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Identification of functional regions of Cbp3p, an enzyme-specific chaperone required for the assembly of ubiquinol-cytochrome *c* reductase in yeast mitochondria[1]

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Abstract

The Cbp3 protein of *Saccharomyces cerevisiae* is an enzyme-specific chaperone required for the assembly of ubiquinol-cytochrome *c* reductase of the mitochondrial respiratory chain. To gain preliminary insight into the role of Cbp3p during assembly, 29 independently isolated mutants were examined to define functional regions of the protein. Mutants were analyzed with respect to respiratory growth, ubiquinol-cytochrome *c* reductase assembly, and steady state amounts of enzyme subunits and Cbp3p. Three regions essential for Cbp3p activity were identified: regions 1 and 3 were required for Cbp3p function, while region 2 was necessary for protein stability. Mutation of Glu134 in region 1 (Cys124 through Ala140) impaired the ability of the Rieske FeS protein to assemble with the enzyme complex. Mutations targeted to region 3 (Gly223 through Asp229) primarily affected the 14 kDa subunit and cytochrome c_1 assembly. Gly223 was found especially sensitive to mutation and the introduction of charged residues at this site compromised Cbp3p functional activity. Region 2 (Leu167 through Pro175) overlapped the single hydrophobic domain of Cbp3p. Mutations within this area altered the association of Cbp3p with the mitochondrial membrane resulting in enhanced protein turnover. The role of the amino-terminus in Cbp3p activity was investigated using *cbp3* deletion strains Δ12–23, Δ24–54, Δ56–96 and Δ12–96. All mutants were respiratory competent, indicating that residues 12–96 were not essential for Cbp3p function, stability or mitochondrial import. Analysis of carboxy-terminal deletion mutants demonstrated that the final 44 residues were not necessary for Cbp3p function; however, alterations in the secondary structure of the extreme carboxy-terminal 17 residues affected assembly protein activity.

Keywords: Organelle biogenesis; Respiratory chain; Chaperone-assisted assembly of multisubunit enzyme

1. Introduction

Ubiquinol-cytochrome *c* reductase is a respiratory chain enzyme of the inner mitochondrial membrane, transferring electrons from reduced ubiquinone to ferricytochrome *c*. The multisubunit enzyme couples electron transport with a proton pumping mechanism which assists in forming an electrochemical gradient across the inner membrane. Energy from the

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gradient is utilized during the production of ATP, in addition to sustaining other mitochondrial functions [1,2].

In the yeast *Saccharomyces cerevisiae*, ubiquinol-cytochrome *c* reductase is composed of 10 non-identical subunits with a defined catalytic center consisting of cytochrome *b*, cytochrome c_1 and the Rieske FeS protein [3–6]. The majority of the remaining subunits are required for enzyme activity, though their roles are not completely defined. The yeast enzyme is functionally and in general structurally similar to its mammalian counterparts, except the bovine and chicken heart enzymes contain an 11th subunit which corresponds to the cleaved presequence of the Rieske FeS protein [7–9].

The mechanism by which the subunits assemble into a functional enzyme is not fully understood. Cytochrome *b* is encoded on mitochondrial DNA and synthesized within the organelle. The remaining subunits are transcribed from nuclear DNA, synthesized in the cytosol, then imported into the mitochondria where they assemble with cytochrome *b* in the inner membrane. Previous studies have shown that two enzyme-specific chaperones, Cbp3p and Cbp4p, are essential for ubiquinol-cytochrome *c* reductase assembly and function during a late stage of the assembly pathway [10,11].

Cbp3p and Cbp4p are localized to the mitochondrial membrane but are not components of the purified enzyme. Strains carrying *CBP3* or *CBP4* nuclear gene deletions are respiratory deficient and lack ubiquinol-cytochrome *c* reductase activity, but show wild type activity levels of other respiratory enzymes. Loss of either assembly protein results in decreased steady state amounts of cytochrome *b*, Rieske FeS protein, plus the 14 kDa and 11 kDa subunits. All four subunits are extremely protease labile in the absence of an assembled enzyme [12–15]. The *cbp3* and *cbp4* null mutants are phenotypically identical, suggesting that the two proteins may function in a similar manner.

Several roles have been proposed for Cbp3p and Cbp4p during enzyme assembly [11]. Cbp3p and Cbp4p may interact with and stabilize a select group of subunits during assembly. The association of subunits into a partially assembled complex could provide binding sites for interactions with other subunits, signaling a continuation of enzyme assembly. Perhaps Cbp3p and Cbp4p bind to the protease labile subunits, shielding them from proteolysis until they can be properly assembled. Another possibility is that the assembly proteins bind newly folded subunits, masking interactive polypeptide surfaces until the correct partner is found. This would prevent unwanted interactions with proteins belonging to other multisubunit enzymes in the mitochondrial membrane.

Cbp3p and Cbp4p are members of a larger, ever expanding collection of proteins which play a role in the assembly/stability of multisubunit enzymes required for electron transport and ATP synthesis. The proteins function as enzyme-specific chaperones, since each is essential for the formation of a specific enzyme complex during its later stages of assembly, yet none are components of the final product. Some examples include the yeast *ATP10*, *ATP11* and *ATP12* genes which encode proteins required for mitochondrial F_0F_1-ATPase assembly [16,17]. *SCO1*, *COX14*, *COX15*, *PET100*, *PET117* and *PET191* are necessary for cytochrome oxidase formation and act to stabilize and/or enhance subunit assembly [18–22]. Yeast succinate reductase (complex II) assembly is dependent on the *TCM62* gene product, and assembly of the membrane segment of NADH-ubiquinone oxidoreductase (complex I) involves two unique chaperone proteins [23,24]. Recently, Bcs1p has been shown to be definitively required for assembly of the Rieske FeS protein and 8.5 kDa subunit into ubiquinol-cytochrome *c* reductase [25,26].

This study explores protein structure–activity relationships of the Cbp3p chaperone. Here we identify three important regions of Cbp3p, two of which are required for ubiquinol-cytochrome *c* reductase assembly; the third is necessary for Cbp3p stability. We describe how mutations in these areas affect enzyme assembly and subunit stability, identify essential features of the protein and define a minimal domain of Cbp3p required for chaperone function. The current work will serve as a basis for further studies into the mechanism by which Cbp3p assists in ubiquinol-cytochrome *c* reductase assembly.

