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Stabilization of Cox1p intermediates by the Cox14p-Coa3p complex

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Abstract

Cox14p and Coa3p have been shown to regulate translation of the mitochondrial *COX1* mRNA and to be required for assembly of cytochrome oxidase. We present evidence that Cox14p and Coa3p stabilize previously identified Cox1p intermediates and that in the absence of either protein, Cox1p aggregates with itself and other mitochondrial gene products, including cytochrome *b*, Var1p and Cox2p. Our evidence suggests that Cox1p assembly intermediates are in close proximity to other mitochondrially translated proteins and that an important function of Cox14p and Coa3p is to prevent Cox1 from entering into unproductive aggregation pathways.

Keywords

Mitochondria; cytochrome oxidase; biogenesis; *Saccharomyces cerevisiae*; Cox14p; Coa1p; Coa3p; Cox1p stability

1. Introduction

Yeast cytochrome oxidase (COX)² is composed of 11 distinct subunits three of which make up the catalytic core of the enzyme, are encoded in mitochondrial DNA. Cox1p, an important catalytic subunit containing the cytochrome *a* and *a₃* centers, is translationally regulated by at least four accessory proteins, Mss51p [1], Cox14p [2], Coa1p [3] and Coa3p [4, 5]³. Newly translated Cox1p interacts with Mss51p, its specific translational activator, and with Cox14p, Coa3p and Coa1p [2, 3, 6–8]. Current models envision translation of Cox1p to be down-regulated until Mss51p, sequestered by its interaction with newly translated Cox1p, is released by a downstream event during COX assembly. The complexity of this regulatory pathway and more generally of COX assembly, is underscored by recent evidence indicating the existence of at least five different Cox1p assembly intermediates [9].

The involvement of Cox14p and Coa3p in regulating Cox1p expression is supported by their physical association with Mss51p and Cox1p and more importantly by the observation that *cox14* and *coa3* mutants, on their own or in combination with other mutations that arrest

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²Abbreviations used: COX, cytochrome oxidase; BN, blue native; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; mtDNA, mitochondrial DNA.

³Coa3p, also called Cox25p, was identified as a component of the Cox1p-Mss51p complex independently in two different laboratories (see references 4 and 5). The standard name Coa3p is used in this communication.

COX assembly, display normal translation of Cox1p [2, 4, 5]. Cox14p and Coa3p, in addition to their function in regulating Cox1p translation have also been inferred to play a role in COX assembly as mutations in either protein produce a COX deficient phenotype [4, 5, 10].

In this communication we report a new function of Cox14p and Coa3p in Cox1p biogenesis, deduced from the radiolabeled products detected following *in organello* labeling of mitochondria from different mutants. Our evidence indicates that one, if not the main, function of both proteins is to prevent Cox1p from forming assembly-incompetent aggregates. The aggregation of Cox1p with itself and with cytochrome *b*, Var1p and Cox2p in *cox14* and *coa3* mutants suggests that newly translated mitochondrial gene products may be in close proximity to one another in the inner membrane.

2. MATERIALS AND METHODS

2.1 Strains and growth media

The genotypes and sources of the strains of *S. cerevisiae* used in this study are listed in Table 1. The compositions of liquid and rich glucose (YPD), galactose (YPGal) and glycerol/ethanol (YEPG) media used to grow wild type and mutant yeast have been described [10].

2.2 Labeling of mitochondrial gene products in isolated mitochondria and purification of Cox1p-HAC on protein C antibody beads

Mitochondria were prepared by the method of Herrmann et al [11] from yeast grown to early stationary phase in YPGal. In some experiments cells were transferred to fresh YPGal containing 2 mg/ml chloramphenicol for 2 hours of additional growth. Mitochondria (500 µg protein) were labeled at 24°C for 30 min with a mixture of ³⁵S-methionine plus cysteine (3,000 Ci/mmol, MP Biochemicals, Solon, OH) in a total volume of 170 µl [12]. Translation was terminated by addition of puromycin to a final concentration of 100 µM. After an additional 10 min of incubation at 24°C, 1.2 volumes of 4% digitonin in extraction buffer was added and the mixture was centrifuged at 100,000 × g_{av} for 10 min. Alternatively, mitochondria were pelleted by centrifugation at 10,000 g_{av} for 10 min and extracted with 3% digitonin as above. The supernatant obtained after the high speed centrifugation was collected and mixed with 50 µl of packed protein C antibody beads (Roche Applied Science, Indianapolis, IN). The beads were rotated at 4°C for 90 min and unadsorbed proteins were removed by centrifugation at 500 × g_{av} for 30 seconds. The beads were then washed 3 times with 10 volumes of a buffer containing 0.5% digitonin, 20 mM Tris-Cl, pH 7.5, 2 mM CaCl₂ and 200 mM NaCl (wash buffer) and eluted with 100 µl a buffer containing 0.5% digitonin, 20 mM Tris-Cl, pH 7.5, 7 mM EDTA and 200 mM NaCl (elution buffer). The beads in elution buffer were rotated at 4°C for 30 min proteins eluted with the EDTA were collected by centrifugation of the beads at 500 × g_{av} for 30 seconds.

