at 23 °C (0.1 M Tris, pH 7.4, 1 mM EDTA) in the presence of thioredoxin or YajL as indicated (24). Reactivation of scrambled RNase was initiated by diluting scrambled RNase in buffer (100 mM Tris, pH 7.4, 1 mM EDTA, 60 μ M dithiothreitol) at 23 °C in the presence of thioredoxin, YajL, or DJ-1 (24). Insulin reduction was assayed by measuring the increase in absorbance at 650 nm as described previously (24).

NADH Dehydrogenase I Assay—NADH dehydrogenase I was measured by following the reduction of 2,6-dichlorophenolindophenol at 600 nm with deamino-NADH as substrate. The assay mixture contained the following in a final volume of 1 ml: 0.1 M Tris, pH 7.5, 100 μ M deamino-NADH (a substrate that is specific for NADH dehydrogenase I (25)), 30 mM KCN, and 10 μ g of membranes prepared by the French press procedure (26).

Aconitase A and B Assay—Extracts from the *yajL* mutant and the parental strain, prepared by ultrasonic disruption of cells (27), were exposed to 300 μ M EDTA (EDTA specifically inactivates AcnB), and at the indicated time points, the remaining aconitase activity was assayed anaerobically by the production of *cis*-aconitate, as described in Ref. 27.

Immunodetection of Mixed Disulfides between YajL, YhbO, DJ-1, and Endogenous *E. coli* Proteins—YajL-, YajL_{C106A}-, YajL_{C47A}-, YhbO-, DJ-1-, and DJ-1_{C106S}-overproducing cells were grown at 37 °C in LB medium, and YajL, YhbO, or DJ-1 was induced for 40 min with 1 mM isopropyl-1-thio- β -D-galactopyranoside. Mid-exponential phase cells were exposed to oxidative stress (0–2 mM hydrogen peroxide for 10 min) and immediately lysed by ultrasonic disruption (7) in a buffer containing 30 mM Tris, pH 7.4, and 30 mM NaCl, with 10 mM *N*-ethylmaleimide added to block disulfide interchange (28). Crude bacterial extracts (supernatants obtained following centrifugation at 8000 \times g) were separated by SDS-PAGE, either in the absence or in the presence of 100 mM dithiothreitol. The proteins were then transferred to nitrocellulose membranes and probed with anti-YajL (Eurogentec), anti-YhbO (Eurogentec), anti-DJ-1 (Abcam) (these antibodies did not cross-react), anti-

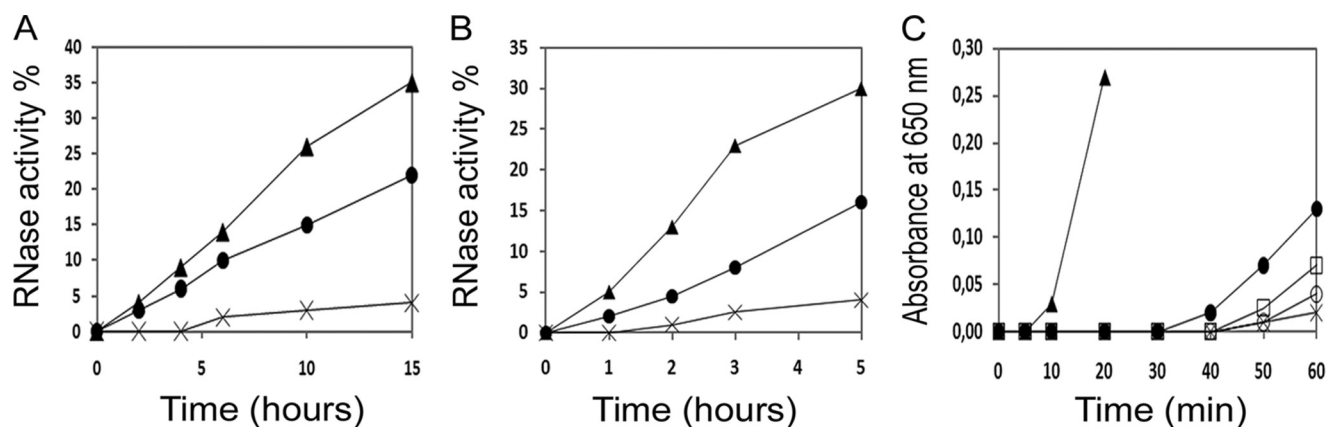


FIGURE 1. **Protein oxidoreductase activities of YajL.** *A*, oxidative folding of reduced, denatured RNase by air. Mixtures containing air-saturated buffer (0.1 M Tris, pH 7.4, 1 mM EDTA) and 30 μ M reduced, denatured RNase alone (crosses) or in the presence of 30 μ M YajL (closed circles) or 30 μ M thioredoxin (triangles) were assayed for RNase activity at the times indicated. *B*, refolding of scrambled RNase. Mixtures containing 60 μ M dithiothreitol and 30 μ M scrambled RNase A alone (crosses) or in the presence of 30 μ M YajL (filled circles) or 30 μ M thioredoxin (triangles) were assayed for RNase activity at the times indicated. *C*, insulin reduction by dithiothreitol. Incubation mixtures containing 0.3 mM dithiothreitol alone (crosses) or in the presence of 5 μ M YajL (filled circles), 5 μ M YajL_{C106A} (open circles), 5 μ M YajL_{C47A} (open squares) or 3 μ M thioredoxin (triangles) were assayed for insulin (170 μ M) reduction, and precipitation was monitored by measuring optical density at 650 nm. These experiments were repeated three times and gave similar results.