2. Materials and methods

2.1. Yeast strains and growth media

Yeast strains used in this study: *cbp3* null mutant, W303ΔCBP3 (*MATa*, ρ^+, *ade2-1*, *his3-11*, *15*, *leu2-3*, *112*, *ura3-1*, *trp1-1*, *can1-100*, *CBP3::HIS*) [10]; parental strain W303-1A (*MATa*, ρ^+, *ade2-1*, *his3-11*, *15*, *leu2-3*, *112*, *ura3-1*, *trp1-1*, *can1-100*) [27]. Media: YPD (2% glucose, 2% peptone, 1% yeast extract), YPGal (2% galactose, 2% peptone, 1% yeast extract), glycerol (3% glycerol, 2% ethanol, 2% peptone, 1% yeast extract), WO (2% glucose, 0.67% yeast nitrogen base without amino acids (Difco), supplemented with amino acids and nutrients as required at 25 μg/ml). Solid media contained 2% agar.

2.2. PCR mutagenesis

CBP3 mutations were introduced by gene-specific random-hit PCR mutagenesis [28]. An approx. 1720 bp *Pst*I-*Bgl*II fragment carrying *CBP3* was subcloned into pRS316 [29] in which the *Nde*I, *Sal*I and *Xho*I sites had been destroyed. The recombinant centromere plasmid (pCBP3/RS316) was used as template for PCR reactions primed with oligonucleotides corresponding to the T7 and T3 regions of the vector. Mutations were introduced during the first two PCR cycles using *Taq* DNA polymerase while enhancing enzyme infidelity with 6 mM $MgCl_2$ plus low concentrations (0.001 mM) of one dNTP in each reaction. Following the second cycle, dTNP concentrations were increased to 1 mM and the introduced mutations amplified for 23 additional cycles.

Mutations were rescued in the *cbp3* null strain by gap repair of the wild type gene on a centromere plasmid [30]. Gapped plasmids were prepared from pCBP3/RS316 by removing a 953 bp *Nde*I-*Hin*dIII fragment containing 681 bp of the 5′ end of the open reading frame plus upstream sequence, or by excising a 766 bp *Nde*I-*Sac*I fragment containing 324 bp of the 3′ open reading frame plus downstream sequence. The *cbp3* null strain was co-transformed with a gapped plasmid plus PCR product (1:4 ratio) in the presence of 100 μg carrier DNA. Transformants prototrophic for uracil were selected, replica plated to glycerol plates and incubated at 23°C, 30°C and 37°C. Glycerol growth was scored over 7 days.

2.3. Isolation of single point mutations from cbp3 mutants carrying more than one mutation

Mutant plasmid C124Y/A140V/M171I was digested with *Hin*dIII plus *Sna*BI, the DNA coding for the C124Y+A140V mutations (780 bp *Hin*dIII-*Sna*BI fragment) was isolated then ligated into pCBP3/RS316, after excision and removal of the corresponding 780 bp *Hin*dIII-*Sna*BI wild type DNA, to create plasmid C124Y/A140V. Using the same cloning strategy, mutation K125E was isolated on a 780 bp *Hin*dIII-*Sna*BI fragment from plasmid carrying K125E/R172G and subcloned into pCBP3/RS316 linearized with *Hin*dIII and *Sna*BI. Mutations E327K/R328K were rescued from a triple mutation strain on a 500 bp *Xba*I-*Sac*I fragment and substituted for the analogous wild type DNA in pCBP3/RS316.

2.4. Construction of N-terminal deletions Δ12–23, Δ25–54, Δ56–96, Δ12–96

Internal deletion Δ12–23 was constructed by digesting pG28/ST3, a multiple copy plasmid containing *CBP3* plus approx. 275 bp upstream and 440 bp downstream genomic sequence [10] with *Sal*I and *Xho*I to remove the 40 bp intervening sequence. The linear DNA was blunt-ended with DNA polymerase I Klenow fragment and religated, creating both *Bsi*EI and *Pvu*I sites at the junction, which were subsequently used to identify recombinant plasmids. Deletion Δ12–23 was transferred to centromere plasmid pRS316 [29] utilizing *Hin*dIII and *Sac*I sites in the multiple cloning region to create Δ12–23/cen. Deletion Δ25–54 was prepared from pCBP3/RS316 by removing a 93 bp *Xho*I-*Eco*RI fragment, filling in 3′ recessed ends using the large Klenow fragment of DNA polymerase I, and religating in the presence of an 8-mer *Xba*I linker (5′-CTCTAGAG-3′). In the final construct (Δ25–54/cen) the tripeptide encoded by the linker, Thr-Leu-Glu, was inserted between Ser24 and Asn55. Deletion Δ56–96 was created by digesting pCBP3/RS316 with *Eco*RI and *Spe*I to remove 129 bp. The resulting DNA was blunt-ended

with DNA polymerase I Klenow fragment, and religated in the presence of a *Xba*I linker (5′-CTCTAGAG-3′). A Leu was inserted between Ser55 and Glu97 in the final construct (Δ56–96/cen). Deletion Δ12–96/cen was constructed by digesting pCBP3/RS316 with *Sal*I and *Spe*I, the approx. 6300 bp linear DNA blunt-ended with Klenow fragment of DNA polymerase I and religated. Mutant genes Δ25–54, Δ56–96 and Δ12–96 were moved to multicopy plasmid Yep352 [31] utilizing *Hin*dIII and *Sac*I sites in the multiple cloning regions of each plasmid. All constructs were verified by DNA sequencing using the Sanger dideoxy method [32].

2.5. Carboxy-terminal deletions

Plasmid pG28/ST3 was linearized at the unique *Bam*HI restriction site in the 3′ end of the open reading frame (codon 320), and 2 μg aliquots were incubated with 0.067 units of Bal31 nuclease for 15 and 30 min. The DNA was blunt-ended with DNA polymerase I, Klenow fragment, and recircularized in the presence of a *Xba*I 8-mer linker (5′-CTCTAGAG-3′). The size of each deletion was estimated by restriction mapping and later confirmed by sequencing through the end point. Deletions were transferred to centromere plasmid pRS316 utilizing the unique *Hin*dIII, *Sac*I restriction sites in the multiple cloning regions of both vectors. Mutants were obtained by transforming the *cbp3* null strain with the deletion plasmids.