2.3 Construction of the plasmid expressing Coa3p with a C-terminal double HA plus protein C tag

Approximately 500 bp of 5' UTR plus the *COA3* coding sequence minus the termination codon was amplified from yeast nuclear DNA with primers *coa3-5* GCGAGATCTCTGTTGTCTCCTGCTAAAAAC and *coa3-HA* GGCGTCGACTCAAGCGTAGTCTGGGACGTCGTATGGTGCCTTCTTCTTGGCTTCGTA. The PCR product was used as a template to amplify the HA tagged gene with primers *coa3-5* and *Coa3-C* GGCTGCAGTCATTTACCATCGATTAATCTTGGATCTACTTGATCTTCTCCTCCAGCGTAGTCTGGGACGTCGTATGG. The resultant product consisted of 500 bp of 5' -UTR followed by the coding sequence, the HA tag, 3 glycine codons and ending

with the protein C epitope. The fusion gene was digested with XbaI and PstI and cloned into YIp351 [13] to yield pG127/ST3, which was linearized at the *LEU2* gene for homologous recombination [14] in a *coa3* null mutant.

2.4 Miscellaneous Procedures

Preparation and ligation of DNA and transformation of *E. coli* were done by standard methods [15]. Yeast was transformed by the lithium acetate method of Schiestl and Gietz [16]. Proteins were separated by SDS-PAGE on different concentrations of polyacrylamide gels in the buffer system of Laemmli [17] and by BN-PAGE on 4–13% polyacrylamide gels as described [18]. Second dimensions SDS-PAGE were run on 12% polyacrylamide gels. Western blots were treated with a monoclonal antibody against the HA tag followed by a second reaction with anti-mouse IgG conjugated to horseradish peroxidase (Sigma, St. Louis, MO). Proteins were detected with SuperSignal chemiluminescent substrate kit (Pierce, Rockford, IL). Protein concentrations were estimated by the Lowry procedure [19].

3. Results

3.1 Association of Coa3p with Cox1 assembly intermediates

Newly translated Cox1p was previously shown to be present in five different high molecular weight complexes (D1–D5), which were inferred from pulse-chase experiments to be precursors of COX [9]. The intermediates were characterized with respect to their content of all currently known COX chaperones except Coa3p. Cox14p interacts physically with Coa3p and shares many of its properties [4, 5]. Cox14p is associated with newly translated Cox1p in four of the precursors (complexes D2–D5) [9]. To ascertain if Coa3p is present in the same intermediates as Cox14p, pull-down assays were performed on pulse-labeled mitochondria from a strain expressing Coa3p with a C-terminal HAC double tag consisting of HA followed by a protein C epitope (W303/COA3-HAC). The presence of this tag did not compromise the ability of yeast to grow on respiratory substrates (Fig. S1A). As controls we used mitochondria of the parental W303-1B strain lacking tagged Coa3p and of MRS/COX1-HAC, which expresses Cox1p with the double HAC tag [9]. Digitonin extracts of mitochondria from strains with tagged Coa3p or Cox1p, when purified on the protein C antibody beads, showed the same enrichment of radiolabeled Cox1p (Cox1p-HAC), Cox2p and Cox3p (Fig. S1B). Only background levels of the radiolabeled products were detected in the eluates of the control W303-1B parental strain. Separation of the eluates on blue native gels disclosed radiolabeled D2–D5 (Fig. S1C), as had previously been observed in the strain expressing tagged Cox14p [9]. The presence or absence of Coa3p in D1 could not be determined because of the very low level of labeling of this intermediate in this experiment.

3.2 Cox1 intermediates in *cox14*, *coa3* and *coa1* mutants

Both Cox14p and Coa3p have been shown to stabilize the Cox1p-Mss51p complex of D3 [2, 4, 5]. To ascertain if the stability of the other Cox1p intermediates also depends on these proteins, a mitochondrial genome containing the COX1-HAC fusion gene was introduced into *cox14*, *coa3* and *coa1* null mutants. Coa1p was of interest as it has been proposed to alter the interaction of Mss51p with Cox1p [4]. Cox1p intermediates were analyzed in the three mutants and in control cells that included respiratory competent strains with and without Cox1p-HAC and a *cox4* mutant blocked in assembly of COX [20]. The steady-state concentrations of Cox1p intermediates were assessed by western analysis of Cox1p-HAC in digitonin extracts of mitochondria following purification on protein C antibody beads and separation on a blue native gel. In agreement with earlier results the antibody against the HA tag detected principally the more abundant intermediates D3 and D4 and the two supercomplexes of the respiratory competent and the *coa1* mutant, which has a leaky growth phenotype on non-fermentable carbon sources. Over-exposure of the blot, however, also

showed the presence of D5/COX and of a weaker D2 signal in the *coa1* mutants (Fig. 1). While D3 and D4 were also present in the eluates of the *cox4* mutant, the other intermediates were either absent or their concentrations were below the sensitivity of the assay. None of the Cox1p intermediates were detected in the *cox14* and *coa3* mutants (Fig. 1). A long exposure of the Western blot, however, revealed a diffuse background in the D3–D5 region of the *coa3* mutant and to a lesser degree also in the *cox14* mutant. Both mutants had a weak discrete signal above the 480 kDa marker and in the region corresponding to the smaller of the two supercomplexes (Fig 1).

