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Shy1p occurs in a high molecular weight complex and is required for efficient assembly of cytochrome *c* oxidase in yeast

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Abstract Surf1p is a protein involved in the assembly of mitochondrial respiratory chain complexes. However its exact role in this process remains to be elucidated. We studied *SHY1*, the yeast homologue of *SURF1*, with an aim to obtain a better understanding of the molecular pathogenesis of cytochrome *c* oxidase (COX) deficiency in *SURF1* mutant cells from Leigh syndrome patients. Assembly of COX was analysed in a *shy1 null* mutant strain by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Steady-state levels of the enzyme were found to be strongly reduced, the total amount of assembled complex being approximately 30% of control. The presence of a significant amount of holo-COX in the *SHY1*-disruptant strain suggests that Shy1p may either facilitate assembly of the enzyme, or increase its stability. However, our observations, based on 2D-PAGE analysis of mitochondria labelled in vitro, now provide the first direct evidence that COX assembly is impaired in a Δ*shy1* strain. COX enzyme assembled in the absence of Shy1p appears to be structurally and enzymically normal. The in vitro labelling studies additionally indicate that mitochondrial translation is significantly increased in the *shy1* null mutant strain, possibly reflecting a compensatory mechanism for reduced respiratory capacity. Protein interactions of both Shy1p and Surf1p are implied by their appearance in a high molecular weight complex of about 250 kDa, as shown by 2D-PAGE.

Key words: *SURF1*; *SHY1*; Cytochrome *c* oxidase; Leigh syndrome; Protein assembly

1. Introduction

Cytochrome *c* oxidase (COX) catalyses the final step of the respiratory chain in which oxygen is reduced to water [1]. This reaction is important for the generation of energy but must be well controlled, because escape of reactive oxygen can occur as an unwanted side product [2]. Proper function of COX therefore involves propagation of the enzyme reaction in which electrons are transferred to oxygen, as well as preventing the escape of toxic intermediates.

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Abbreviations: COX, cytochrome *c* oxidase; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; BN-PAGE, blue native polyacrylamide gel electrophoresis

COX contains 13 subunits of which the three largest are encoded by the mitochondrial genome [3]. Irrespective of their coding sequence, all subunits of the complex are guided to the mitochondrial inner membrane and positioned in the appropriate orientation. Beside these protein subunits, also two heme groups and several metal ions have to be incorporated to obtain a functional complex [3].

A surprisingly large number of factors is needed for the proper assembly of COX ([4] for review). Characterisation of yeast cells deficient in COX activity has led to the identification of a number of genes whose products are required for wild-type function of the enzyme. Proteins involved in the maturation of heme (*COX10*, *COX11*) [5] and in the incorporation of copper atoms (*SCO1*, *SCO2* and *COX17*) [6] have been identified. *OXA1* appeared to be required for obtaining the correct membrane orientation of Cox2p [7]. However the role and mechanism of action of most of the assembly factors including *COX18*, *COX20*, *PET197*, *COX14*, *COX15* and *SHY1* remain to be elucidated.

Human homologues of most of the yeast COX assembly factors have been found. Mutations in *SCO1* [8], *SCO2* [9], *COX10* [10] and *SURF1* [11,12] have been linked to different human diseases. Leigh syndrome caused by mutations in *SURF1* (the human homologue of the yeast gene *SHY1*) is by far the most frequently reported defect and is characterised by a deficiency in COX activity [11,12].

The *SURF1* gene is highly conserved in a number of species [13]. The protein is located in mitochondria where it is probably anchored to the mitochondrial inner membrane by two transmembrane domains [11,14]. A construct containing only the two transmembrane helices and the central domain was able to compensate for the COX negative phenotype [14]. Although it is likely that *SURF1* affects the stability and or assembly of COX [15], its exact role remains so far unclear. The yeast homologue of *SURF1* (*SHY1*) was originally found to be a mitochondrial protein required for respiration. A Δ*shy1* strain displays in addition to a partial COX deficiency an elevated concentration of cytochrome *c* and an increased complex III activity [16]. Therefore in contrast to human *SURF1*, *SHY1* was not solely linked with COX. Together with the lack of complementation of a *SHY1* deletion strain by *SURF1* this has led to the suggestion that Shy1p and Surf1p may perform similar, but non-overlapping functions [14]. However this needs not necessarily be so. The sequence similarity between human *SURF1* and yeast *SHY1*, especially of some conserved domains is particularly striking [13] and

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several factors might contribute to the lack of species exchangeability. Changes in cytochrome *c* concentration and complex III activity might reflect a compensatory mechanism in yeast.