2.6. Site-directed mutagenesis

The G(223)A mutation was constructed using PCR-based site-directed mutagenesis. Sense primer 5′-TCCAATACGCAACTGCGCGCTGCGATATTT-3′, which introduced a codon change of Gly to Ala and created a *Bss*HII restriction site, was partnered with the vector hybridizing T7 primer to amplify a 770 bp fragment from template pCBP3/RS316 using Deep Vent DNA polymerase. A complementary primer, 5′-AAATATCGCAGCGCGCAGTTGCGTATTGAA-3′, was paired with vector hybridizing T3 primer to amplify 950 bp of DNA upstream from the mutation. The 770 bp DNA, digested with *Bss*HII and *Sac*I, plus the 950 bp fragment, digested with *Bss*HII and *Hin*dIII, were ligated simultaneously into centromere plasmid pRS316 linearized with *Sac*I and *Hin*dIII. W303ΔCBP3 was transformed with the recombinant plasmid to create mutant Gly(223)Ala.

2.7. Preparation of yeast mitochondria and alkaline extraction of membrane proteins

Mitochondria were prepared by the method of Faye et al. [33] except that zymolyase was used to convert the cells to spheroplasts and 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to the spheroplast lysis buffer and upon final resuspension of the organelles. For alkaline extraction of membrane proteins, mitochondria were treated with 100 mM ice cold Na_2CO_3 (pH 11.7) at a final concentration of 0.75 mg/ml and incubated on ice for 30 min in the presence of protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, 5 μ/ml aprotinin, 1 μg/ml pepstatin, 2 μg/ml chymostatin) [34,35]. Membranes were separated from soluble material by centrifugation for 90 min at $100\,000 \times g_{ave}$. Pellets were washed in 20 mM Tris–HCl, pH 7.5, 1 mM EDTA plus protease inhibitor cocktail, membranes reisolated by centrifugation as above for 30 min, then resuspended in wash buffer.

2.8. Sucrose gradient centrifugation

Mitochondria (4 mg) were solubilized with dodecyl maltoside (0.8 mg/mg protein) for 30 min on ice and the soluble fraction isolated following centrifugation at $107\,000 \times g_{ave}$ for 90 min [5]. The supernatant was brought to 100 mM NaCl, incubated at 4°C for 60 min, then loaded onto a 3.8 ml gradient of 7–50% sucrose in 50 mM Tris–HCl (pH 8.0), 1 mM $MgSO_4$, 0.1 mg/ml dodecyl maltoside, 100 mM NaCl. Gradients were centrifuged at $485\,049 \times g_{ave}$ for 5.25 h and 16 fractions collected.

2.9. Miscellaneous methods

Yeast were transformed by the lithium acetate/polyethylene glycol method described by Ito et al. [36] when repairing gapped plasmids with mutagen-

ized PCR products, or by Elble [37] for all other transformations. Standard protocols were used for DNA manipulations including DNA purification on agarose gels, ligation of restriction fragments, *Escherichia coli* transformation and isolation of recombinant plasmids [38]. Mitochondrial proteins (40 μg) were separated on 12 or 17% SDS–polyacrylamide gels [39], electrophoretically transferred to nitrocellulose membranes [40] and challenged with polyclonal antisera directed against ubiquinol-cytochrome *c* reductase subunits or Cbp3p [10,13]. Ubiquinol-cytochrome *c* reductase activity was measured as previously described [11]. Protein concentrations were estimated by the method of Lowry [41].

3. Results

3.1. Mutagenesis and mutant screening

Random point mutations were introduced into *CBP3* by PCR mutagenesis and mutations rescued in vivo by gap repair of *CBP3* on a centromere plasmid (see Section 2). The effects of mutations on protein function were assessed by examining the growth rates of transformants on glycerol, a non-fermentable carbon source. Out of 5000 transformants screened, approx. 170 exhibited respiratory impaired growth and were either unable to grow, or grew slowly, on glycerol at 23°C, 30°C or 37°C. Mutants

Table 1
Phenotype of *cbp3* point mutants

Mutant	Growth on glycerol[a]	Ubiquinol-cytochrome *c* reductase activity (37°C) (mol/min/mg)[b]	Mutational region No.
W303 (wild type)	wild type	4.15 (±0.06)	none
Glu(134)Gly	ts[c] (slow growth at 37°C)	1.33 (±0.08)	1
Lys(125)Glu/Arg(172)Gly	ts (no growth at 37°C)	0.00	1, 2
Lys(125)Glu[d]	ts (slow growth at 37°C)	0.36 (±0.01)	1
Cys(124)Tyr/Ala(140)Val/Met(171)Ile	ts (no growth at 37°C)	0.00	1, 2
Cys(124)Tyr/Ala(140)Val[e]	ts (very slow growth at 37°C)	0.16 (±0.01)	1
Met(171)Ile[e]	ts (negligible growth at 37°C)	0.00	2
Tyr(131)Asp/Asp(229)Tyr	ts (no growth at 37°C)	0.00	1, 3
Asp(229)Tyr[f]	ts (no growth at 37°C)	0.00	3
Leu(167)Pro (two mutants isolated)	ts (negligible growth at 37°C)	0.00	2
Arg(172)Gly (six mutants isolated)	ts (no growth at 37°C)	0.00	2
Pro(175)Ser/Gly(223)Arg (two mutants isolated)	slow growth at 23 and 30°C, very slow growth at 37°C	0.17 (±0.01)	2, 3
Gly(223)Arg	slow growth at all temperatures, progressively slower as temperature increases	0.25 (±0.02)	3
Gly(223)Glu	slow growth at all temperatures, no growth at 37°C	0.00	3
Ala(227)Glu	ts (negligible growth at 37°C)	0.00	3
Ser(154)Pro/Gly(223)Arg	slow growth at all temperatures, progressively slower as temperature increases	0.30 (±0.02)	3 and non-cluster mutation
Pro(325)Leu	slow growth at all temperatures, progressively slower as temperature increases	0.28 (±0.02)	non-cluster mutation

[a]Growth on glycerol was assessed following replica plating mutants to solid medium containing glycerol. Mutants were scored for growth over a 7 day period at 23, 30 and 37°C.
[b]Values are averages of three independent assays performed on two or more preparations of mitochondria.
[c]Temperature sensitive.
[d]Isolated from mutant Lys(125)Glu/Arg(172)Gly.
[e]Isolated from mutant Cys(124)Tyr/Ala(140)Val/Met(171)Ile.
[f]Isolated from mutant Tyr(131)Asp/Asp(229)Tyr.