Mitochondria of the same strains, grown to early stationary phase with and without an additional 2 hours growth in the presence of chloramphenicol, were labeled *in organello*, extracted with digitonin and Cox1p-HAC purified on protein C antibody beads. All the eluates from the antibody beads except that of control mitochondria with untagged Cox1p, when separated by SDS-PAGE, showed the expected enrichment of Cox1p-HAC, Cox2p and Cox3p (Fig. 2A, 2C). Interestingly, there was also an enrichment of Var1p, cytochrome *b* and Cox2p in the *cox14* and *coa3* mutants. To a lesser extent this was also true of the *coa1* mutant. The blue native gel of the eluates revealed radiolabeled Cox1p intermediates D1–D5 in mitochondria of all the strains grown in the absence of chloramphenicol except the control respiratory competent W303- 1A (with untagged Cox1p) and the *cox14* and *coa3* mutants (Fig. 2B). The latter two mutants had a band at the position of the smallest intermediate D1 but lacked any discernable bands corresponding to D2–D5. Instead the *cox14* and *coa3* mutants showed diffuse radioactivity extending from the D1 intermediate to the very top of the gel (Fig. 2B). Very similar results were obtained when mitochondria were isolated from cells that had been grown for 2 hours in the presence of chloramphenicol, except that D1 was reduced to undetectable levels (Fig. 2D). The additional growth in chloramphenicol was included to permit mitochondria to accumulate nuclear gene products that might be limiting and hence cause Cox1p to aggregate. The size heterogeneity of Cox1p-HAC appears to be specific to *cox14*, *coa3* and partially to the *coa1* mutant, as the *cox4* (Figs. 2) and other mutants blocked in COX assembly fail to show this phenotype [9]. Aggregation of Cox1p-HAC was also seen when the fraction purified on protein C antibody beads from the *cox14* mutant, was size-fractionated by sedimentation in a sucrose gradient (Fig. S2).

The enrichment of Var1p and cytochrome *b* in the antibody eluates of the *cox14*, *coa3* and *coa1* mutants suggested that Cox1p also aggregates with COX-unrelated proteins. To ascertain if Cox1p aggregates with the other mitochondrial gene products randomly, the eluates from the control strain (expressing untagged Cox1p) and from the *cox14* and *coa1* mutants were separated in the first dimension by BN-PAGE and in the second dimension by SDS-PAGE. The 2-D gel of the eluate from the *cox14* mutant revealed that the radiolabeled bands corresponding to Cox1p, cytochrome *b*, Cox2p, and Cox3p were similarly distributed across the length of the first dimension (Fig. 3). The sucrose gradient analysis of the *cox14* mutant confirmed aggregation of cytochrome *b*, Cox2p and Cox3p. This is consistent with random aggregation of Cox1p with itself and other newly translated mitochondrial gene products. Identical results were obtained with the *coa3* mutant (not shown). The 2-D gel of the eluate from the *coa1* mutant showed that most of Cox1p migrated as discrete bands corresponding to the supercomplexes and the different Cox1p intermediates. With the exception of a small fraction of radiolabeled Cox3p present in the supercomplexes, most of Cox3p migrated as a streak in the region between COX/D5 and D1 of the *coa1* mutant (Fig. 3, lower panel). This is consistent with some aggregation of Cox1p with Cox3p, which normally is present only in the supercomplexes and COX/D5 (Fig. S1C, middle panel).

3.3 Evidence that the Cox1p aggregates of *cox14* and *coa1/coa3* mutants are formed in mitochondria

The Cox1p aggregates observed in *cox14* and *coa3* mutants may have originated in mitochondria or could have been formed after extraction with digitonin. Since non-specific interaction of hydrophobic proteins is a time dependent process, we reasoned that if the aggregates are formed after extraction, their sizes should increase upon incubation of the extracts prior to purification on protein C antibody beads. Mitochondria from the respiratory competent control strain and from *cox14* and *coa3* mutants were labeled and fractionated under the same conditions except that in one case the extracts were incubated 20 hours at 4°C before purification on antibody beads. The labeled products of the respiratory control strain MRS/COX1-HAC were nearly identical in the two samples (Fig. 4A, B), indicating the Cox1p intermediates are very stable and are neither degraded nor do they aggregate anew or further in the digitonin extract. In the *cox14* mutant, however, the overnight incubation of the extract led to a substantial reduction in the amount of radiolabeled Cox1p-HAC. This was evident from the level of Cox1p-HAC detected following separation of the extracts by SDS-PAGE as well as the radioactivity seen in the radioautogram of the blue native gel (Figs. 4A, B). Even though this experiment indicated an increased susceptibility of the aggregates to proteolytic degradation, the relative size distribution of the Cox1p aggregates was not noticeably affected by the overnight incubation of the digitonin extract.

4. DISCUSSION

Newly synthesized Cox1p interacts with its translational activator Mss51p [1, 6] to form an assembly intermediates that have been proposed to be stabilized by Cox14p and Coa3p [2, 4, 5]. At least 4 different Cox1p intermediates have been identified that contain Mss51p, Cox14p and, as shown in the present study, also Coa3p. Some Cox1p intermediates also contain Coa1p and the nuclear encoded subunits Cox5ap and Cox6p [4, 9]. The sequestration of Mss51p prevents it from exercising its role in Cox1p translation until it is disengaged from the complex at a later assembly step [2, 6, 11]. This regulatory loop is currently believed to be the mechanism by which mitochondrial translation of Cox1p is coupled to its subsequent utilization for COX assembly, much in the same way as translation of certain proteins on chloroplast ribosomes depends on their assembly into photosynthetic complexes [21].