AcnB (from Dr. J. R. Guest), and anti-NuoG (from Dr. T. Friedrich) antibodies.

Characterization of YajL Covalent Substrates—YajL-, YajL_{C106A}-, and YajL_{C47A}-overproducing strains were grown at 37 °C in 400 ml of LB medium, and YajL was induced by the addition of 1 mM isopropyl-1-thio- β -D-galactopyranoside for 40 min. Mid-exponential cells were exposed to 5 mM hydrogen peroxide for 5 min and immediately treated with 10% TCA. TCA pellets were resolubilized for 2 h at 37 °C in 6 ml of buffer A (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM iodoacetamide, 1% SDS), and YajL from each strain was purified via its polyhistidine tag on a nickel column (His-Select nickel affinity gel from Sigma, 1-ml bed volume) equilibrated in 50 mM sodium phosphate, pH 8.0, 100 mM NaCl, 10 mM iodoacetamide, and 0.3% SDS. YajL was eluted from each column with an imidazole gradient (20–200 mM), and the eluates were analyzed by SDS-PAGE (under nonreducing or reducing conditions) and by nonreducing-reducing two-dimensional SDS-PAGE as follows. Proteins were resolved by SDS-PAGE (10% polyacrylamide) in the first dimension; after electrophoresis, gel lanes were cut and immersed in SDS sample buffer containing 100 mM DTT at 30 °C for 30 min. Each gel strip was placed at the top of a 12% SDS-polyacrylamide gel and bridged by a 0.6% agarose gel in 100 mM Tris, pH 6.8. After electrophoresis, proteins were silver-stained and characterized by MALDI/TOF/TOF mass spectrometry (Applied Biosystems proteomics analyzer) as described previously (7).

RESULTS

Protein Oxidoreductase Activities of YajL—Protein disulfide isomerases, such as thioredoxin, PDI, and DsbC, catalyze three typical redox reactions: oxidative folding of reduced RNase, refolding of scrambled RNase, and reduction of insulin disulfide bridges (29, 30). YajL catalyzed the oxidative folding of reduced RNase by air with an efficiency ~2-fold lower than that of thioredoxin (Fig. 1A) and reactivated scrambled RNase (a randomly oxidized RNase) with an efficiency ~3-fold lower than that of thioredoxin (Fig. 1B).

YajL (5 μ M) also catalyzed insulin reduction in a dithiothreitol-dependent manner, leading to insulin precipitation after 30 min, whereas 3 μ M thioredoxin or dithiothreitol alone led to insulin precipitation at 12 and 50 min, respectively (Fig. 1C). YajL_{C106A} was unable to stimulate insulin reduction by dithiothreitol (Fig. 1C), and YajL_{C47A} displayed a lower activity than wild-type YajL (Fig. 1C).

Thus, YajL displays the three characteristic activities of protein disulfide isomerases, but its protein oxidoreductase activities are weaker than those of thioredoxin, DsbC, and PDI. Moreover, Cys-106 is required, in accordance with the reactivity of Cys-106 in DJ-1 and its low pK_a (19). The weaker activity of the C47A mutant, relative to that of wild-type YajL, suggests that this cysteine might play a similar structural function to that of Cys-46 in DJ-1 involved in dimer formation (the position of Cys-47 in the three-dimensional structure of YajL (9) makes it unlikely that it functions like the resolving cysteine of thioredoxins). We investigated the quaternary structures of YajL, YajL_{C106A}, and YajL_{C47A} by gel permeation on a Bio-Gel P200 column, as described under "Experimental Procedures." We found that although YajL and YajL_{C106A} migrated as dimers, with apparent molecular masses of 52 and 50 kDa, respectively, YajL_{C47A} migrated as a monomer, with an apparent molecular mass of 26 kDa. This result is in accordance with a role of Cys-47 in YajL dimer formation, which is similar to that of cysteine 46 in DJ-1 dimer formation (not shown).

Formation of Mixed Disulfides between YajL and *E. coli* Proteins—To test the formation of mixed disulfides between YajL and *E. coli* proteins *in vivo*, YajL-overproducing bacteria were lysed (either before or after hydrogen peroxide stress) in buffer containing *N*-ethylmaleimide. The crude bacterial extracts were analyzed by nonreducing or reducing SDS-PAGE followed by electrotransfer onto nitrocellulose membranes and immunodetection with anti-YajL antibodies (28). When cellular extracts were electrophoresed under nonreducing conditions, anti-YajL antibodies decorated monomeric YajL, dimeric YajL, and more than 10 additional bands. These bands were not

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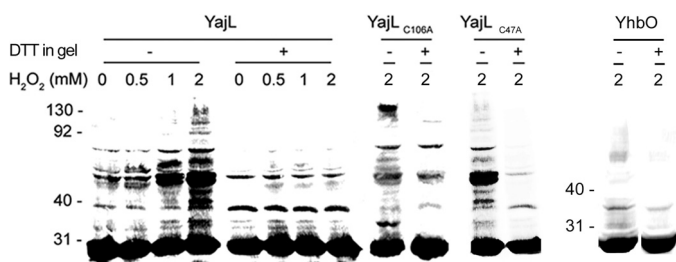


FIGURE 2. Formation of mixed disulfides between YajL and cytoplasmic proteins. YajL-, YajL_{C106A}-, YajL_{C47A}-, and YhbO-overproducing strains were incubated with 0–2 mM hydrogen peroxide for 10 min, and the lysates were separated on SDS-polyacrylamide gels under nonreducing or reducing (+100 mM DTT) conditions. The proteins were then transferred to a nitrocellulose membrane and labeled with anti-YajL antibodies.

detected in the unstressed extract, and their intensity increased with increasing hydrogen peroxide concentrations (Fig. 2). Under reducing conditions, antibodies reacted with monomeric YajL and with a low amount of nonreduced dimer, but most of the additional bands were no longer present (Fig. 2), suggesting that under nonreducing conditions, the additional bands represent mixed disulfides of YajL and its protein substrates.