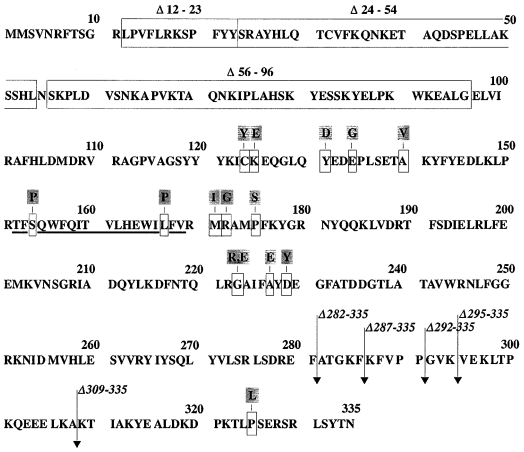

Fig. 1. Schematic presentation of Cbp3p mutants. The primary translation product of *CBP3* is shown in single-letter code. Large open boxes signify the three amino-terminal deletions with the deleted residues indicated above each box (Δ12–23, Δ24–54 and Δ56–96). Gray boxes indicate the amino acid substitutions identified in the independently isolated point mutants, with a corresponding white box indicating the targeted residue. The heavy black line indicates the location of the putative hydrophobic domain (residues 152–169). Arrows define the carboxy-terminal deletions.

incapable of glycerol growth were crossed to a rho^0 tester strain lacking mitochondrial DNA. Respiratory competent diploids issuing from the crosses indicated that the mutations did not reside in mitochondrial DNA.

Mutants which grew slowly on glycerol or exhibited temperature dependent respiratory growth were propagated vegetatively in liquid YPD and single colonies replica plated to glycerol and ura$^-$ plates. Co-segregation of the respiratory impaired phenotype and uracil prototrophy confirmed plasmid dependence and stability of the mutation. Plasmid DNA isolated from the mutants was reintroduced into the *cbp3* null strain. Back-transformants displaying the original mutant phenotype identified mutations as plasmid localized rather than nuclear in origin.

3.2. Identification of mutation sites

DNA sequence analysis of 19 independently isolated mutants revealed 26 mutations affecting 14 different residues (Table 1). In general, mutations were clustered within three regions of the protein (Fig. 1). Mutational cluster 1 (Cys124 to Ala140) was located on the amino-terminal side of the sole putative hydrophobic domain (residues 152–169). The second grouping (Leu167 to Pro175) overlapped the hydrophobic region, and the third collection included Gly223 through Asp229. The clustering of mutations, together with the independent isolation of specific mutations multiple times, suggested that the targeted areas may represent functionally significant regions of the assembly protein. Two residues were particularly sensitive to mutation. Arg(172)Gly occurred in seven independently isolated strains, while Gly(223)Arg was isolated four times, Gly(223)Glu once. All *cbp3* mutants demonstrated severely reduced to negligible levels of ubiquinol-cytochrome *c* reductase activity (Table 1).

Several genes carried more than one mutation. To determine the contribution of each to the overall phenotype, individual mutations were isolated on DNA restriction fragments and substituted for the analogous wild type DNA in pCBP3/RS316 (see Section 2). Recombinant plasmids were introduced into the *cbp3* null strain and respiratory growth rates plus ubiquinol-cytochrome *c* reductase activity of the resulting transformants assessed (Table 1). A mutation

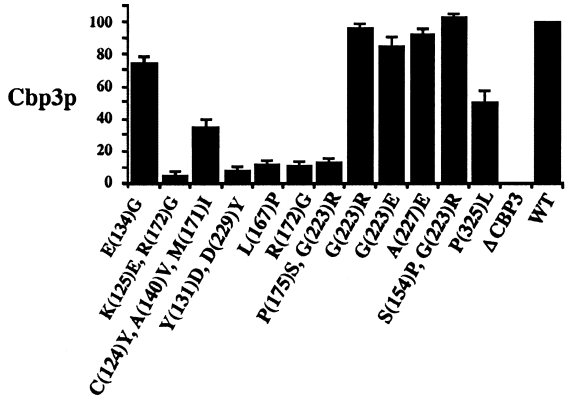

Fig. 2. Western analysis of Cbp3p in *cbp3* point mutants. Mitochondria were prepared from respiratory competent strain W303-1A (WT), *cbp3* null mutant (ΔCBP3), and 12 *cbp3* point mutants generated by random PCR mutagenesis. All strains were grown at 37°C. Total mitochondrial proteins were separated on 12% SDS–polyacrylamide gels, transferred to nitrocellulose membranes and challenged with Cbp3p polyclonal antibodies. Antigen–antibody complexes were decorated with ^{125}I-protein A and visualized by autoradiography. Signal intensities were quantitated using a Pharmacia Biotech ImageMaster VDS. The values reported have been normalized relative to wild type and are averages of three or more independent experiments. Different exposures were used for densitometric scanning.

