Assembly of mitochondrial ubiquinol-cytochrome *c* oxidoreductase complex in yeast *Saccharomyces cerevisiae:* The role of Cbp3p and Cbp4p assembly factors

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Abbreviations

ADH	Alcohol dehydrogenase
APS	Ammoniumperoxidisulphate
ATP	Adenosine triphosphate
bc_1-complex	Ubiquinol-cytochrome *c* oxidoreductase
C-terminus	Carboxyl-terminus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleosidtriphosphate
DSP	Dithiobis(succinimidylpropionate)
DTT	Dithiothreitol
EDTA	Ethylendiamine-tetraacetic acid
$FADH_2$	Flavin adenine dinucleotide (reduced form of FAD)
IM	Inner membrane
IMS	Intermembrane space
ISP	Rieske Fe-S protein
mt	mitochondrial
N- terminus	Amino-terminus
OD	Optical density
OM	Outer membrane
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride
RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N`,N`,N`,N`- Tetramethylethylenediamine
TM	Transmembrane
TOM	The Translocase of the Outer Membrane of Mitochondria
Tris	Tris(hydroxymethyl)aminomethane
v/v	Volume per volume
w/v	Weight per volume

Amino acids

A	Ala	alanine	M	Met	methionine
C	Cys	cysteine	N	Asn	asparagine
D	Asp	aspartate	P	Pro	proline
E	Glu	glutamate	Q	Gln	glutamine
F	Phe	phenylalanine	R	Arg	arginine
G	Gly	glycin	S	Ser	serine
H	His	histidine	T	Thr	threonine
I	Ile	isoleucine	V	Val	valine
K	Lys	lysine	W	Trp	tryptophan
L	Leu	leucine	Y	Tyr	tyrosine

Aim of the work

Ubiquinol-cytochrome *c* reductase (complex III) is a respiratory chain enzyme of the inner mitochondrial membrane, transferring electrons from reduced ubiquinone to ferricytochrome *c*. In *Saccharomyces cerevisiae*, ubiquinol-cytochrome *c* reductase is composed of 10 non-identical subunits with defined catalytic centers consisting of cytochrome *b*, cytochrome c_1 and the Rieske FeS protein. Correctly assembled and functional complex III is an essential prerequisite for oxidative energy metabolism. Complex III deficiency has been reported to be associated with several neurodegenerative diseases. However, the assembly process is not fully understood and requires the involvement of different chaperones. Three non-subunit proteins, which are important for assembly and/or stability of complex III have been detected. The role of Bcs1p in assembly of Rieske FeS and Qcr10p into complex III was already shown. The role of two chaperones, Cbp3p and Cbp4p, is not known.

This work will be focused on the characterization of both Cbp3p and Cbp4p proteins and their homologs in human and in *Schizosaccharomyces pombe*. It is planned to develop a yeast model to characterize *hCBP3* mutations if some would be identified. The topology of both proteins, their ability to form homomers and/or heteromers, as well as their potential to substitute for each other will be analysed. Experiments to exclude the possible roles of the two assembly factors in lipid metabolism (e. g. cardiolipin synthesis) will be performed. To define the roles of the two proteins in complex III assembly, the interactions of the two proteins with selected subunits of complex III will be investigated using TAP-method or co-immunoprecipitation. Moreover, to identify the pathway of complex III assembly and the steps, in which Cbp3p and/or Cbp4p act, the assembly of complex III subunits in wild type, *cbp3* and *cbp4* null mutants will be monitored using the technique of Blue Native PAGE (BN-PAGE).