Analysis of cell lysates from the YajL_{C106A}- and YajL_{C47A}-overproducing strains revealed that YajL_{C106A} and YajL_{C47A} formed small and intermediate amounts, respectively, of mixed disulfides (Fig. 2). This suggests that Cys-106 is the main cysteine involved in disulfide formation between YajL and client proteins (the formation of a mixed disulfide between DJ-1 and ASK1 also requires Cys-106 and is only slightly decreased in the C46A mutant (31)).

The oxidative stress response protein YhbO (32) is a YajL homolog that contains a nucleophilic cysteine (Cys-104) as part of a putative Cys-His-Asp catalytic triad (5) but does not belong to the YajL/DJ-1 subfamily (it belongs instead to the PfpI peptidase subfamily, and in contrast with YajL and DJ-1, it does not display classical chaperone properties (32)). Note that YhbO did not form significant amounts of mixed disulfides with *E. coli* proteins (Fig. 2), suggesting that this function is specific to YajL and to DJ-1 (see below).

To examine the stability of mixed disulfides *in vivo*, the cells were exposed to 3 mM hydrogen peroxide, and increasing DTT concentrations (0–10 mM) were added for 15 min prior to cell lysis. The amounts of mixed disulfides between YajL and client proteins decreased with increasing DTT concentrations (Fig. 3), suggesting that their formation is reversible.

Together, these results suggest that upon oxidative stress, YajL forms mixed disulfides with many cytoplasmic proteins via its conserved cysteine 106. Protein disulfide isomerases with a CXXC active site do not form stable mixed disulfides with substrate proteins because mixed disulfides formed via their first cysteine are quickly resolved by the second cysteine. Mutants of the second cysteine have been used to characterize these disulfides (33). Several endoplasmic reticulum PDIs with a monothiol active site can form stable mixed disulfides with client proteins. Erp44 retains incompletely folded immunoglobulins and adiponectin in the endoplasmic reticulum (28); PDILT (protein disulfide isomerase-like of the testis) engages in disulfide-dependent interactions with a few uncharacterized substrates in testis (34); and AGR2 (anterior gradient homolog

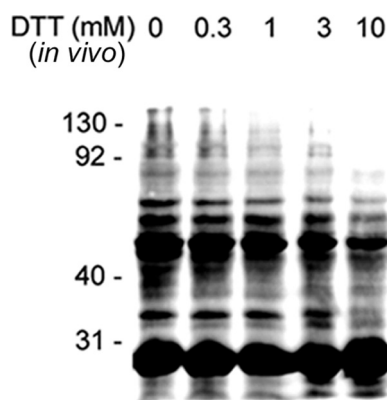


FIGURE 3. *In vivo* reversal by DTT of mixed disulfide formation between YajL and its substrate proteins. YajL-overproducing cells were submitted to a 3 mM hydrogen peroxide stress for 10 min and then treated with DTT at the indicated concentrations for 15 min. Lysates were resolved in SDS-polyacrylamide gel under nonreducing conditions, transferred to a nitrocellulose filter, and labeled with anti-YajL antibodies.

2) forms mixed disulfides with the cysteine-rich intestinal glycoprotein MUC2 that forms the protective mucus gel lining the intestine (35). Each of these covalent chaperones is involved in oxidative protein folding in the endoplasmic reticulum (36). By contrast, YajL, which is located in the cytoplasm, forms mixed disulfide with many proteins upon oxidative stress and likely protects them against oxidative stress and aggregation, in accordance with its roles in oxidative stress resistance and protein solubilization (7).

Characterization of YajL Covalent Partners—The YajL-, YajL_{C106A}-, and YajL_{C47A}-overproducing cells were exposed to 3 mM hydrogen peroxide for 5 min and then immediately treated with TCA. TCA pellets were resolubilized in buffer, and YajL, YajL_{C106A}, and YajL_{C47A} (and their covalently linked substrates) were purified via binding of their polyhistidine tag to nickel affinity columns. They were eluted with imidazole, and the eluates were analyzed by SDS-PAGE (either nonreducing or reducing (28)) and by nonreducing-reducing two-dimensional SDS-PAGE (33, 37).

In nonreducing SDS-PAGE (Fig. 4A), the YajL eluate displayed two primary bands that represent monomeric and dimeric YajL and a number of additional bands in the upper part of the gel. The YajL_{C106A} eluate did not display significant amounts of these additional upper bands, suggesting that it is impaired in forming mixed disulfides, whereas the YajL_{C47A} eluate displayed a significant number of additional bands (Fig. 4A). The addition of the reducing agent dithiothreitol to the YajL eluate before electrophoresis led to the disappearance of most of these bands (Fig. 4A), which suggests that the upper bands corresponded to YajL bound to unknown proteins.