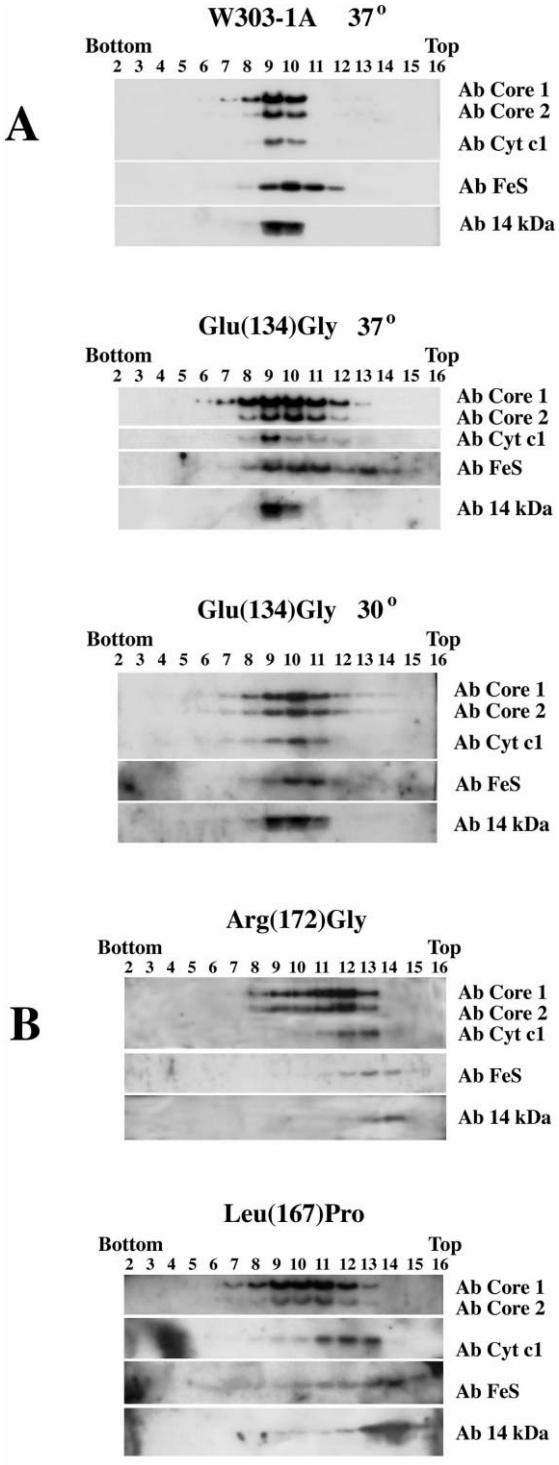

Fig. 3. Assembly of ubiquinol-cytochrome *c* reductase in *cbp3* point mutants. Mitochondria (4 mg) prepared from strains grown at 37°C were treated with dodecyl maltoside. Solubilized extracts were isolated by centrifugation and applied to a 3.8 ml sucrose gradient. Proteins were separated by high-speed centrifugation and 16 fractions collected. Samples were electrophoresed on 12% or 17% SDS–polyacrylamide gels and proteins visualized by Western blotting using polyclonal antisera specific to the individual enzyme subunit, as described in the legend to Fig. 2. (A) Region 1 mutant Glu(134)Gly grown at both 30°C and 37°C, W303-1A (wild type); (B) Region 2 mutants Arg(172)Gly and Leu(167)Pro; (C) Region 3 mutants Gly(223)Arg, Gly(223)Glu and Ala(227)Glu. Top and Bottom indicate the orientation of the gradient, numbers represent the fractions collected. The antibodies used are shown in the right margin of the figure. Ab, antibody; Cyt c1, cytochrome c_1; FeS, Rieske FeS protein.

conferring a stronger phenotype often masked the contribution of others (K125E/R172G compared to K125E). Frequently, dissection of multiple lesions revealed silent mutations (not shown) which were always located outside the defined mutational clusters.

3.3. Steady state amounts of Cbp3p in point mutants

To determine the effect of mutations on protein stability, Cbp3p steady state levels were examined. The analysis revealed a correlation between the

amount of immunodetected Cbp3p and location of the mutation (Fig. 2). The assembly protein was at or near wild type levels in mutants carrying single lesions in either region 1 or region 3, suggesting the respiratory impaired phenotype was due to loss of Cbp3p function rather than increased susceptibility of mutant protein to proteolysis. Strains with region 2 mutations showed decreased steady state amounts of Cbp3p, implying enhanced protein turnover. It is noteworthy that mutations affecting protein stability lie in or close to the hydrophobic domain. A region 2 phenotype dominated when combined with other mutations in multiple lesion strains (Fig. 2). As anticipated, all temperature sensitive mutants exhibited wild type Cbp3p levels at permissive growth temperatures (not shown).

3.4. *Assembly of ubiquinol-cytochrome c reductase and steady state amounts of enzyme subunits in cbp3 point mutants*

Ubiquinol-cytochrome *c* reductase assembly was examined in *cbp3* point mutants following sedimentation of detergent solubilized mitochondrial extracts through sucrose gradients. Subunits of an intact, assembled enzyme co-migrated to the middle fractions of the gradient in a wild type strain, but not in the mutants (Fig. 3). The sedimentation pattern of enzyme subunits was generally similar among mutants from the same cluster.

Region 1 mutations E134G, K125E and C124Y/A140V conferred a temperature sensitive phenotype with mutants demonstrating slow respiratory growth at the non-permissive temperature (Table 1). Mutant E134G exhibited wild type amounts of Cbp3p and ubiquinol-cytochrome *c* reductase subunits (Fig. 4) (core 1 and core 2 not shown), suggesting the mutation affected Cbp3p function. To explore this further, E134G mutant mitochondrial extracts were separated on a sucrose column and gradient fractions analyzed by Western blotting. Fig. 3A shows the co-migration of enzyme subunits as an assembled complex, with the exception of the Rieske FeS protein. The latter was concentrated into two areas of the gradient, co-migrating with assembled enzyme (middle fractions) plus another peak in the upper portion of the gradient (fraction 13). Cytochrome *b* and the 11 kDa subunit were found exclusively in the middle gradient fractions co-sedimenting with assembled subunits (not shown). The results suggest that the E134G mutation, either directly or indirectly, altered the ability of the Rieske FeS protein to assemble into the enzyme complex. This partial assembly of the Rieske FeS subunit would account for the slow respiratory growth of the mutant at 37°C and small amount of ubiquinol-cytochrome *c* reductase activity. When mutant E134G was grown at the permissive temperature and mitochondrial extracts similarly analyzed, all immunodetected Rieske FeS protein co-migrated with the assembled subunits (Fig. 3A).

Region 2 mutants were temperature sensitive for respiratory growth, exhibited reduced amounts of Cbp3p, and decreased cytochrome *b*, 14 kDa, 11 kDa, and Rieske FeS protein levels comparable to those observed in the *cbp3* null strain (Fig. 4). The large core subunits were present at wild type levels (not shown). Mutations L167P and R172G led to diminished assembly of the enzyme complex as detected on sucrose gradients. Fig. 3B shows the Rieske FeS and 14 kDa proteins sedimenting near the top of the gradient as unassembled monomers. The large core 1 and core 2 subunits co-migrated as a lighter subcomplex in mutant R172G compared to wild type, and cytochrome c_1 did not associate with the core subunits in either mutant. The amounts of cytochrome *b* and the 11 kDa subunit were not sufficient to allow their detection in the gradient fractions. In addition, ubiquinol-cytochrome *c* reductase activity was absent in the mutants (Table 1). Taken together, the results suggest the respiratory chain enzyme is not assembled in region 2 mutants.