1. INTRODUCTION

Mitochondria are essential organelles present in nearly all eukaryotic cells. They are separated from the cytosol by two membranes that are very different in protein and lipid composition. The outer membrane is composed of about half lipid and half protein. Transmenbrane channels formed by the protein mitochondrial (mt) porin make this membrane freely permeable to most small molecules (< 4,000 to 5,000 MW). Many proteins are translocated into mitochondria in an energy dependent process via the TOM complex in outer membrane and the TIM complex in the inner membrane. The inner membrane of mitochondria is less permeable and contains more proteins (80 %) than lipids (20 %). The surface area of inner membrane is increased by protruding into the matrix forming invaginations called *cristae.* The cristae are the sites where the reactions necessary for generating energy during cellular respiration are carried out. *Saccharomyces cerevisiae*, a facultative anaerobic yeast is an excellent model organism to study the mitochondrial enzymes involved in oxidative phosphorylation and ATP synthesis. *S. cerevisiae* can survive in the absence of oxidative phosphorylationbecause it possesses the ability of radical alteration of the metabolism depending on the availability of a fermentable carbon source. This property of the yeast, *S. cerevisiae* enables an easy detection of the respiratory defective phenotype.

1.1. The Respiratory Chain

The respiratory chain is localised in the inner mitochondrial membrane and is a configuration of four multi-subunit complexes (complex I-IV) that in terms of function are coupled with electron transfer and proton pumping. The main function of the respiratory chain is the generation of ATP by oxidative phosphorylation (OXPHOS). Electrons are donored to respiratory chain from different substrates. While electrons are passed down the chain, protons are pumped into the mt intermembrane space, generating thereby a proton gradient across the inner membrane, which is the driving force for phosphorylation of ADP to ATP catalysed by complex V (for review see Saraste, 1999). Complex I oxidises NADH molecules, transfer electrons to ubiquinone (coenzyme Q, CoQ) and pumps protons to the IMS. The link between the tricarboxylic acid cycle and the respiratory chain is mediated via Complex II. It converts succinate to fumarate, producing reducing equivalents as $FADH_2$ and transfering electons to ubiquinone. Ubiquinol:cytochrome *c* oxidoreductase (bc_1 complex or complex III) is a central component of the respiratory chain, which transfers electrons from

reduced ubiquinone to ferricytochrome *c* (cyt *c*) and additional protons are pumped into the IMS. Electron flow continues from cyt *c* through complex IV to molecular oxygen and this transfer is also associated with proton uptake into the IMS. Electron flow through OXPHOS complexes is schematically shown in Fig. 1.

S. cerevisiae shows several important differences in the respiratory chain. *S. cerevisiae* does not contain complex I, but instead possesses several NADH dehydrogenases, two of which are able to transfer electrons to ubiquinone. Furthermore, a third NADH dehydrogenase is present in the outer mitochondrial membrane, which transfers electrons directly to cytochrome *c*. Another difference is that *S. cerevisiae* contains an enzyme that transfers electrons from L-lactate to cyt *c*, called L-lactate:cytochrome *c* oxidoreductase or cytochrome b_2, an enzyme unique to yeast (de Vries and Marres, 1987).