Cox14p and Coa3p have also been implicated in COX biogenesis as mutations in the respective genes elicit a severe COX deficiency. The relationship between the functions of Cox14p and Coa3p in regulating translation of Cox1p and COX assembly has not been delineated. The studies described in this communication help to account for the COX assembly defect of *cox14* and *coa3* mutants. The observed polymerization of Cox1p into a heterogeneous collection of different sized aggregates indicates that Cox14p, Coa3p and to a lesser degree Coa1p play an important role in stabilizing Cox1p intermediates. A reasonable interpretation, and one consistent with all the data, is that in the absence of either Cox14p or Coa3p, the Cox1p-Mss51p complex is either prevented from forming, or is readily dissociated and as a result Cox1p is free to be channeled into irreversible and unproductive aggregation pathways.

Our evidence indicates that the Cox1p aggregates present in the antibody purified fractions of *cox14* and *coa3* mutants are most likely formed *in situ* following their translation in mitochondria rather than after solubilization of Cox1p with digitonin. This is supported by the similarity in the size-distribution of the Cox1p aggregates in the eluates obtained from the digitonin extract that had been purified on the antibody beads immediately after extraction or after an added period of incubation at 4°C. Most of the aggregates migrate below the 720 kDa marker. If the aggregation had occurred after solubilization, the overnight

incubation would have been expected to cause further polymerization of Cox1p into larger aggregates.

It is significant that the Cox1p aggregates include mitochondrial gene products unrelated to COX. This would suggest that Cox1 and some of the other mitochondrially translated proteins detected in the aggregates are in close proximity to one another in the mitochondrial inner membrane. The presence in the Cox1p aggregates of Var1p, the only endogenously expressed protein component of mitochondrial ribosomes, is not altogether surprising as this water soluble protein has been shown to be translated also at the inner membrane [22]. One possibility is that the mitochondrial gene products of COX and the bc₁ complex are inserted into a physically identical or distinct but proximal compartment of the inner membrane where the partner subunits are matured and assembled. Biogenesis of the two complexes may, therefore, be not only temporally but also physically coordinated to yield a supercomplex of bc₁ and COX. According to this model, the absence of ATP synthase subunits in the Cox1p aggregates suggests that assembly of this complex occurs in a separate compartment. This is consistent with the absence of evidence for an association of the ATP synthase with the bc₁/COX supercomplexes (Heinemeyer et al, 2007; Mileykovskaya et al, 2012).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Perez-Martinez X, Broadley SA, Fox TD. Mss51p promotes mitochondrial Cox1p synthesis and interacts with newly synthesized Cox1p. *EMBO J.* 2003; 22:5951–5961. [PubMed: 14592991]
2. Barrientos A, Zambrano A, Tzagoloff A. Mss51p and Cox14p jointly regulate mitochondrial Cox1p expression in *Saccharomyces cerevisiae*. *EMBO J.* 2004; 23:3472–3482. [PubMed: 15306853]
3. Pierrel F, Bestwick ML, Cobine PA, Khalimonchuk O, Cricco JA, Winge DR. Coa1 links the Mss51 post-translational function to Cox1 cofactor insertion in cytochrome c oxidase assembly. *EMBO J.* 2007; 26:4335–4346. [PubMed: 17882260]
4. Mick DU, Vukotic M, Piechura H, Meyer HE, Warscheid B, Deckers M, Rehling P. Coa3 and Cox14 are essential for negative feedback regulation of *COX1* translation in mitochondria. *J Cell Biol.* 2010; 191:141–154. [PubMed: 20876281]
5. Fontanesi F, Clemente P, Barrientos A. Coa3 teams up with Mss51, Ssc1, and Cox14 to regulate mitochondrial cytochrome c oxidase subunit 1 expression and assembly in *Saccharomyces cerevisiae*. *J Biol Chem.* 2011; 286:555–566. [PubMed: 21068384]
6. Perez-Martinez X, Butler CA, Shingu-Vazquez M, Fox TD. Dual functions of Mss51 couple synthesis of Cox1 to assembly of cytochrome c oxidase in *Saccharomyces cerevisiae* mitochondria. *Mol Biol Cell.* 2009; 20:4371–4380. [PubMed: 19710419]
7. Fontanesi F, Soto IC, Horn D, Barrientos A. Mss51 and Ssc1 facilitate translational regulation of cytochrome c oxidase biogenesis. *Mol Cell Biol.* 2010; 30:245–259. [PubMed: 19858289]
8. Mick DU, Fox TD, Rehling P. Inventory control: cytochrome c oxidase assembly regulates mitochondrial translation. *Nat Rev Mol Cell Biol.* 2011; 12:14–20. [PubMed: 21179059]
9. McStay GP, Su CH, Tzagoloff A. Modular assembly of yeast cytochrome oxidase. *Mol Biol Cell.* 2013 in press.
10. Glerum DM, Koerner TJ, Tzagoloff A. Cloning and characterization of *COX14*, whose product is required for assembly of yeast cytochrome oxidase. *J Biol Chem.* 1995; 270:15585–15590. [PubMed: 7797555]