Respiratory growth phenotypes varied among region 3 mutants. Mutant G223R grew progressively slower on glycerol as the ambient temperature increased whereas the G223E mutation was more deleterious to protein function, and produced a respiratory deficient phenotype at 37°C. Mutant A227E was temperature sensitive. Strain dependent differences were observed in the steady state amounts of enzyme subunits. Mutant G223R showed wild type levels of all subunits tested except cytochrome *b*. Mutations G223E and A227E resulted in decreased amounts of cytochrome *b*, Rieske FeS protein, 14 kDa and 11 kDa subunits which were either greater than or equivalent to, as in the case of the Rieske FeS pro-

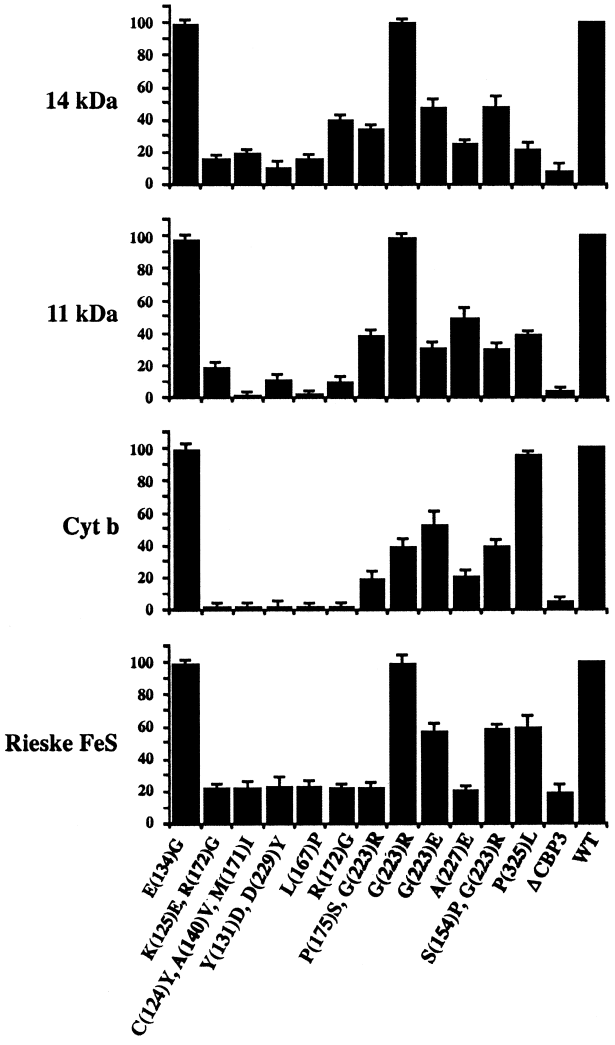

Fig. 4. Western analysis of ubiquinol-cytochrome *c* reductase subunits in *cbp3* point mutants. Mitochondria were prepared from respiratory competent strain W303-1A (WT), *cbp3* null strain (ΔCBP3), and *cbp3* point mutants grown at 37°C. Total mitochondrial proteins were separated on 12% or 17% SDS–polyacrylamide gels. Subunits were visualized by Western blotting using polyclonal antisera and the signal intensities quantitated as described in the legend to Fig. 2. Cyt b, cytochrome *b*.

tein in mutant A227E, the *cbp3* null strain (Fig. 4). The levels of core 1 and core 2 were approximately wild type.

Ubiquinol-cytochrome *c* reductase was not fully assembled in region 3 mutants. Several features distinguish region 3 from region 2 mutants. In the former, wild type or near wild type quantities of Cbp3p were immunodetected at 37°C (Fig. 2). Second, the 14 kDa subunit was largely assembled in region 3 mutants. In mutant G223R, the 14 kDa protein was immunodetected in fraction 9, analogous to wild type (Fig. 3C). Combined with a slow growth phenotype, which correlated to approx. 6% wild type enzymatic activity, the presence of some assembled enzyme can be inferred. When Gly223 was replaced with negatively charged Glu, the 14 kDa subunit appeared equally distributed between peak fractions 9 and 13–14. This finding suggests some enzyme subunits were assembled. However, since strain G223E was respiratory deficient at 37°C and ubiquinol-cytochrome *c* activity was absent, assembly was probably not complete. The 14 kDa subunit was likewise distributed into two areas of the gradient in mutant A227E, but again the mutant showed negligible respiratory growth at 37°C and lacked enzyme activity.

Core subunits 1 and 2 co-sedimented through the mutant gradients as a lighter subcomplex. Cytochrome c_1 co-migrated with the core subunits in G223E mutant extracts, but was not associated with the large core proteins in either the G223R or A227E strain. The majority of the Rieske FeS protein was immunodetected in the upper gradient fractions in all three mutants. Since the Rieske FeS subunit is one of the last subunits to associate with the complex [13,26], region 3 mutations may not directly affect its assembly. In the absence of a precomplex formed from partner subunits, assembly of the Rieske FeS protein will not occur.

Five independently isolated mutants carried an amino acid substitution at Gly223, suggesting the residue may be functionally important. To examine the requirement for an uncharged residue at position 223, alanine was introduced using PCR-based site-directed mutagenesis (see Section 2). When the *cbp3* null strain was transformed with the G223A mutation on a centromere plasmid the resulting transformants were respiratory competent, with growth rates on glycerol and amounts of mutant Cbp3p (not shown) similar to wild type. The findings indicate a preference for an uncharged residue with minimal bulk at this critical position of the protein.

3.5. *Cbp3p is a peripherally associated membrane protein*

Cbp3p remains associated with the mitochondrial membrane following disruption of the organelle in

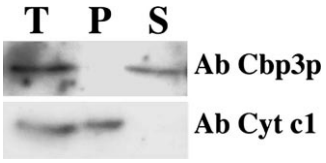

Fig. 5. Alkaline extraction of wild type mitochondria. Wild type mitochondrial proteins (150 μg) were treated with 100 mM Na_2CO_3 (pH 11.7) at a final concentration of 0.75 mg/ml in the presence of protease inhibitors. Membranes were separated from the supernatant fraction by high-speed centrifugation. Total (T) mitochondrial proteins (30 μg) plus equivalent volumes of soluble fraction (S) and washed, resuspended membranes (P) were electrophoresed on 12% SDS–polyacrylamide gels followed by Western analysis, as described in the legend to Fig. 2. The antibodies used are indicated in the right margin of the figure. Ab, antibody; Cyt c1, cytochrome c_1.

the presence of high salt, and is released from the membrane with detergents [10]. To determine whether Cbp3p is a transmembrane protein, mitochondria were extracted under alkaline conditions [34,45]. Fig. 5 demonstrates that Cbp3p was completely extracted into the supernatant following treatment with sodium carbonate, while cytochrome c_1, an integral membrane subunit of ubiquinol-cytochrome *c* reductase, remained with the membrane. The results indicate that the hydrophobic area of Cbp3p does not constitute a transmembrane domain but rather fosters a peripheral association of the protein with the membrane.