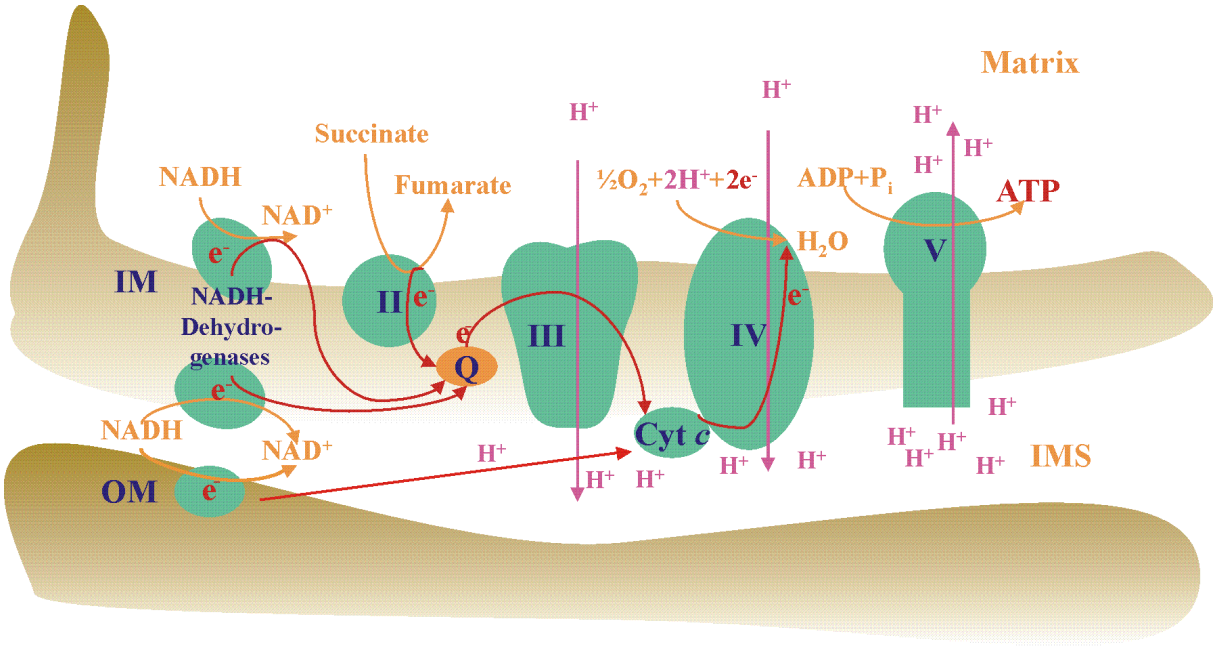

Figure 1. Electron flow through the yeast respiratory chain. Electrons (e^-) are transfered from two NADH-dehydrogenases of IM and from Complex II to ubiquinone (Q). Complex III oxidises ubiquinol by transfering e^- to cytochrome *c* (Cyt *c*) in the IMS and pumps protons (H^+) into the IMS. Electrons from a third NADH-dehydrogenase of the OM are transfered directly to Cyt *c*. Reduced Cyt *c* gives e^- to Complex IV and additional H^+ are pumped into the IMS. The proton gradient is used by Complex V to produce ATP. Electron flow is followed by red arrows and the proton transfer is shown by pink arrows.

A special feature of the respiratory chain is, that from a genetic point of view, it results from coordinate expression of the nuclear (n) genome and the mt genome. The mitochondrial DNA (mtDNA) of baker`s yeast, *S. cerevisiae* is 85.8 kb long and encodes subunits I, II and III of cytochrome *c* oxidase (*COX1*, *COX2* and *COX3*), apocytochrome *b* (*COB*), subunits 6, 8 and

9 of ATPase (*ATP6*, *ATP8* and *ATP9*) and a ribosome-associated protein (*VAR1*). It also contains a number of unidentified reading frames (URFs) and intron-related open reading frames (ORFs). In addition, the mitochondrial genome specifies the small and large ribosomal RNAs (SSU rRNA and LSU rRNA), 24 transfer RNAs (tRNAs) and the 9S RNA (Rpm1r) component of RNase P (Foury *et al.*, 1998). All genes are encoded and transcribed from one strand, except for $tRNA^{Thr1}_{CUN}$ (de Zamaroczy and Bernardi, 1985).

Formation and assembly of the OXPHOS enzyme complexes require a multitude of specific nuclearly encoded proteins. For example, in the case of cytochrome *c* oxidase (COX or complex IV) more than 30 genes have been identified, which are required for specific steps in the formation of the enzyme (Pel *et al.*, 1992; Barrientos *et al.*, 2002). Similar factors have been reported for ATP synthase and complex III. Factors required for assembly of complex III are described later.

Two models for the molecular organization of the respiratory chain are discussed. In the first, components of the respiratory chain are free to diffuse laterally and independently of each other in the membrane plane and electron transfer is coupled to diffusion-based collisions between the complexes (Gupte *et al.*, 1984). In the other model, respiratory components are arranged in macromolecular structures (so called “supracomplexes”) to enhance electron flow. Most of the studies favour the second model and indicate that respiratory chain complexes form one functional and physical unit (Boumans *et al.*, 1998).