11. Herrmann, JM.; Foelsch, H.; Neupert, W.; Stuart, RA. Isolation of yeast mitochondria and study of mitochondrial protein translation. In: Celis, JE., editor. *Cell Biology: A Laboratory Handbook*. Vol. I. San Diego: Academic Press; 1994. p. 538-544.
12. Rak M, Gokova S, Tzagoloff A. Modular assembly of yeast mitochondrial ATP synthase. *EMBO J*. 2011; 30:920–930. [PubMed: 21266956]
13. Hill JE, Myers AM, Koerner TJ, Tzagoloff A. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. *Yeast*. 1986; 2:163–167. [PubMed: 3333305]
14. Rothstein RJ. One-step gene disruption in yeast. *Methods Enzymol*. 1983; 101:202–211. [PubMed: 6310324]
15. Sambrook, J.; Fritsch, EF.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*. NY: Cold Spring Harbor Laboratory Press; 1989.
16. Schiestl RH, Gietz RD. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr Genet*. 1989; 16:339–346. [PubMed: 2692852]
17. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227:680–685. [PubMed: 5432063]
18. Wittig I, Braun HP, Schagger H. Blue native PAGE. *Nat Protoc*. 2006; 1:418–428. [PubMed: 17406264]
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951; 193:265–275. [PubMed: 14907713]
20. Dowhan W, Bibtus CR, Schatz G. The cytoplasmically-made subunit IV is necessary for assembly of cytochrome c oxidase in yeast. *EMBO J*. 1985; 4:179–184. [PubMed: 2990892]
21. Choquet Y, Wostrikoff K, Rimbault B, Zito F, Girard-Bascou J, Drapier D, Wollman FA. Assembly-controlled regulation of chloroplast gene translation. *Biochem Soc Trans*. 2001; 29:421–426. [PubMed: 11498001]
22. Fiori A, Mason TL, Fox TD. Evidence that synthesis of the *Saccharomyces cerevisiae* mitochondrially encoded ribosomal protein Var1p may be membrane localized. *Eucaryot Cell*. 2003; 2:651–653.
23. Heinemeyer J, Braun HP, Boekema EJ, Kouril R. A structural model of the cytochrome c reductase/oxidase supercomplex from yeast mitochondria. *J Biol Chem*. 2007; 282:12240–12248. [PubMed: 17322303]
24. Mileykovskaya E, Penczek PA, Fang J, Mallampalli VK, Sparagna GC, Dowhan W. Arrangement of the respiratory chain complexes in *Saccharomyces cerevisiae* supercomplex III₂IV₂ revealed by single particle cryo-electron microscopy. *J Biol Chem*. 2012; 287:23095–23103. [PubMed: 22573332]

Highlights

- Coa3p is associated with the same four Cox1p assembly intermediates as Cox14p.
- Cox14p and Coa3p stabilize four Cox1p assembly intermediates.
- Cox1p aggregates with other mitochondrial gene products in *cox14* or *coa3* mutants.
- Aggregated Cox1p is incompetent in assembling into cytochrome oxidase.
- Some newly translated mitochondrial gene products are proximate in the membrane.