Mutations clustered on the edge of the hydrophobic domain may reduce Cbp3p functional activity by altering the association of the protein with the membrane and/or modifying local three-dimensional structure. To assess the effect of a region 2 mutation on membrane association/stability, the detergent solubility of Cbp3p in mutant R172G was examined. Fig. 6 shows that approximately one-half of wild type Cbp3p was extracted from the mitochondrial membrane in the presence of dodecyl maltoside. In contrast, mutant Cbp3p was completely solubilized and found exclusively in the supernatant. Computer analyses predict that the Gly substitution increases the hydrophobicity of the area [42], which may alter the protein–membrane interaction resulting in an enhanced susceptibility to proteolytic attack.

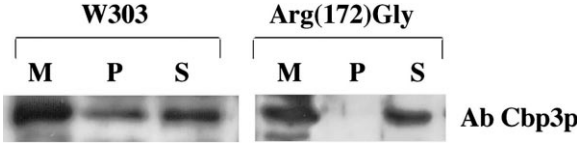

Fig. 6. Solubilization of Cbp3p in mutant and wild type strains. Mitochondria (4 mg) prepared from wild type (W303) and *cbp3* mutant (Arg172Gly) strains grown at 37°C were treated with dodecyl maltoside (0.8 mg/mg protein) for 30 min on ice and solubilized proteins isolated following centrifugation at $107\,000 \times g_{ave}$ for 90 min. Supernatants were carefully removed and the pellets resuspended to the original volume of the sample. Total mitochondrial (M) proteins (40 μg) plus equivalent volumes of supernatant (S) and resuspended pellet (P) were separated by electrophoresis on 12% SDS–polyacrylamide gels, transferred to nitrocellulose membranes and immunoblotted using Cbp3p-specific antisera. The Cbp3p antibody is indicated in the right margin of the figure. Ab, antibody.

3.6. A large amino-terminal domain is not essential for Cbp3p function

To examine the importance of the amino-terminal domain with respect to protein function, *cbp3* deletions Δ12–23 (deletion of residues 12–23), Δ24–54 and Δ56–96 were constructed and introduced into the *cbp3* null strain on centromere plasmids (Fig. 1). Transformants carrying the deletions were respiratory competent, except Δ12–23/cen and Δ56–96/cen grew slightly slower on glycerol than wild type. When Δ12–23 and Δ56–96 were transferred to multiple copy plasmids, the resulting transformants displayed wild type respiratory growth indicating that overproduction of the mutant proteins fully compensated the deletion effect. It is possible that respiration rates may remain unchanged if the individual deletions eliminate functionally redundant domains or only partially remove an internal import sequence. To test this, a deletion of the entire area was constructed. Transformants carrying the Δ12–96 gene on a centromere plasmid exhibited wild type respiratory growth, implying that this large block of 85 residues is not needed for Cbp3p function or protein import.

3.7. Secondary structure of the extreme carboxy-terminus is important for protein function

To determine the role of the carboxy-terminal domain in protein function, five deletions in the 3′ end of the gene were constructed and introduced into the *cbp3* null mutant on multiple copy plasmids (see Section 2). The expressed proteins lacked either the ter-

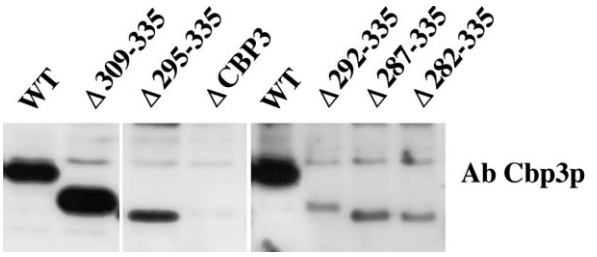

Fig. 7. Western analysis of Cbp3p in the carboxy-terminal deletion mutants. Mitochondria were prepared from respiratory competent strain W303-1A (WT), *cbp3* null strain (ΔCBP3), and *cbp3* carboxy-terminal deletion mutants. Total mitochondrial proteins (40 μg) were separated on 12% SDS–polyacrylamide gels followed by Western analysis, as described in the legend to Fig. 2. The Cbp3p antibody is indicated in the right margin of the figure. Ab, antibody; WT, W303-1A; ΔCBP3, W303ΔCBP3.

minal 27 residues (Δ309–335), 41 residues (Δ295–335), 44 residues (Δ292–335), 49 residues (Δ287–335) or 54 residues (Δ282–335) (Fig. 1). Mutant Δ292–335 was respiratory competent, suggesting the carboxy-terminal 44 residues are not essential for Cbp3p activity. Removal of an additional five residues (Δ287–335) resulted in slowed glycerol growth, with further truncations resulting in respiratory deficient strains. Cbp3p was immunodetected at wild type levels in mutant Δ309–335, and at reduced amounts in the remaining strains (Fig. 7). It may be that loss of more than 27 carboxy-terminal residues promotes protein instability, encouraging proteolysis. Alternatively, the truncations may have eliminated antibody epitopes. If the former were the case then it is interesting to note that reduced amounts of Cbp3p in Δ295–335 and Δ292–335 were sufficient to sustain wild type respiratory growth. When Δ295–335 and Δ309–335 were expressed from centromere plasmids the resulting mutants were respiratory competent, emphasizing that overproduction of the truncated proteins was not required for activity.

If Cbp3p function is entirely independent of the extreme 44 carboxy-terminal residues, then mutations in this region should be silent. However, the slow respiratory growth of mutant P325L refutes this assumption. Examination of secondary structure alterations may explain the contradiction. Computer analyses predict the native protein forms an α-helix between residues 301 and 318 with the remaining 17 residues (319–335) existing in a coiled configuration (agreement among multiple programs [43–50]). The P325L substitution favors formation of a short helical segment, from residues 323 to 329, within the terminal coiled region. Conceivably, this structural modification may alter protein tertiary conformation effecting a decline in respiratory function.

To determine if the disrupting factor is the proposed change in secondary structure or simply the introduction of mutations into this area in general, a strain carrying two amino acid replacements predicted to preserve the coiled configuration between residues 319 and 335 was constructed (mutant E327K/R328K). When the mutant gene was introduced into the *cbp3* null strain on a centromere plasmid, the resulting transformants exhibited wild type respiratory growth. The finding suggests that maintenance of the native secondary structure within the final 17 carboxy-terminal residues may be important for Cbp3p function.