To obtain insight in the molecular action of *SURF1* we studied the yeast homologue *SHY1* [16] and compared our findings with the situation in man. In this study we addressed two main questions. First, in what way does Shy1p affect assembly, stability or intrinsic activity of COX? Second, does Shy1p act as a monomer or is there association with other components involved in the COX assembly process?

Using blue native two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) we demonstrate that there is a reduced amount of fully assembled COX in a Δ*shy* strain. Pulse-chase experiments using radioactive methionine show that this decrease is caused by a slower assembly. Still there is a significant amount of fully assembled COX present which has normal electron transfer activity. When assessing the molecular weight of Shy1p with 2D-PAGE Shy1p ran as a monomer, dimer and as a high molecular weight complex of approximate 250 kDa. Similar results were obtained using human cells.

2. Materials and methods

2.1. Cell lines, strains and media

Crude mitochondria from patients with Leigh syndrome and from healthy individuals were used in the study [15]. The *Saccharomyces cerevisiae* strain was W303/1A with the nuclear genotype: MATa, ade2-1, his3-11, -15, leu2-3, -112, ura3-1, trp1-1[ρ^+] and its disruptant shy1::*URA3*. Yeast cells were grown in YPGal (2% galactose, 1% peptone, 1% yeast extract). Solid media contained 2% agar. The standard temperature for yeast growth was 28°C.

Escherichia coli strain DH5α was used [17]. *E. coli* cells were grown in LB medium (1% bactotryptone, 0.5% yeast extract, 1% NaCl) supplemented with ampicillin (100 μg/ml) when necessary for plasmid selection.

2.2. Generation of SHY1-HA and SURF1 containing yeast expression vectors

Human *SURF1* cDNA [15] was cloned into p425TEF [18]. *SHY1* was amplified by means of PCR on chromosomal DNA (*S. cerevisiae* FY 1679-28C) using the primers 5′-GGG GAC TAG TTT AAT TAA GAT GTC TCT ACT AGG CGC CAG-3′ and 5′-CCC GGC GGC CGC CGC CGC GCC CAT ATA TTT CCT TGA ATG CTT-3′. The introduced restriction sites *Spe*I and *Not*I were used to ligate this fragment into pGH4 [19], a modified version of p413TEF [18] which already contained an in-frame GFP-HA sequence. In a next step the GFP fragment could be removed by deleting an *Asc*I fragment.

2.3. Electrophoresis, Western blotting and 'in-gel activity'

Standard sodium dodecyl sulphate (SDS)–PAGE was performed according to Laemmli [20] and 2D-PAGE was done by the method of Schägger and von Jagow [21]. We used a 10 kDa protein ladder as reference (Gibco). Following electrophoresis, proteins were blotted to nitrocellulose [22]. Immunoreactive material was visualised by chemoluminescence (ECL™, Amersham) according to the instructions of the manufacturer. In-gel activity assays were performed as described by Zerbetto and colleagues [23].

2.4. Preparation of mitochondrial fractions for blue native polyacrylamide gel electrophoresis (BN-PAGE)

The crude mitochondrial pellets of human fibroblasts were prepared using digitonin [24]. Yeast cells were grown on 20 ml YPGal medium (1% yeast extract, 1% peptone, 2% galactose) overnight to an OD_{600} of approximately 2.0. Mitochondrial pellets were prepared using glass beads to break the yeast cells followed by differential centrifugation [25]. Mitochondrial pellets were stored at −70°C until further processing for 2D-PAGE.

2.5. Antibodies

Polyclonal antiserum against Shy1p was kindly donated by Dr. A. Tzagoloff [16] and the polyclonal antibody used against Surf1p was described by Tiranti and colleagues [15]. Yeast prohibitin antibody was as described before [26]. The monoclonal antibody 12CA5 for the detection of HA-tagged SHY1 was obtained from Boehringer Mannheim. The monoclonal antibodies to yeast subunits Cox1p, Cox2p, Cox3p and Cox4p as well as the monoclonal antibodies to human subunits Cox1p and Cox2p were obtained from Molecular Probes.