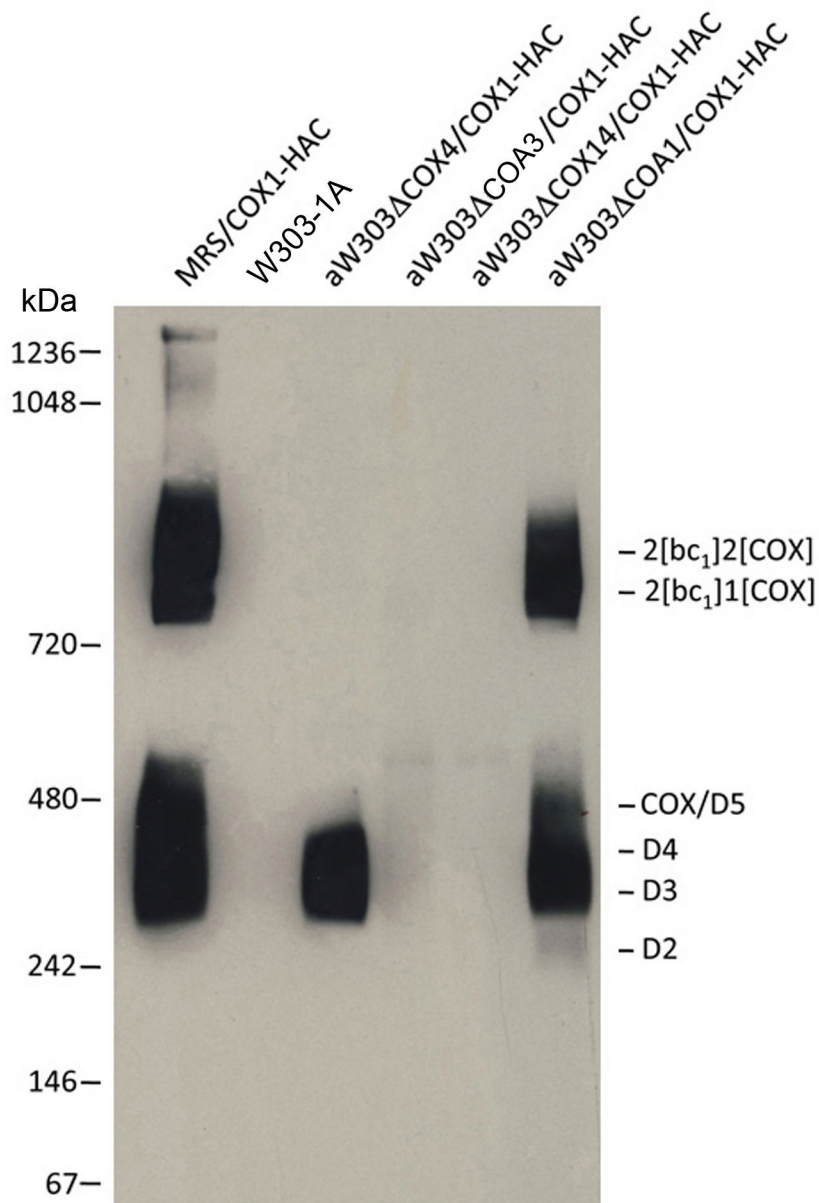


Figure 1. Supercomplexes, COX and Cox1p intermediates in different COX mutants. Mitochondria of the parental strain W303-1A expressing untagged Cox1p, of MRS/COX1-HAC and of *cox4*, *cox14*, *coa3* and *coa1* mutants, each harbouring the *COX1-HAC* fusion gene were extracted with digitonin and fractionated as described in Materials and Methods. The fraction eluted from the protein C antibody beads with EDTA was separated by BN-PAGE on 4–13% polyacrylamide gel, transferred to a PVDF membrane and probed with a monoclonal antibody against the HA tag followed with peroxidase coupled anti-mouse antibody. Cox1p-HAC was detected with the Super Signal chemiluminescent substrate kit (Pierce, Rockford, IL).