1.2. Complex III

Enzymes of the cytochrome (cyt) bc_1 complex family are central components of all the main energy transduction systems of the biosphere, accoording to maintenance of the proton gradient and of energy transmission. They all catalyze essentially the same reaction, the transfer of reducing equivalents from a quinol in the lipid phase (ubiquinol in the classical bc_1 complexes) to a higher-potential acceptor protein in the aqueous phase. This electron transfer is coupled to transfer of 1 H^+/e^- across the membrane. The mitochondrial complexes arose from bacterial antecedents, and the catalytic core of three subunits [cyt c_1, cyt *b* and the Rieske iron sulfur protein (ISP)] is highly conserved (Woese, 1987; Degli Esposti *et al.*, 1993). The latter two subunits span the archaeal/bacterial divide and are clearly more ancient than the cyt c_1 subunit, whose function is carried by a wide variety of different structures (Hunte *et al.*, 2003).

1.2.1. The catalytic mechanism of the bc_1 complex

The electron (e^-) and proton (H^+) pathways within the bc_1 complex are described by the proton-motive Q-cycle mechanism, which was originally proposed by Mitchell (1975). To explain the electron and proton pathway in bc_1 complex was not so easy, because of one putative difficulty: ubiquinol is a 2 e^- donor whereas all prosthetic groups of bc_1 complex are 1e^- carriers. Already in 1972 Wikstrom and Berden postulated that the electron transfer pathway through bc_1 complex is not a linear chain, but a branched pathway in which two e^- from ubiquinol diverge upon its oxidation, one reducing cytochrome *b* and the other cytochrome c_1. This idea later on led to Mitchell´s formulation of the Q-cycle mechanism.

1.2.2. The proton motive Q-cycle

An essential feature of the proton motive Q-cycle mechanism is that there are two distinct ubiquinone-reaction centers, the Q_{out} and Q_{in} site (Zhu *et al.*, 1982). These sites are located on the opposite sites of the membrane and are also called center P or center o, and center N or center i, respectively. According to the sequential model of Q-cycle (Fig. 2), ubiquinol (QH_2) binds to the Q_{out} site of the bc_1 complex. After deprotonation the first electron is released to reduce the Rieske Fe-S cluster (reaction 1) and is subsequently transferred to cytochrome c_1 (reaction 2) and then to cytochrome *c*. A rather unstable ubisemiquinone (Q^-), formed after the first deprotonation, immediately reduces the low potential cytochrome *b* heme (b_L) and a second proton is released into the intermembrane space. The electron from b_L heme is later on transferred to the high potential heme b_H (reaction 3) and then reduces ubiquinone at the Q_{in} site to form stable ubisemiquinone (reaction 4a). To complete the Q-cycle, another ubiquinol must be oxidized on Q_{out} site and the same set of reactions are repeated except the last step. In the last step, the Q-cycle is completed by reduction of ubisemiquinone (reaction 4b), which is still present at the Q_{in} site to form ubiquinol that can again bind to Q_{out} site. Thus, reaction 1 occurs twice during one Q cycle, whereas reaction 4b occurs only once.

Although the mechanism of Q-cycle is generally understood, there is no evidence for a semiquinone intermediate at Q_{out} center. Supported with numerous experimental observations Trumpower, 2002) has proposed a concerted, alternating sites mechanism of ubiquinol oxidation. A concerted mechanism of ubiquinol oxidation is inferred from the finding that there is reciprocal control between the high potential and low potential redox components involved in ubiquinol oxidation. The potential of the Rieske iron–sulfur protein controls the rate of reduction of the *b* cytochromes, and the potential of the *b* cytochromes controls the

rate of reduction of the Rieske protein and cytochrome c_1. The concerted reaction begins when ubiquinol binds to His-181 of the Rieske protein, which is one of the ligands to the redox active iron of the Fe-S cluster to form a ubiquinol–imidazolate complex. The ubiquinol–imidazolate complex then forms a hydrogen bond to Glu-272 of cytochrome *b*, docked within electron transfer distance of the b_L heme, so that electrons are transferred from the quinol–imidazolate complex to the Rieske cluster and the b_L heme simultaneously. As cytochrome *b* is reduced, the hydrogen bond to Glu-272 is broken, allowing the iron–sulfur protein to move between cytochromes *b* and c_1. The iron–sulfur protein will remain predominately reduced since its potential is higher than that of cytochrome c_1. Cytochrome b_L immediately reduces cytochrome b_H, which then reduces ubiquinone to ubisemiquinone at the Q_{in} site. The second turnover at center Q_{out} is dependent upon the redox state of cytochrome b_L and the iron–sulfur protein, and will occur only when both are oxidized (for review see Berry and Huang, 2003; Crofts, 2004).