4. Discussion

In this study we identify three protein regions important for Cbp3p function during ubiquinol-cytochrome *c* reductase assembly. These are delineated by Cys124-Ala140, Leu167-Pro175 and Gly223-Asp229. The first and third are required for Cbp3p function, while the second, Leu167-Pro175, is essential for protein stability. Mutants within each grouping share common phenotypic properties. While the mutant screen reported here was fairly extensive, it was not exhaustive. Most likely there are residues outside of the defined regions, or even others within the regions, which when mutated would elicit a respiratory deficient phenotype attributable to alterations in three-dimensional structure or additional functional sites.

Cbp3p is a peripherally associated membrane protein which is readily extracted from the lipid bilayer under alkaline conditions. Region 2 (Leu167 through Pro175) partially overlaps the singular stretch of hydrophobic residues within the protein (Thr152 to Val169). Mutations in this area significantly reduce the steady state amounts of Cbp3p and destabilize the interaction of the assembly protein with the membrane. Secondary structure analyses suggest the

hydrophobic domain initiates with a short helical segment and β-turn followed by a longer α-helix which extends beyond the area to include Ala173. When arranged in a helical wheel, one side of the large α-helix is hydrophobic, with the exception of Arg172 located near the edge of the hydrophobic face. Replacement of Arg172 with Gly increases the overall hydrophobicity of the region and expands the hydrophobic face of the predicted α-helix. This could account for the enhanced detergent solubility of Cbp3p in the mutant. The phenotype displayed by mutant L167P further emphasizes the importance of region 2 in protein stability. The mutation is predicted to decrease hydrophobicity of the surrounding area and break the large helix approximately at its middle, a structural change which could destabilize the protein leading to the observed proteolytic degradation.

In mutant E134G (functional region 1) all tested subunits are fully assembled into the enzyme complex with the exception of the Rieske FeS protein. The partial assembly of the Rieske FeS subunit and slow respiratory growth rate exhibited by the mutant correlates with ubiquinol-cytochrome *c* reductase activity levels of approx. 30% wild type. Cbp3p and enzyme subunit levels are equivalent to wild type at the non-permissive growth temperature. Two of the untested subunits, the 7.3 and 8.5 kDa proteins, most likely approach wild type amounts since normally the loss of any single subunit, with the exception of the 17 kDa protein, would result in Rieske FeS protein degradation [13,15,51–55]. Similarly, the amount of 17 kDa should be approximately wild type since lack of this subunit at 37°C results in reduced Rieske FeS levels [56]. Possibly the protease labile subunits, cytochrome *b*, 11 kDa and 14 kDa, are protected from degradation in mutant E134G because enzyme assembly approaches completion. Unassembled Rieske FeS protein may similarly be stabilized through an association with other subunit(s), such as the 8.5 kDa protein [55]. The observed state of enzyme assembly in the mutant supports the general belief that the Rieske FeS protein is one of the last subunits to assemble into the complex [13,26]. The defective Cbp3p in mutant E134G may retard the rate of this final assembly step.

The two outstanding features of region 3 mutations are the partial to complete assembly of the 14 kDa subunit into a high molecular weight complex and the presence of wild type amounts of mutant protein. This is in direct contrast to region 2 mutants which reduce the stability of Cbp3p resulting in largely unassembled 14 kDa subunit. Region 3, underlined in the sequence 'LRGAIFAYDEG', is bordered by charged residues. Gly223 appears to be a critical residue, with the respiratory competent phenotype of mutant G223A emphasizing the requirement for a small, uncharged amino acid at this position in the protein. Introduction of additional charges into the area impairs Cbp3p function, perhaps by interfering with ionic associations of residues at its boundaries. For example, in mutant G223R the 14 kDa subunit associates with a collection of subunits in the middle fractions of the gradient, while the bulk of cytochrome c_1 and Rieske FeS protein are found in an unassembled state. The unassembled Rieske FeS protein most likely reflects a secondary mutational effect since its incorporation does not normally occur unless a precomplex of partner subunits is initially formed [26]. Insertion of a negatively charged residue, either G223E or A227E, is more detrimental to Cbp3p activity. Approximately one-half of the 14 kDa protein associates with a high molecular weight complex in both mutants, while the remainder migrates in the top fractions of the gradient as unassembled monomer. The large complex may not represent fully assembled enzyme but an assembly intermediate, since mutant G223E is respiratory deficient at higher growth temperatures and ubiquinol-cytochrome *c* reductase activity is lacking. Because the wild type dimeric enzyme approaches 500 kDa, absence of small subunits would not result in noticeable changes in its migration on sucrose gradients.

Interestingly, residues 12–96 are not required for protein function or import. Polypeptides destined for the mitochondria often contain an amino-terminal targeting signal which may be proteolytically cleaved from the precursor following import into the organelle. The calculated molecular weight of the *CBP3* gene product is similar to the estimated molecular weight of the mitochondrial protein, which could suggest that only a few residues are removed upon import. Perhaps just the initial Met is post-translationally cleaved, as is the case for ubiquinol-cytochrome *c* reductase subunits 14 kDa, 11 kDa and

7.3 kDa and cytochrome oxidase subunits VII and VIIa [57,58]. Whether one or more of the first 11 amino-terminal residues serve as an import signal and/or are post-translationally cleaved during translocation is a question presently under examination.

There is a strong interdependence between cytochrome *b*, the 14 kDa and 11 kDa subunits. Biochemical and genetic evidence suggests that the three proteins form a subcomplex prior to enzyme assembly [13,53,55,59–61]. Members of the cytochrome *b* subcomplex are highly susceptible to proteolytic degradation if one of the partners is mutated or absent, or if enzyme assembly is aborted due to other causes [12–15]. In fact, in the absence of either Cbp3 or Cbp4 assembly proteins, steady state amounts of all three subunits are greatly reduced. It is possible that Cbp3p and Cbp4p are involved in assembly of the cytochrome *b* subcomplex. In support of this hypothesis, we have recently demonstrated a direct interaction between Cbp4p and the 11 kDa plus 14 kDa subunits and a physical association between Cbp3p and Cbp4p (unpublished results). The state of enzyme assembly observed in mutant E134G may also suggest participation of Cbp3p in subcomplex recruitment, since in the presence of this mutation an assembly defect is manifested just prior to Rieske FeS protein addition. The cause of this assembly defect is currently under investigation.

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