2.6. Pulse-chase experiments

Pulse-chase experiments in isolated mitochondria were performed as described before [25]. In brief, isolated mitochondria were incubated in optimised protein-synthesising medium D in the presence of 8 μl/ml TRAN^{35}S-LABEL™ (1175 Ci/mmol; 10.5 mCi/ml; containing 70% L-[^{35}S]methionine and 15% L-[^{35}S]cysteine; ICN Biomedicals, Inc.). Labelling was allowed to continue for 30 min and 250 μl samples were taken at 10 min and 30 min. An excess of cold methionine (final concentration 0.2 M) was added after 30 min to start the chase. Samples were taken at 30 min and 60 min chase. Pulse-chase experiments in whole cells were performed using cycloheximide to stop cytoplasmic translation [25]. In short, pulse labelling was started by adding [^{35}S]methionine to a final concentration of 50 μCi per ml. After 30 min cells were collected by centrifugation and washed twice. Cells were chased by adding excess unlabelled methionine. Mitochondrial fractions for BN-PAGE were prepared as described above.

2.7. Miscellaneous

E. coli transformations were carried out by electroporation with the *E. coli* pulser (Bio-Rad). *S. cerevisiae* was transformed by using the one-step method [27].

3. Results and discussion

Shy1p was first characterised as a protein required for respiration in yeast [16]. Database comparison revealed similarity to one of a set of genes present in an evolutionarily conserved cluster known as SURF. *SHY1* is a yeast homologue of *SURF1*. The product of this gene is known to be involved in the biogenesis of COX, since Leigh patients with deficiency in the enzyme possess a mutated form of the gene [12]. Differences exist in phenotypes of a yeast Δ*shy1* mutant and Leigh patients, but the degree of protein sequence conservation suggests that both Shy1p and Surf1p play largely similar if not identical roles in COX biogenesis. Whether they are required primarily for assembly or maintaining stability of assembled complex is not known as is also the exact role played by either protein.

The steady-state levels of assembled COX in a Δ*shy1* strain and in a wild-type strain were analysed by 2D-PAGE (Fig. 1). The disruptant strain contains a complex that according to its migration in a native gel exactly resembles the fully assembled complex. This complex contains at least subunits I, II, and III (Fig. 1), and therefore very likely represents fully assembled COX. Low molecular weight immunoreactive material was clearly increased in the disruptant strain (indicated in Fig. 1). These species likely represent unassembled or partially degraded subunits. The strong signal in the upper part of the gel from Δ*shy1* likely comes from Cox1p aggregates. In bovine COX it is well documented that hydrophobic COX subunits especially Cox1p and Cox3p tend to aggregate, even in the presence of SDS [28]. In contrast to the situation in yeast, in human patient fibroblasts there are significant amounts of partially assembled intermediates present. Yeast may be better in degrading redundant possibly harmful assembly intermedi-

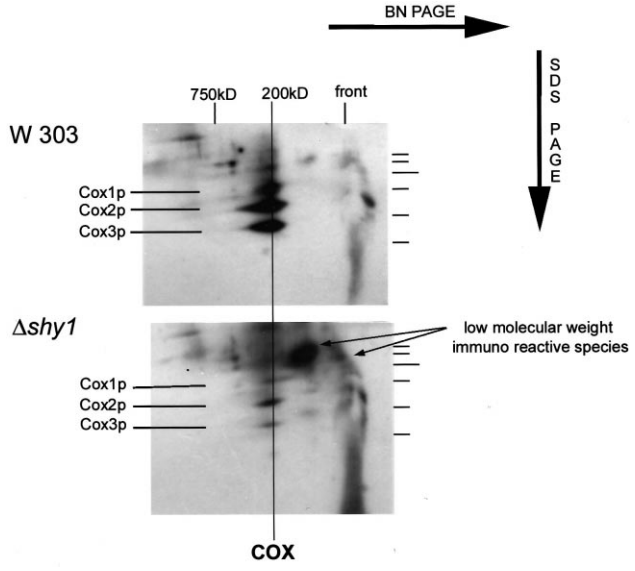

Fig. 1. Steady-state levels of COX in a Δ*shy1* and its corresponding wild-type strain (W303). Western blot analysis of blue native two-dimensional gels were incubated with monoclonal antibodies against subunit I (Cox1p), subunit II (Cox2p) and subunit III (Cox3p) of COX. Holo-cytochrome *c* oxidase is indicated as COX. Arrows indicate low molecular weight immunoreactive species (see also text). Molecular weights in the first dimension based on the molecular mass of known complexes: prohibitin (1000 kDa), ATP synthase (750 kDa) and COX (200 kDa) are indicated. In the second dimension a 10 kDa protein ladder was used (Gibco), the 50 kDa standard can be recognised by the longer mark.

ates or the lack of assembly intermediates might simply reflect differences in stability during the isolation procedure.