The YajL eluate was also analyzed by nonreducing-reducing two-dimensional SDS-PAGE, in which the first dimension was nonreducing and the second was reducing. As shown in Fig. 4B, the eluate displayed a major off-diagonal line that represents covalent partners released from YajL and weaker diagonals that represent covalent partners released from YajL migrating as oligomers in the first dimension. The proteins in each spot on these off-diagonal lines were analyzed by mass spectrometry. In all, more than 50 proteins were characterized (Table 1). They included ribo-

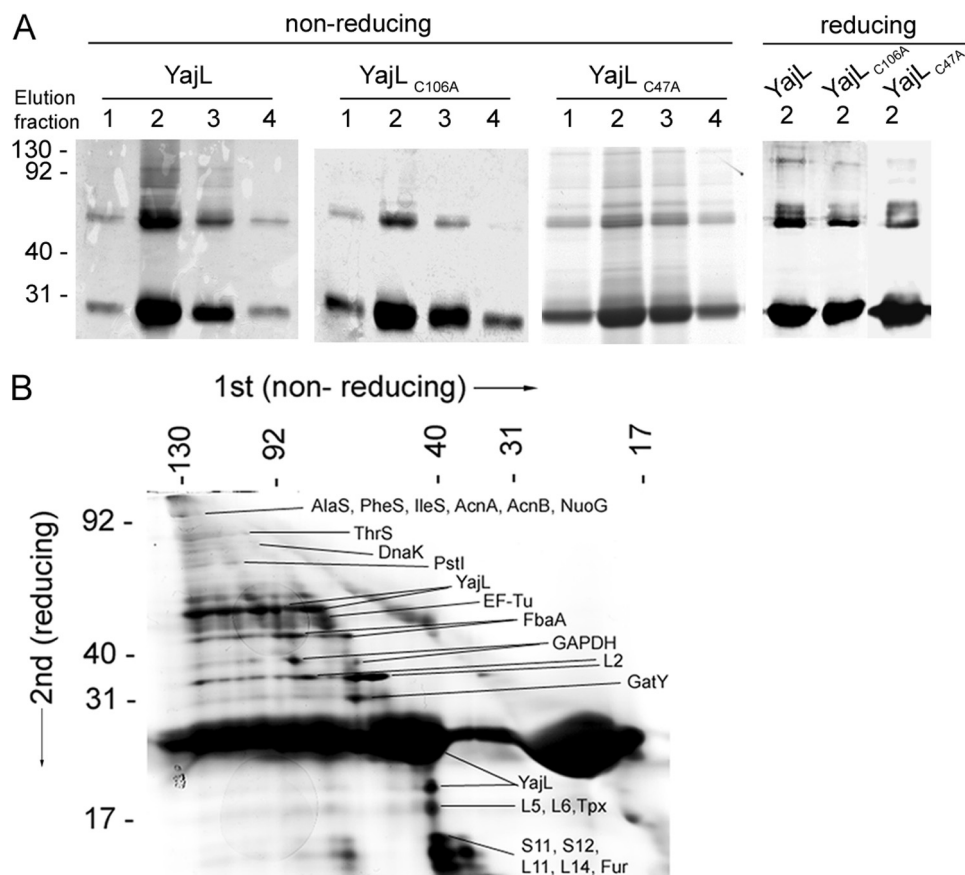


FIGURE 4. Characterization of YajL covalent partners. *A*, SDS-PAGE analysis of mixed disulfides between YajL and client proteins. The YajL, YajL_{C106A}, and YajL_{C47A} overproducers were submitted to 3 mM hydrogen peroxide for 5 min and treated with TCA. TCA pellets were solubilized, and mixed disulfides between YajL and client proteins were purified on nickel affinity columns and analyzed by nonreducing or reducing SDS-PAGE (the gels were silver-stained). The nickel columns were eluted by a step gradient (elution fractions 1–4) containing 20, 50, 100 and 150 mM imidazole, respectively. *B*, characterization of YajL substrates by nonreducing-reducing (diagonal) two-dimensional SDS-PAGE. The first dimension was performed under nonreducing conditions (10% polyacrylamide), and the separation in the second dimension was performed under reducing conditions (12% polyacrylamide). The gel was silver-stained, and proteins were identified by mass spectrometry. Several of the YajL covalent substrates are indicated by arrows.

somal proteins S1, S2, S3, S4, S8, S10, S11, S12, S19, L2, L5, L6, L10, L11, L12, L13, L14, L27, L28, the Ala-, Ile-, Leu-, Thr-, and Phe-tRNA synthetases, the RNA polymerase subunits RpoA and RpoB, the chaperones DnaK, DnaJ, GroEL, and ClpB, the peptidase PepN, the protein translocase subunit SecA, the catalase KatE, the peroxidases AhpC, Tpx, and Bcp and a number of metabolic enzymes that belong to the thiol proteome, including glyceraldehyde-3-phosphate dehydrogenase, acetaldehyde dehydrogenase, the aconitases AcnA and AcnB, and the FeS clusters containing subunits NuoG, SdhB, and FdoG of NADH dehydrogenase, succinate dehydrogenase, and formate dehydrogenase-O, respectively.

Most of the YajL substrates belong to the thiol proteome, a set of thiol- or disulfide-containing proteins that are retained on activated thiol-Sepharose (shown in boldface characters in Table 1) (21). Several of these proteins form disulfides after menadione treatment of bacteria (underlined in Table 1), whereas others (S4, L14, AdhE, GAPDH, and Tpx (22)) harbor H₂O₂-mediated thiol modifications or are heavily oxidized and accumulate in proteolysis-deficient bacteria (AceE, RpoB, EF-Tu, and DnaK (38)).

Deficits in Several Covalent YajL Substrates in yajL Mutant—Many of the covalent YajL substrates described in this study aggregate or display reduced activities in the *yajL* mutant,

which suggests that YajL is important *in vivo* for their biogenesis or their protection against denaturation. Ten, among the 19 ribosomal substrates of YajL, aggregate in the *yajL* mutant (7) (S1, S2, S3, S8, S10, L2, L5, L6, L10, and L11). Moreover, YajL stimulates 3-fold the refolding of urea-denatured ribosomal protein S1 *in vitro* (7).