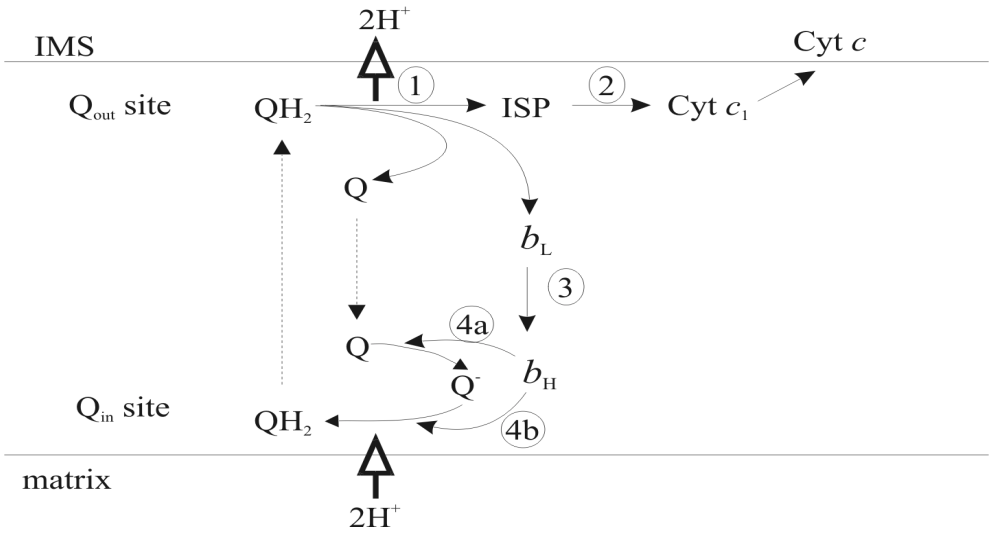

Figure 2. The proton motive Q-cycle through the bc_1 complex. The electrons from ubiquinol (QH_2) are released upon deprotonation. One e^- is transfered to cytochrome *c* (Cyt *c*) via cytochrome c_1 (Cyt c_1) while the second e^- reduces the quinone in the first Q-cycle and the ubisemiquinone in the second Q-cycle. Detailed description of the Q-cycle reactions (depicted by circled numbers) is in the text.

1.2.3. Relevance of the dimeric structure for catalysis

The catalytic mechanism of the bc_1 complex, as described by the Q-cycle, does not consider the dimeric structure of this complex. De Vries *et al.*, (1983) proposed a `double Q-cycle´ model which incorporates different behavior for each of the two monomers. Linke *et al.*, (1986) suggested that dimeric bc_1 complex contains only one Q_{in} site and both monomers

catalyze single e^- transfer at Q_{out} site and co-operate at the Q_{in} site in reducing ubiquinone to ubiquinol. A lot of knowledge about the catalytic mechanism of the bc_1 complex is derived from studies with specific inhibitors. There are numerous inhibitors the bc_1 complex that are thought to mimic ubiquinol or ubisemiquinone and thus by binding to Q_{in} or Q_{out} sites inhibit the enzyme. Some of these inhibitors act with a stoichometry of 0.5 per cytochrome c_1, indicating that one molecule of inhibitor is sufficient to fully inhibit the dimeric enzyme (Gutierrez-Cirlos and Trumpower, 2002). In the same year Trumpower (2002) provided evidence for half-of-the-sites reactivity, where only half of the bc_1 dimer is active at any one time and ubiquinol oxidation alternates between the two halves of the dimer. This mechanism is supported by the observation that a crystal structure of the yeast bc_1 complex co-crystallized with cytochrome *c* shows only one molecule of cytochrome *c* bound to the dimeric enzyme, and ubiquinone is present in only half of the dimer (Lange and Hunte, 2002). A mechanism in which only one half of the dimer is active allows for the possibility that, under some conditions, both halves of the dimer are active and that the activity of the bc_1 complex may be regulated by switching between a half-of-the-sites active and a fully active enzyme.