The amount of this holo-COX is approximately 30% of wild-type cells. This result is in line with the measured enzyme activity of COX which is also approximately 30% of the wild-type strain [16]. Fully assembled COX can also be found in Leigh patient fibroblasts albeit at reduced levels [15]. Since the amount of fully assembled COX and residual enzyme activity are both reduced to approximately 30% of the control, the reduced enzymatic activity appears to be due to a decrease in protein amount rather than an altered intrinsic COX activity. To test this we performed an in-gel activity assay as described by Zerbetto and colleagues [23]. A blue native gel containing Δ*shy1* and wild-type samples was cut into two halves. The lower half was incubated with substrates for histological staining for COX and the top half of the gel was incubated with substrates for complex V staining (Fig. 2A). The specific bands were quantified by scanning and the percentage of COX activity as a percentage of ATPase activity (internal control) was found to be 36% of the wild-type (Fig. 2B). This is comparable with the differences in amounts of fully assembled complex. We therefore conclude that the electron transfer activity of COX in a Δ*shy1* strain is normal and that assembly occurs to what in all other respects appears to be a normal enzyme.

Synthesis and turnover of mitochondrially encoded subunits were studied by pulse-chase labelling in isolated mitochondria. In the Δ*shy* cells there was a remarkable increase of newly synthesised mitochondrial subunits (Fig. 3A). When the same blot was probed with antibodies against COX subunits, the steady-state levels appeared to be as expected namely there is a decrease in a Δ*shy1* strain (Fig. 3B). Similar results were obtained when whole cells were labelled in the presence of a specific inhibitor of cytoplasmic translation (cycloheximide). This marked increase in newly synthesised COX subunits compared to wild-type cells possibly reflects a compensatory attempt of the cell to restore full respiratory capacity. This increased translation is not due to an increase in amount of mitochondria, because in the pulse-chase experiment in isolated mitochondria the amount of protein was kept constant and still there was an increased translation. The increased mitochondrial translation is not restricted to COX subunits but holds for all the mitochondrially encoded gene products, suggesting an increase of transcript levels. It has been demonstrated in *S. cerevisiae* that the increase in transcript levels which occurs upon release from glucose repression is mainly caused by an increased transcription and not by an increased mitochondrial DNA replication [29]. Despite the significantly increased translation there is still not a full restoration of COX. This increased translation of mitochondrially encoded subunits, however, explains the increase of complex III activity in a Δ*shy1* strain [16]. A net increase of in vitro mtDNA translation products can also be observed in *SURF1 null* fibroblasts (data not shown). Because synthesis is higher but steady-state levels are lower, this implies that there is an increased breakdown of the newly synthesised subunits, otherwise steady-state levels should also be increased in a Δ*shy1* strain.

To study the assembly of COX in more detail we performed a pulse-chase experiment in combination with 2D electrophoresis. Mitochondrially encoded gene products were selectively labelled by treating cells with cycloheximide. Incorporation of label into the individual subunits and holo-enzyme can then be resolved and quantified (Fig. 4). When we focus on Cox3p

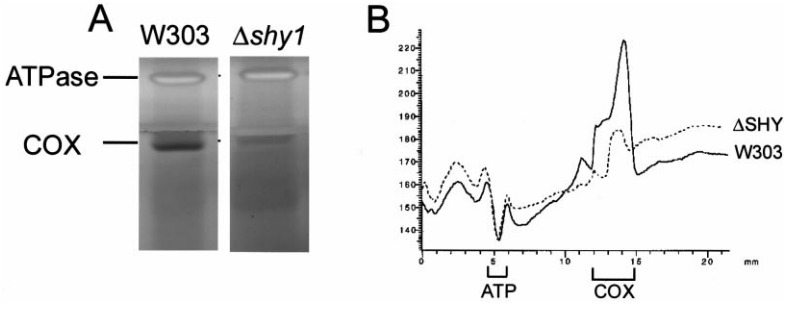

Fig. 2. The 'in-gel' COX activity and ATP synthase activity assays of a Δ*shy1* and W303 strain. A: The top part of the gel was stained for ATP synthase activity and the bottom part was stained for COX activity (see also Section 2). B: Quantification of the gel bands was done using the computer program Image Quant®. The horizontal axis displays distance on the gel (mm), the vertical axis represents absorbance of the gel in arbitrary units. Because the ATP synthase activity gives white precipitates in the gel this gives a negative absorbance on the gel.