We measured GAPDH activities in crude extracts from the mutant and parental strains (as described in Ref. 22) and found that the activity of the mutant strain was only 36% of the activity of the parental strain (not shown). The two *E. coli* aconitases, AcnA (oxidative stress-resistant) and AcnB (oxidative stress-sensitive) (39), covalently interact with YajL (they contain 3 essential cysteines, each involved in FeS cluster binding). They can be differentiated in bacterial extracts because AcnB is inactivated by EDTA, whereas AcnA is not. We found that 80% of the aconitase activity of a parental strain extract was inactivated by EDTA, suggesting that (as reported previously (27)) AcnB represents the main aconitase activity in *E. coli*. In contrast, the aconitase activity of the *yajL* mutant extract was low and resistant to EDTA, suggesting that its weak aconitase activity was that of AcnA and that AcnB was inactive (Fig. 5A) (transcriptional profiling of the *yajL* mutant shows that its AcnB mRNA is normally

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TABLE 1

Proteins that form mixed disulfides with YajL

The protein accession number and Mascot score of each protein are index- indicated, except for ribosomal proteins for which only the Mascot score is indicated. Mascot scores reflect results obtained by combining peptide mass fingerprinting and MS/MS. Proteins printed in boldface belong to the thiol proteome (*i.e.* thiol- and disulfide-containing proteins isolated on activated thiol-Sepharose (21)); underlined proteins contain disulfide(s) after menadione treatment (21); proteins in italics possess cysteines essential for metal or FeS cluster binding; proteins with asterisks are heavily oxidized upon oxidative stress (22, 38); and shaded ribosomal proteins form aggregates in the *yajL* mutant (7).

Ribosomal proteins	S1* ₇₉ , S2 ₄₆₅ , S3 ₅₅ , S4* ₁₁₄ , S8 ₁₆₄ , S10 ₂₁₃ , S11 ₆₂ , S12 ₇₂ , S19 ₁₀₀ , L2 ₁₆₄ , L5 ₁₃₁ , L6 ₆₄ , L10 ₈₄ , L11 ₉₂ , L12 ₁₆₄ , L13 ₇₈₀ , L14* ₁₃₀ , L27 ₁₁₇ , L28 ₁₀₁
Aminoacyl-tRNA ligases	<i>AlaS</i> _{GI16130604, 122} , <i>IleS</i> _{GI16128020, 165} , LeuS* _{GI16128625, 133} , <i>ThrS</i> _{GI16129675, 244} , <i>PheS</i> _{GI16129670, 223}
Translation factors	TufA _{GI16131218, 250} , TufB _{GI16131810, 183}
Transcription	RpoA _{GI16131174, 64} , RpoB* _{GI16131817, 79} , Hns _{GI16129198, 62} , <i>Fur</i> _{GI16128659, 95} , LacI _{GI49176012, 73} , MalT _{GI16131294, 60} , Crp _{GI16131236, 108}
Chaperones, Peptidases	DnaK* _{GI1786196, 112} , DnaJ _{GI16128009, 260} , GroEL* _{GI16131968, 88} , ClpB* _{GI16130513,} 108, <i>SecA</i> _{GI16128091, 82} , <i>PepN</i> _{GI16128899, 118}
Peroxidases, catalases	Tpx* _{GI16129285, 454} , AhpC _{GI16128588, 80} , KatE _{GI49176140, 412} , Bcp _{GI16130405, 102}
Metabolism	Gapdh* _{GI16129733, 66} , AdhE* _{GI16129202, 80} , PtsI _{GI16130315, 68} , Ald _{GI16129376, 76} , AceE* _{GI16128107, 367} , SucA _{GI16128701, 76} , TnaA _{GI87082323, 112} , AcnA _{GI16129237, 88} , AcnB* _{GI16128111, 92} , SdhB _{GI16128699, 74} , NuoG _{GI145693161, 86} , FdoG _{GI16131734, 66} , <i>FbaA</i> _{GI16130826, 108} , <i>RluC</i> _{GI16129049, 118}