1.2.4. Structure of the bc_1 complex

In the yeast *S. cerevisiae* complex III is composed of 10 non-identical subunits including the catalytic centre composed of cytochrome *b* (Cobp), cytochrome c_1 (Cyt1p) and the Rieske FeS protein (Rip1p) (Katan *et al.*, 1976), the two core proteins Cor1p and Cor2p as well as five supernumerary subunits (Qcr6p - Qcr10p). All subunits, except for cytochrome *b*, are encoded by nuclear genes. Gene inactivation studies show that the functional bc_1 complex cannot be formed in the absence of supernumerary subunits except for Qcr10p (Crivellone *et al.*, 1988); however the functional roles of these subunits are not well understood. Detailed information about the subunits of yeast bc_1 complex are summarised in Table 1.

The crystal structures of cytochrome bc_1 complex demonstrate that the enzyme is present as an intertwined homodimer (Zhang *et al.*, 1998; Hunte *et al.*, 2000) (Fig. 3). The centre of the complex is formed per monomer by eight transmembrane helices of cytochrome *b*, and attached to these central domain are the single transmembrane anchors of the Rieske protein and cytochrome c_1, the two other catalytic subunits. The extrinsic domains of the latter are located in the intermembrane space. On the same side of the membrane, Qcr6p or the so-called hinge protein is bound. Single transmembrane helices of subunits Qcr8p and Qcr9p are

attached at the periphery of the catalytic core. Qcr10p, a loosely associated subunit of the yeast complex is not present in the structure (Hunte *et al.*, 2000). The matrix portion of the complex is formed by two large subunits, Cor1p and Cor2p, the so-called core proteins, and by Qcr7p. The core proteins have high sequence similarity with soluble, matrix processing peptidases and are thought to be evolutionarily related to these Zn-binding proteases (Braun and Schmitz, 1995). Core proteins do not have proteolytic activity in most species, with the exception of plants (Braun and Schmitz, 1995).

Subunit (Synonyms)	**Molecular weight (MW)[1]**	**Prostethic groups**
Cor1p (Qcr1)	47,4 kDa	-
Cor2p (Qcr2)	38,7 kDa	-
Cytbp (COB)*	43,6 kDa	*b*-type hemes (b_{562} and b_{566})
Cyt1p (Cytc_1)	27,8 kDa	cytochrome c_1
Rip1p (Rieske Fe-S)	20,1 kDa	[2Fe-2S]
Qcr6p	14,4kDa	-
Qcr7p	14,5 kDa	-
Qcr8p	10,8 kDa	-
Qcr9p	7,3 kDa	-
Qcr10p	8,8 kDa	-

Table 1. Subunits of the yeast bc_1 complex. The bc_1 complex of *S. cerevisiae* is composed of 10 non-identical subunits.

[1] Molecular weights correspond to the mature subunits of the bc_1 complex as given by the Yeast Protein Database (YPD) (Costanzo *et al.*, 2000).

* mitochondrially encoded

One of the striking features of the frst bc_1 structures was the finding that the extrinsic domain of the Rieske protein is mobile. The movement of the Rieske domain facilitates the transfer of the iron-sulfur cluster from a position close to heme b_L to a position close to heme c_1. The resulting orientations of the cofactors are well suited for electron transfer and underly the Q cycle mechanism and quinol oxidation at Q_{out} site proposed by Trumpower (2002) (see also section 1.2.2.). Additionally, there is some evidence that the orientation of the domain is related to redox condition (Brugna *et al.*, 2000).