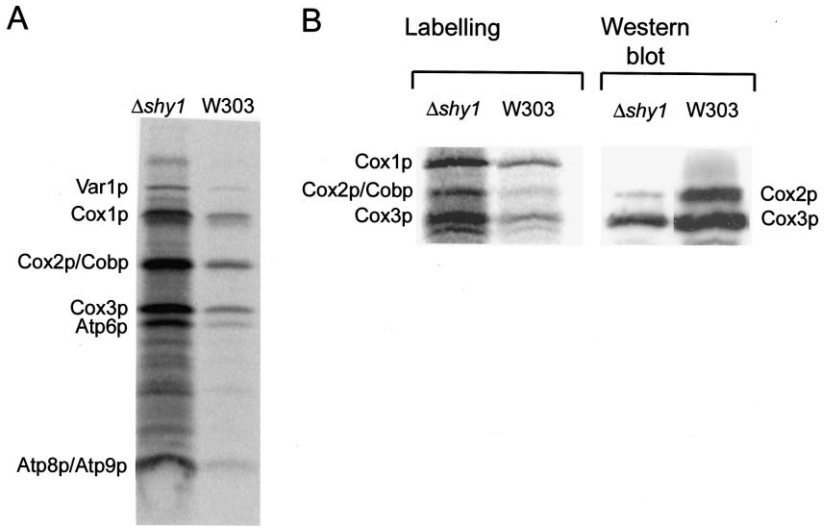

Fig. 3. Synthesis of mitochondrially encoded proteins. A: Isolated mitochondria of wild-type (W303) and *shy1* null mutant cells were labelled with [^{35}S]methionine for 20 min. 25 μg of proteins were loaded on a gel and separated by SDS–PAGE. B: Labelled mitochondrial translation products were separated by SDS–PAGE and blotted to nitrocellulose. First, labelled proteins were detected using a phosphorimager and subsequently the same blot was incubated with Cox2p and Cox3p antibodies to detect steady-state levels of these subunits.

and compare the wild-type cells with the Δ*shy1* cells, three main differences can be observed. First there is more smearing in a Δ*shy1* strain. Possibly this smear might represent incorrectly assembled, partially aggregated forms. Second there is more label in unassembled subunits in the *shy1*-disruptant strain. Third there is less label incorporated in holo-enzyme in Δ*shy1* cells compared to wild-type cells. In the *shy1*-disruptant strain label is much slower incorporated into holo-COX. This leads to the conclusion that the assembly of COX is impaired when Shy1p is not present. This is the first direct evidence that Shy1p is involved in COX assembly.

It has been demonstrated that mutants that have a *bc1* complex activity of 30% of the wild-type enzyme still display 75% growth rate of the wild-type strain on glycerol medium and even mutants that have only 5% of the wild-type activity still show growth on glycerol [30]. Because COX has a control strength rather similar to the *bc1* complex (approximate 0.2) [31] this means that 30% of COX activity should be sufficient to sustain approximately normal mitochondrial respiration and growth. The *SHY1*-disruptant strain displays a COX activity which is approximately 30% of that of control cells, yet Δ*shy1* cells completely fail to grow on non-fermentative carbon sources. These findings strongly suggest that additional factors contribute to the lack of growth on non-fermentative carbon sources. Possibly the imbalance of subunits and the handling of unassembled products that might affect the mito-