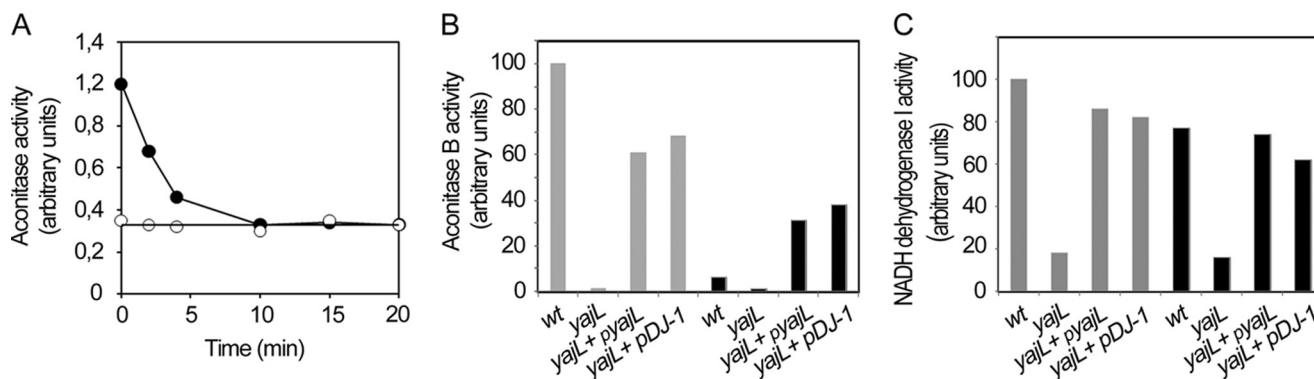


FIGURE 5. Deficits in aconitase B and NADH dehydrogenase I activities in *yajL* mutant. *A*, aconitase A and B. Bacterial extracts were exposed to 300 μ M EDTA (aconitase B is selectively inhibited by EDTA treatment), and at the indicated time points, the remaining aconitase activity was assayed. An activity of 1 represents 0.12 μ mol of *cis*-aconitate/min/mg of protein. *B*, AcnB activity in the *yajL* mutant and its rescue by the *yajL* and DJ-1 plasmids. The AcnB activities of the parental strain, the *yajL* mutant, and the *yajL* mutant transformed with the *YajL*- or the DJ-1-overproducing plasmids were measured in extracts from unstressed (gray bars) or oxidatively stressed (black bars) bacteria. An activity of 100 represents 0.10 μ mol of *cis*-aconitate/min/mg of protein. *C*, NADH dehydrogenase I activity in the *yajL* mutant and its rescue by the *yajL* and DJ-1 plasmids. The NADH dehydrogenase I activities of the parental strain, the *yajL* mutant, and the *yajL* mutant transformed with the *YajL*- or the DJ-1-overproducing plasmids were measured in membrane extracts from unstressed (gray bars) or oxidatively stressed (black bars) bacteria. An activity of 100 represents 0.18 μ mol of deamino-NADH/min/mg of protein.

expressed).⁴ The aconitase B defect of the *yajL* mutant was partially rescued (up to 61 and 68%, respectively, of the activity of the parental strain) by the *YajL*- and DJ-1-overproducing plasmids (Fig. 5*B*). In oxidatively stressed cells (2 mM hydrogen peroxide for 10 min), the AcnB activities of the parental strain and the mutant were severely reduced, and the AcnB defect of the mutant was rescued by the *YajL*- and DJ-1-overproducing plasmids (up to 31 and 38%, respectively of the unstressed parental strain (Fig. 5*B*)).

⁴ V. Gautier, A. Malki, T. Caldas, N. Messaoudi, M. Mihoub, A. Landoulsi, and G. Richarme, manuscript in preparation.

NuoG, the major FeS cluster-containing subunit of NADH dehydrogenase I (40), is a covalent substrate of *YajL* (*NuoG* possesses 15 essential cysteines involved in binding its four FeS clusters). The NADH dehydrogenase I activity (tested by using deamino-NADH (25)) of membranes from the *yajL* mutant was 18% of that of membranes from the parental strain, suggesting that the *YajL* defect is highly detrimental to NADH dehydrogenase I (Fig. 5*C*) (transcriptional profiling of the *yajL* mutant shows that its *NuoA-N* genes (coding for NADH dehydrogenase I) are normally expressed).⁴ The *YajL*- and DJ-1-overproducing plasmids efficiently rescued the NADH dehydrogenase I defect of the *yajL* mutant, either before stress (up to 86 and