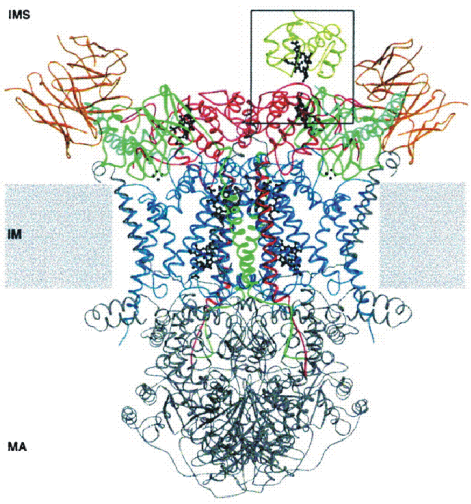

Figure 3 Crystal structure of the bc_1 complex from yeast mitochondria. The dimeric complex is viewed parallel to the plane of the inner membrane (IM) that separates the intermembrane space (IMS) from the matrix (MA). Protein subunits are depicted in ribbon representation with respective colors: Cyt *c* (yellow), Cyt c_1 (red), cytochrome *b* (blue), Rip1p (green), Qcr6p (cyan), and Fv fragment (orange). All other subunits are colored in gray. Redox cofactors (ball-and-stick representation) are colored in black. The position of the inner membrane is indicated as gray boxes. Note that Cyt *c* (depicted in the frame) is not a subunit of the bc_1 complex and only one Cyt *c* molecule is attached to the complex. Figure was adapted from Lange and Hunte (2002).

1.3. Role of lipids in bc_1 complex stability and assembly

Interestingly, phospholipid molecules that are tightly bound within the transmembrane region are found in the structure of yeast bc_1 complex. Their well-defned binding sites suggest specifc roles in assembly and function of the complex (Fig.4). Phospholipids are essential for the activity of several membrane proteins (Dowhan, 1997), including complex III (Schagger *et al.*, 1990). Increased delipidation of the bc_1 complex leads to a gradual decrease in enzyme activity up to complete inactivation and destabilization of the complex. A characteristic lipid of the inner mitochondrial membrane is cardiolipin, which has been shown to be essential for complex III activity (Gomez and Robinson, 1999). Cardiolipin is associated with Cobp and is required for the formation of supracomplexes between complexes III and IV (Zhang *et al.*, 2002). A role of other phospholipids such as phosphatidylethanolamin (PE) in bc_1 complex dimer formation or phosphatidylinositol (PI) in stabilization of the transmembrane anchor of Rip1p and in stabilization of bc_1 complex has been proposed (Lange *et al.*, 2001).

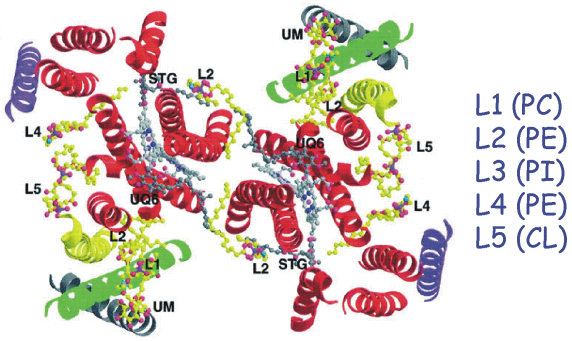

Figure 4. Specific, tightly bound phospholipids of the yeast bc_1 complex. Binding of phospholipids (yellow) and detergent (yellow) in the transmembrane region of bc_1 complex viewed from the matrix side on the membrane plane. The core of the dimeric complex is made up of eight helices of Cobp (red) per monomer. Single helices of Rip1p (green), Cyt1p (yellow), Qcr8p (purple) and Qcr9p (gray) are attached on each side at the periphery. The heme *b* cofactors (light gray) as well as ubiquinone (UQ6) and Stigmatellin (dark gray) are shown. The helices are depicted as ribbon drawings, and further molecules as ball-and-stick representation. (PC-phosphatidylcholin, PI-phosphatidylinositol, PE-phosphatidylethanolamin, CL-cardiolipin) (Lange *et. al.*, 2001)

1.4. Assembly of the bc_1 complex

Components of respiratory electron transfer chain are multi-component complexes, which have to assemble both at the right time and in the right place.