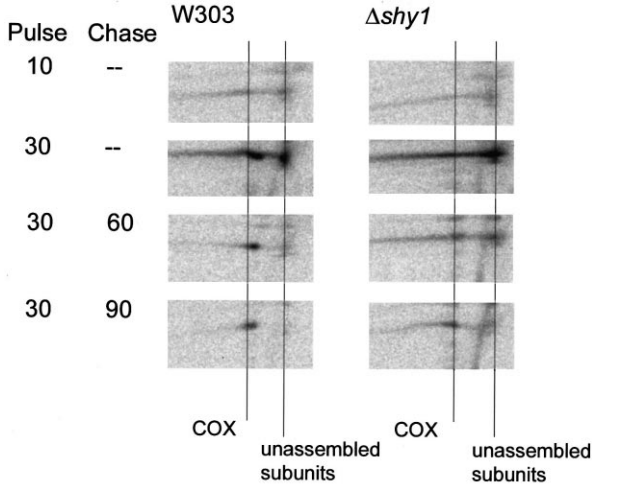

Fig. 4. Synthesis, stability and assembly of mitochondrially encoded Cox3p. Wild-type (W303) and *shy1* null mutant cells were pulse-labelled with [^{35}S]methionine for 10 min and 30 min in the presence of cycloheximide. After adding a large excess of unlabelled methionine, the cells were chased for 30 min and 60 min. Mitochondrial pellets were run on 2D-PAGE as described before. Labelled proteins were detected using a phosphorimager. Lines indicate the electrophoretic mobility of holo-cytochrome *c* oxidase (COX) and unassembled subunits. Note: only the parts of the gels containing Cox3p are shown.

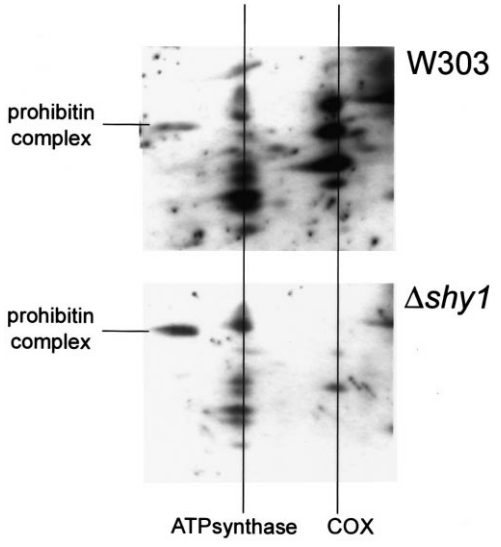

Fig. 5. Prohibitin complex levels are increased in a Δ*shy1* mutant compared to wild-type cells (W303). Western blots of 2D gels (as described before) were incubated with an anti-prohibitin antibody (anti-Phb1p). For better comparison, the blots were also incubated with Cox1p, Cox2p, Cox3p antibodies and a polyclonal antibody against F1-ATP synthase.

chondrial inner membrane integrity is such an additional factor contributing to the growth phenotype. It has been demonstrated that prohibitins form an assembly chaperone complex that is capable of binding newly synthesised COX subunits [26]. This prohibitin complex may play a particularly important role in situations in which there is an imbalance of mitochondrially or nuclear encoded subunits. For this reason we compared the amount of prohibitin complex in the Δ*shy1* strain. In support of this idea is the finding of a distinct increase of the prohibitin complex in the Δshy1 strain compared with wild-type (Fig. 5).

In order to obtain information about the molecular organisation of Shy1p we performed two-dimensional electrophoretic analyses. After isolation of wild-type and Δ*shy1* strain mitochondria, proteins were separated using 2D-PAGE. After transfer to nitrocellulose, blots were probed with an anti-Shy1p antibody (Fig. 6A). In the wild-type cells, a specific signal of the expected molecular weight (45 kDa) appeared. However, in the (native) first dimension Shy1p runs as high molecular weight complex of about 250 kDa, implying that Shy1p is associated with other components (or itself) in order to form a high molecular weight complex. To confirm this result we transformed a Δ*shy1* strain with a HA-tagged Shy1 protein. In this case the major immunoreactive spot of Shy1p has a molecular weight of 250 kDa in the first dimension (Fig. 6B). There are also minor spots of approximately 50 kDa and 100 kDa, which possibly reflect Shy1p monomer and dimer respectively. Although Shy1p behaved as a monomer by sedimentation in sucrose gradient [16], the mild solubilisation and conditions of protein separation used in 2D-PAGE allowed detection of a high molecular weight complex.