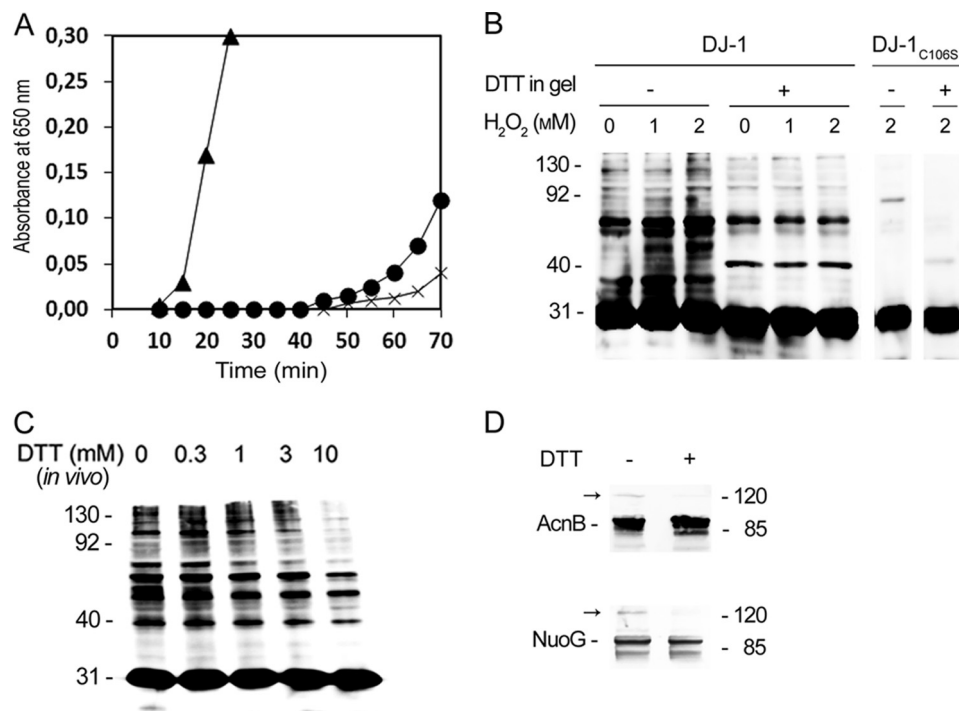


FIGURE 6. Covalent chaperone activities of DJ-1. *A*, insulin reduction by dithiothreitol. Mixtures containing 0.3 mM dithiothreitol alone (crosses), 0.3 mM dithiothreitol plus 5 μ M DJ-1 (filled circles), or 3 μ M thioredoxin (triangles) were assayed for insulin (170 μ M) reduction, and precipitation was monitored by measuring optical density at 650 nm. *B*, mixed disulfides between DJ-1 and cytoplasmic proteins. The DJ-1- and DJ-1_{C106S}-overproducing strains were incubated with hydrogen peroxide for 10 min, and the lysates were separated on SDS-polyacrylamide gels under nonreducing or reducing (+100 mM DTT) conditions. The proteins were then transferred to a nitrocellulose membrane and labeled with anti-DJ-1 antibodies. *C*, *in vivo* reversion by DTT of mixed disulfide formation between DJ-1 and its substrate proteins. DJ-1-overproducing cells were submitted to a 3 mM hydrogen peroxide stress for 10 min and then treated with DTT at the indicated concentrations for 15 min. Lysates were resolved in SDS-polyacrylamide gel under nonreducing conditions, transferred to a nitrocellulose filter, and labeled with anti-DJ-1 antibodies. *D*, immunodetection of mixed disulfides between DJ-1 and AcnB or NuoG. Bacterial lysates were prepared, electrophoresed, transferred to nitrocellulose membranes as described for panel *B*, and probed with anti-AcnB or anti-NuoG antibodies. Mixed disulfides between DJ-1 and AcnB or NuoG are indicated by an arrow.

82%, respectively, of the activity of the parental strain) or after oxidative stress (up to 74 and 62%, respectively, of the activity of the unstressed parental strain (Fig. 5C)).

Protein Disulfide Isomerase and Covalent Chaperone Activities of DJ-1—We checked whether DJ-1 also displayed protein disulfide isomerase activities. Like YajL, DJ-1 (5 μ M) catalyzed insulin reduction in a dithiothreitol-dependent manner, leading to insulin precipitation after 45 min, whereas 3 μ M thioredoxin or dithiothreitol alone led to insulin precipitation at 15 and 55 min, respectively (Fig. 6A). Thus, like YajL, DJ-1 is able to reduce insulin. Its insulin-reducing activity is much weaker, however, than that of thioredoxins.

To test the covalent chaperone activity of DJ-1, we investigated the formation of mixed disulfides between DJ-1 and *E. coli* proteins *in vivo*. DJ-1-overproducing bacteria (6) were lysed (either before or after hydrogen peroxide stress) in buffer containing *N*-ethylmaleimide, as described above for the YajL-overproducing strains. Crude bacterial extracts were analyzed by nonreducing or reducing SDS-PAGE followed by electrotransfer onto nitrocellulose membranes and immunodetection with anti-DJ-1 antibodies (28). When cellular extracts were electrophoresed under nonreducing conditions, anti-DJ-1 antibodies decorated monomeric DJ-1, dimeric DJ-1, and many additional bands. As for YajL, these bands increased with increasing hydrogen peroxide concentrations (Fig. 6B). Under reducing conditions, antibodies reacted with monomeric DJ-1

and with a low amount of nonreduced dimer, but most of the additional bands were no longer present (Fig. 6B), suggesting that under nonreducing conditions, the additional bands represent mixed disulfides of DJ-1 and *E. coli* proteins. The formation of mixed disulfides between DJ-1 and cellular proteins was strictly dependent on its reactive cysteine 106 because it was negligible in the DJ-1_{C106S}-overexpressing strain (Fig. 6A).

The formation of mixed disulfides between DJ-1 and *E. coli* cytoplasmic proteins in oxidative stressed cells (3 mM hydrogen peroxide for 10 min) was reversed *in vivo* by DTT. When increasing DTT concentrations were added for 15 min prior to cell lysis, the amounts of mixed disulfides between DJ-1 and cellular proteins decreased with increasing DTT concentrations (Fig. 6C). Thus, DJ-1, like YajL, reversibly forms mixed disulfides *in vivo* with cellular proteins upon oxidative stress.

Immunodetection confirmed that DJ-1 formed mixed disulfides with two YajL substrates, AcnB (94 kDa) and NuoG (100 kDa). Antibodies raised against these proteins not only reacted with a band corresponding to AcnB or NuoG, but also with a band corresponding to a mixed disulfide between DJ-1 and either AcnB or NuoG (migrating at around 125 kDa, and indicated by an arrow) (Fig. 6D). Interestingly, as already mentioned above (Fig. 5, B and C), the DJ-1-producing plasmid was as efficient as the YajL plasmid in rescuing the aconitase B and NADH dehydrogenase 1 defects of the *yajL* mutant.

DISCUSSION

In the present study, we show that YajL and DJ-1 display protein oxidoreductase and covalent chaperone activities. The protein oxidoreductase activities of YajL are weaker than those of thioredoxin, DsbC, and PDI. For example, its protein disulfide isomerase activity is 3-fold lower than that of thioredoxin and 75-fold lower than that of PDI (29, 30). They are similar, however, to those of single cysteine mutants of thioredoxins or PDI (41) and of monothiol PDI, which function as covalent chaperones, such as Erp44 (28, 42). The remarkable efficiency of disulfide isomerases that possess a CXXC active site is explained by the specific reactivity of their first thiol group, which allows for the rapid formation of a mixed disulfide with a substrate protein and by the vicinity of the second thiol, which facilitates escape from the mixed disulfide and release of the substrate protein (18, 34). YajL, which does not possess vicinal cysteines, might function like single cysteine variants of protein disulfide isomerases by combining the reactivity of an exposed cysteine and of a peptide binding site. A low molecular weight thiol such as glutathione or a second thiol of YajL (either in the same molecule or in another) could fulfill the function of the missing thiol. A single catalytic thiol group was considered during early experiments with PDI, when the presence of an active site disulfide in PDI had not yet been demonstrated (29, 30).