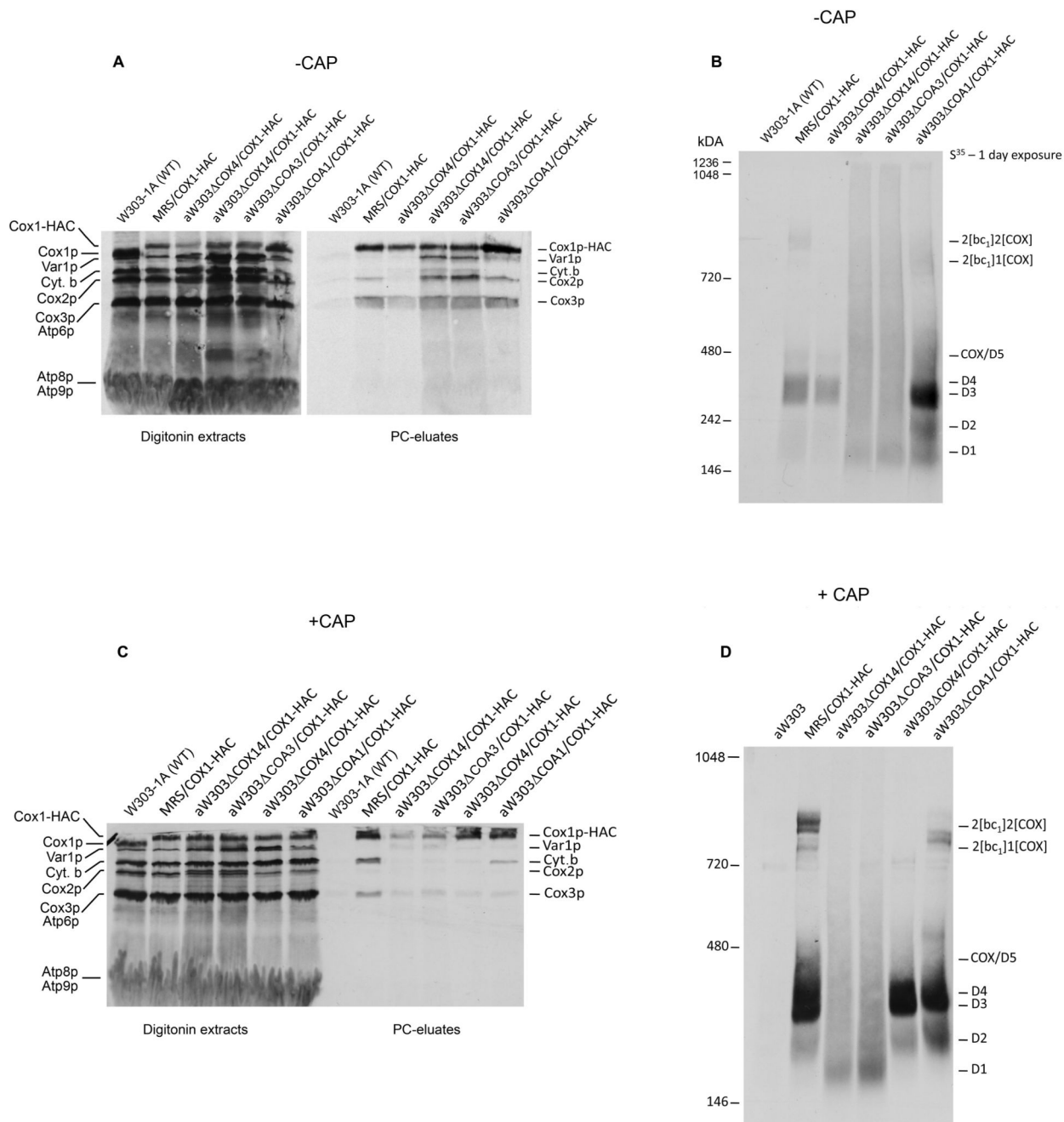


Figure 2. Aggregates of Cox1p in *cox14*, *coa3* and *coa1* mutants. **(A)**, **(C)**. Mitochondria of the indicated strains grown in YPGal, with and without a final 2 hour incubation in fresh medium containing 2 mg/ml chloramphenicol, were labeled for 30 min and chased for 10 min after addition of puromycin. The mitochondria were extracted with 3% digitonin and purified on protein C antibody beads as described in Materials and Methods. The digitonin extracts (10%) and the EDTA eluates (45%) were separated by SDS-PAGE on a 17% polyacrylamide gel, transferred to nitrocellulose and exposed to X-ray film. The radiolabeled proteins are identified in the margins. **(B)**, **(D)**. The remainder of the eluates

from the antibody beads was separated by BN-PAGE on a 4–13% polyacrylamide gel. The radiolabeled bands are marked in the margin.

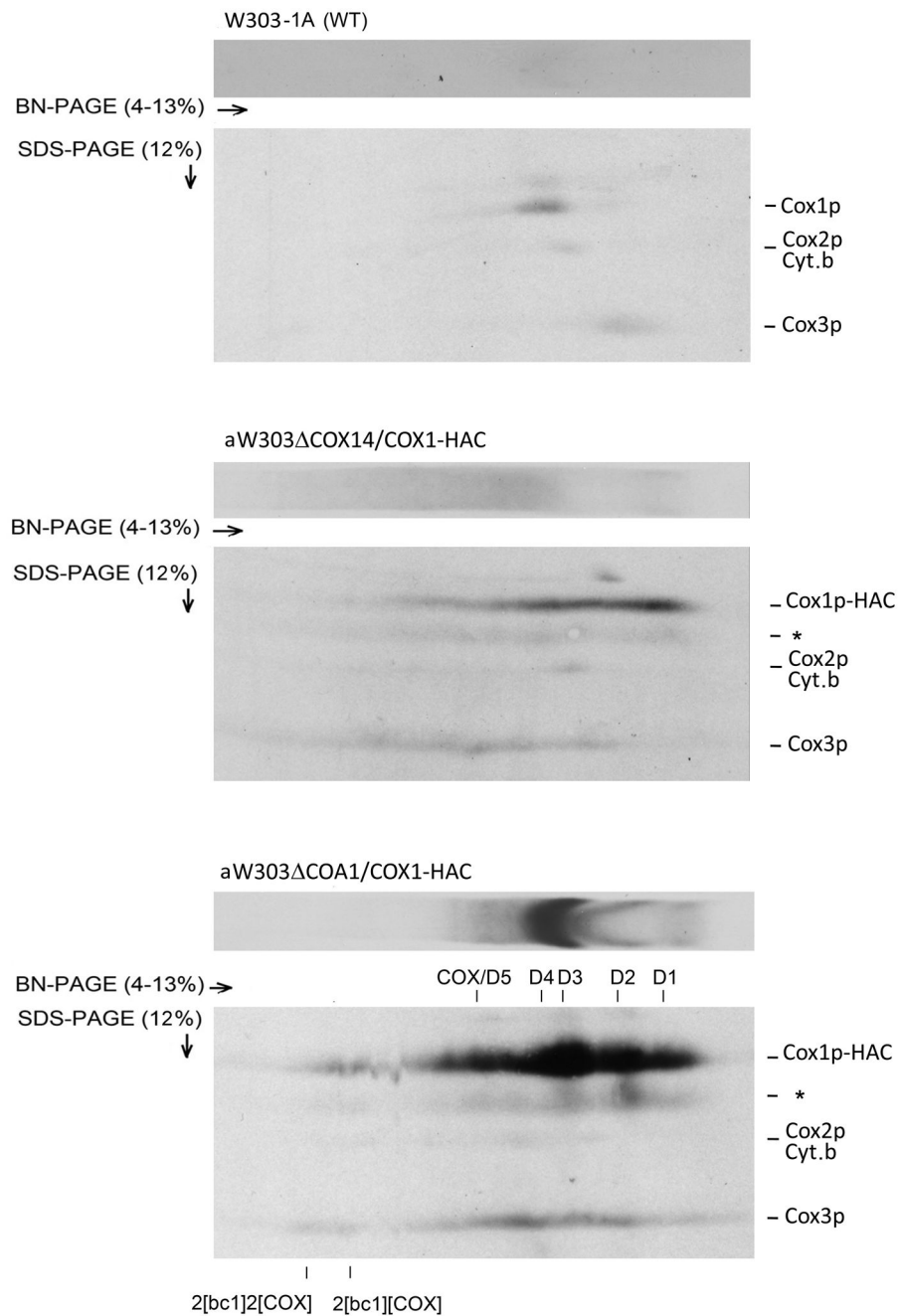


Figure 3. Two-dimensional BN- and SDS-PAGE analysis of newly translated mitochondrial gene products associated with the Cox1p aggregates in *cox14* and *coa1* mutants. Mitochondria of the control strain with wild type mtDNA and of mutants expressing Cox1p-HAC were labeled and fractionated as in Fig. 2. The fractions purified on the protein C antibody beads were separated in two dimensions, transferred to a PVDF membrane and exposed to X-ray film. The band marked with an asterisk is probably a degradation product of Cox1p-HAC.