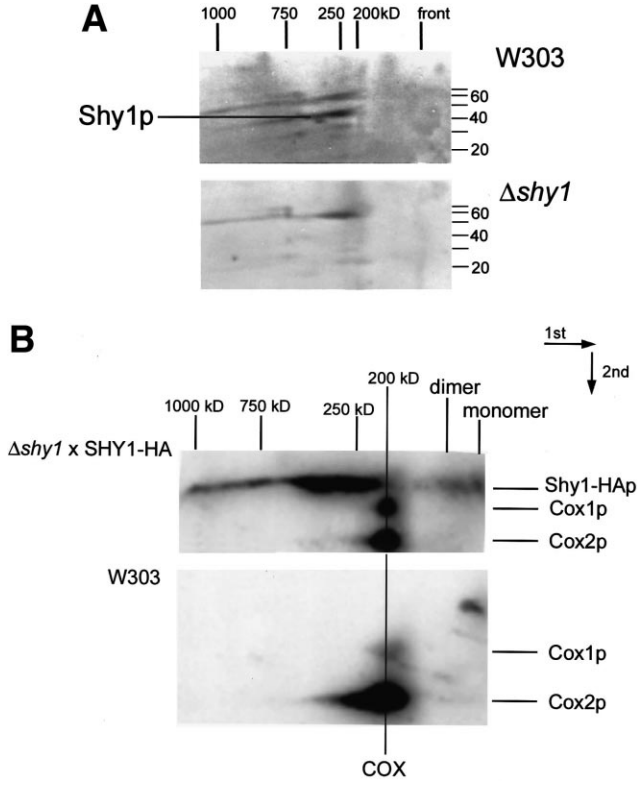

Fig. 6. Western blot of 2D-PAGE of mitochondrial samples from W303 and Δ*shy1* strains. A: Blots were probed with anti-Shy1p polyclonal antibody (Shy1p). B: 2D-PAGE blots of mitochondria from a wild-type strain (W303) and a Δ*shy1* strain transformed with a SHY construct tagged with a HA epitope (see Section 2). The blots were incubated with antibodies against Cox1p, Cox2p and HA. Molecular weight markers are used as in Fig. 1, 20 kDa, 40 kDa and 60 kDa are indicated.

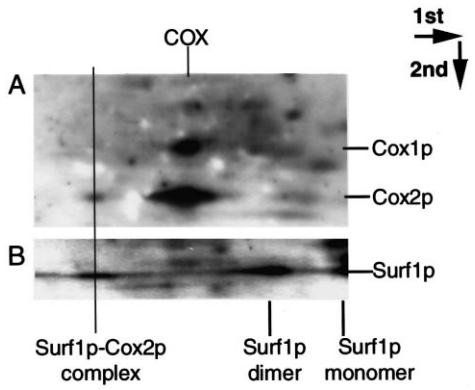

Fig. 7. Human Surf1p also appears in a high molecular weight complex. Fibroblasts, from patients with *surf1* null mutation, transfected with *SURF1*-HA were analysed by Western blots of 2D-PAGE gels, probed with an anti-human Cox1 and Cox2p antibody (A) and with anti-Surf1p antibody (B). COX refers to holo-cytochrome *c* oxydase.

To obtain information about the molecular status of the human Surf1p, we carried out a 2D blot of human fibroblasts that had been transfected with a HA-tagged *SURF1* construct (Fig. 7). A high molecular weight spot and two lower molecular weight species possibly corresponding with the monomer and dimer can be observed. When the same blot was probed with anti-COX antibodies, material reactive with anti-Cox2p antibody migrated at the same high molecular weight as the Surf1p complex. Although it is attractive to speculate that Cox2p may be associated with a high molecular weight *SURF1* complex, direct verification must await more rigorous assays for the presence of such a complex and for evidence of interactions between the proteins. We have attempted to obtain additional information on components associating with Surf1p, by co-immunoprecipitation experiments using the C-terminal antibody (cf. Surf1p [15]). Unfortunately, however, under the mild non-denaturing conditions necessary for demonstrating associations, we were unable to precipitate Surf1p (results not shown). The nature of these interactions is still not clear, but the co-migration of Cox2p with one of the *SURF1* complexes suggests an interaction with this COX subunit. A Cox2p interaction would make sense because from studies in human fibroblasts it has been implied that the COX assembly was disturbed at the level of Cox2p incorporation [15]. Further investigations are underway to address this question.

Our data clearly demonstrate that Shy1p is a COX assembly chaperone. It forms high molecular weight complexes, indicating interactions with other mitochondrial components, among these possibly Cox2p. Still the exact molecular working mechanism of Shy1p (and Surf1p) needs to be resolved.

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