YajL forms mixed disulfides with many cytoplasmic proteins via its conserved cysteine 106, and formation of these mixed disulfides is increased after an oxidative stress. Protein disulfide isomerases with a CXXC active site do not form stable mixed disulfides with substrate proteins because mixed disulfides formed via their first cysteine are quickly resolved by the second cysteine. Mutants of the second cysteine have been used to characterize these disulfides (33). Several endoplasmic reticulum PDIs with a monothiol active site can form stable mixed disulfides with client proteins. Erp44 retains incompletely folded immunoglobulins and adiponectin in the endoplasmic reticulum (28); PDILT engages in disulfide-dependent interactions with a few uncharacterized substrates in testis (34); and AGR2 (anterior gradient homolog 2) forms mixed disulfides with the cysteine-rich intestinal glycoprotein MUC2 that forms the protective mucus gel lining the intestine (35). Each of these covalent chaperones is involved in oxidative protein folding in the endoplasmic reticulum (36). By contrast, YajL, which is located in the cytoplasm, forms mixed disulfide with many proteins upon oxidative stress and likely protects them against oxidative stress and aggregation, in accordance with its roles in oxidative stress resistance and protein solubilization (7).

Several of the proteins that form mixed disulfides with YajL possess one or more cysteine(s) in their active site. The peroxidases AhpC, Tpx, and Bcp each contain 1–2 active site cysteines (43), and GAPDH (whose activity is reduced in the *yajL* mutant) performs covalent catalysis via its cysteine 150. Moreover, GAPDH undergoes oxidation of its reactive cysteine (22), which results in protein aggregation and apoptosis (44).

YajL substrates comprise many ribosomal proteins, several aminoacyl-tRNA synthetases, and EF-Tu, which is reflected by the occurrence of translational defects in *yajL* mutants (decrease in translation accuracy and dissociation of 70 S ribo-

somes into 50 S and 30 S subunits after oxidative stress (17)). Ribosomal proteins are more sensitive to oxidative stress than previously recognized (7, 45). Among the YajL substrates, S1, S2, S3, S4, S11, L5, L6, L10, L12, L27, and L28 belong to the thiol proteome (21), and S4 and L14 harbor hydrogen peroxide-mediated thiol modifications (22). This suggests that many ribosomal proteins contain oxidative stress-sensitive thiols. Moreover, 10 (S1, S2, S3, S8, S10, L2, L5, L6, L10, and L11) of the 19 ribosomal proteins that covalently interact with YajL aggregate in the *yajL* mutant (7), indicating that the covalent chaperone activity of YajL has a functional role *in vivo* in preventing ribosomal protein aggregation.

Other YajL substrates displayed reduced activity in the *yajL* mutant, suggesting that YajL is important for their biogenesis or their protection against denaturation. AcnB and NuoG, two YajL substrates containing 3 and 15 cysteines, respectively, involved in binding their FeS cluster(s) displayed severely reduced activities in the *yajL* mutant (less than 5 and 18%, respectively, of their activities in the parental strain). Moreover, the aconitase B and NADH dehydrogenase 1 defects of the *yajL* mutant were rescued by YajL- or DJ-1-overproducing plasmids, suggesting that YajL and DJ-1 are involved in protecting these proteins against oxidative stress.

Mixed disulfides of YajL and client proteins likely represent an intermediate state in protection against oxidative stress. It is likely that they are resolved by low molecular weight thiols or by the thioredoxin or glutaredoxin system at the expense of NADPH, similarly to the disulfides in peroxidases (43).

DJ-1 also exhibits protein disulfide isomerase and covalent chaperone activities that depend on its cysteine 106. It reduces insulin with kinetics similar to those of YajL; it forms mixed disulfides with *E. coli* proteins in oxidative stressed cells; and several of its substrates are the same as those of YajL. It has been recently reported that DJ-1 forms a mixed disulfide with apoptosis signal-regulating kinase 1 (31). This result might be representative of a more general covalent chaperone function of DJ-1, as suggested by the results of the present study. The covalent chaperone activities of YajL and DJ-1 might explain the pleiotropic defects of their mutants, including: (i) ribosomal protein aggregation and translational defects in *yajL* mutants (7, 17); (ii) the colocalization of Hsp70 with DJ-1 upon oxidative stress and decreased Hsp70 pools in DJ-1 mutants (46); and (iii) catalase, peroxidase, aconitase, and respiratory chain complex I deficiencies in *yajL* and *DJ-1* mutants (aconitase and complex I contain FeS clusters) (4, 19). They are also consistent with reports of noncovalent interactions between DJ-1 and the eukaryotic counterparts of YajL covalent substrates, which might covalently interact with DJ-1 as well. These include (i) GAPDH (DJ-1 was cloned as a GAPDH interactor) (4); (ii) catalase, aconitase, and ribosomal proteins (47); and (iii) glutathione peroxidase 2 (48, 49).

Finally, the results described in this work shed light on the oxidative stress-dependent chaperone functions of YajL and suggest that DJ-1 might also function as a covalent chaperone. They demonstrate an essential role for cysteine 106 and identify many thiol-containing substrates whose defects may be responsible for several phenotypes of *yajL* and DJ-1 mutants, including translational defects, protein aggregation, oxidative stress sen-

sitivity, and metabolic deficiencies, with emphasis on the thiol and FeS cluster proteomes.

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