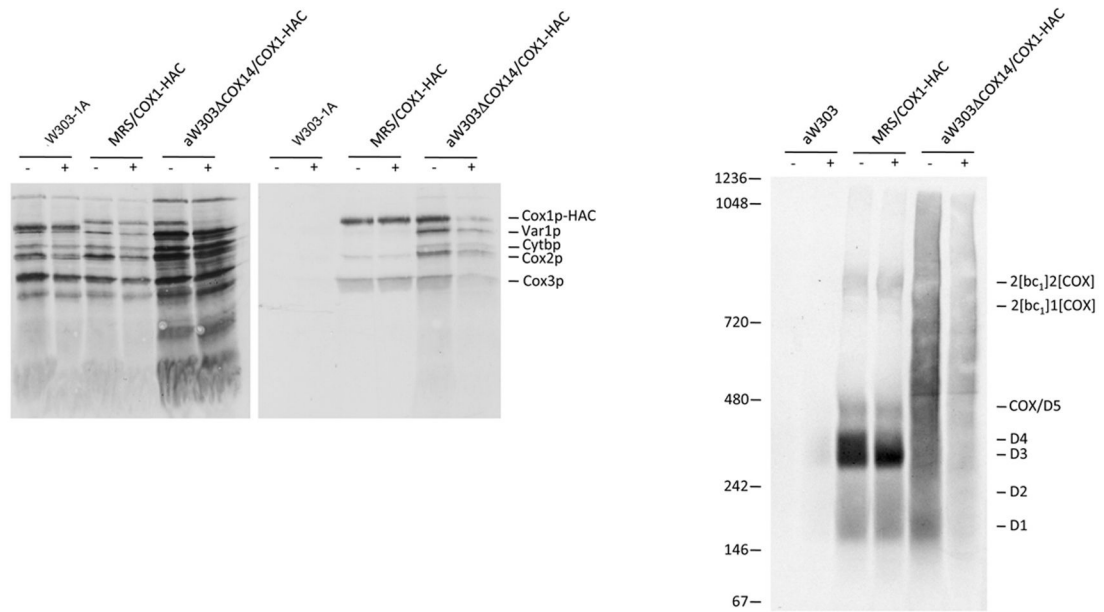


Figure 4.

Effect of incubation of a digitonin extract on the state of aggregation of Cox1p in a respiratory competent strain and in a *cox14* mutant. Mitochondria labeled for 30 min and chased with puromycin were extracted with digitonin and the extracts purified on protein C antibody beads with (+) and without (-) a prior 20 hours incubation at 4°C. (A) Samples of the digitonin extracts (10% of total) and eluates from the beads (45% of total) were separated by SDS-PAGE on a 17% polyacrylamide gel by SDS-PAGE, transferred to a nitrocellulose membrane and exposed to X-ray film. (B) The rest of each sample was applied to a 4–13% blue native gel and after electrophoresis, transferred to a PVDF membrane and exposed to X-ray film. The identity of the different radiolabeled products and of molecular weight standards is indicated in the margin.

Table 1

Genotypes and Sources of *Saccharomyces cerevisiae* Strains

Strain	Genotype	mtDNA	Source
W303-1A	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</i>	+	A
W303-1B	MAT <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</i>	+	A
MRS-3B	MAT <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3</i>	+(S288C)	[9]
aW303 COA3	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coa3::HIS3</i>	+	This study
aW303 COX4	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox4::URA3</i>	+	This study
aW303 COX4/COX1-HAC	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox4::URA3</i>	<i>COX1-HAC</i> allele	This study
aW303 COX14/COX1-HAC	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox14::TRP1</i>	<i>COX1-HAC</i> allele	This study
aW303 COA3/COX1-HAC	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coa3::HIS3</i>	<i>COX1-HAC</i> allele	This study
aW303 COA1/COX1-HAC	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coa1::HIS3</i>	<i>COX1-HAC</i> allele	This study
DFK/COX1-HAC ⁻	MAT <i>kar1-1 ade2-10 arg8::URA3 leu2 ura3-52 lys2</i>	-(pCOX1/ST12)	[9]
MRS/COX1-HAC	MAT <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3</i>	<i>COX1-HAC</i> allele	[9]
aW303 COX4/COX1-HAC	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox4::URA3</i>	<i>COX1-HAC</i> allele	This study
aW303 COX14/COX1-HAC	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox14::TRP1</i>	<i>COX1-HAC</i> allele	This study
aW303/COA3-HAC	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coa3::HIS3 leu2::COA3-HAC</i>	+	This study
aW303/COA1-HAC	MAT <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coa1::HIS3 URA3::COA1-HAC</i>	+	[9]

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