The process of bc_1 complex assembly is not fully understood. When centrifuged in a sucrose gradient containing 0.1% Triton-X100 and 0.5 M KCl, complex III is split in three distinct size fractions, one consisting of Rip1p, the second of Cor1p and Cor2p, and the third of Cobp, Cyt1p, Qcr7p and Qcr8p (Crivellone *et al.*, 1988). Zara *et al.*, (2004) analysed the composition of cytochrome bc_1 subunits in mitochondrial membranes of strains bearing single and double deletion mutations in the genes encoding complex III subunits. These data suggest that subunits Qcr7p and Qcr8p along with Cobp associate to an 68 kDa subcomplex at an early step in the assembly pathway and play an important role in the structural organization of the bc_1 complex. A second set of subunit interactions is defined by the Cor1p/Cor2p subcomplex (84 kDa). Cyt1p has been proposed to be in a subcomplex (58 kDa) with the two supernumerary subunits Qcr6p and Qcr9p. In contrast, deletion of the *QCR10* gene has no effect on the composition of the other bc_1 complex subunits and is probably added late in the assembly pathway. All these results agree with and extend the model of Berden *et al.*, (1988) for the bc_1 complex assembly, in which the existence of three distinct subcomplexes is proposed. However, it is not known in which order the subunits interact to form the putative subcomplexes and in which sequence the subcomplexes are assembled into a functional

enzyme. The model of assembly process is schematically shown in Fig. 5. Recently a further level of organization for respiratory chain complexes has been reported. The complexes are not randomly distributed within the inner mitochondrial membrane, but assemble into supramolecular structures. Complex III exists in three forms: the free dimer and two supracomplexes comprising in addition one or two complex IV monomers (Schagger and Pfeiffer, 2000). Whereas formation of the dimeric forms of complex III and complexes IV appears not to be dependent on their ability to form supracomplexes, supracomplex formation requires the presence of the assembled bc_1 complex and complex IV (Cruciat *et al.*, 2000).

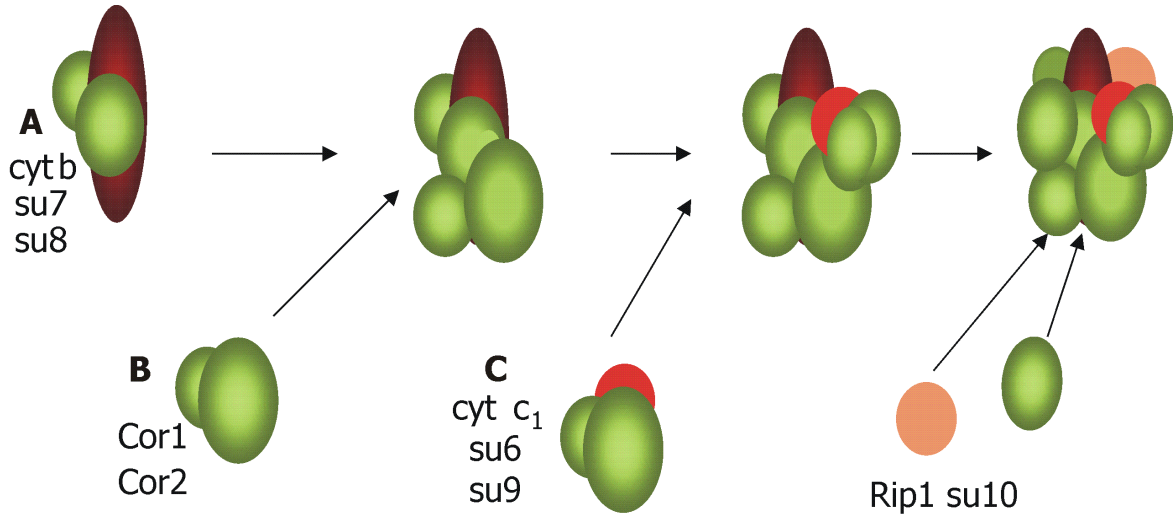

Figure 5. Model for bc_1 complex assembly. In the first step the formation of three subcomplexes (A, B, C) is proposed. However, it is not known in which order the subcomplexes interact to form a functional enzyme. The assembly pathway shown in this model is based on the proposal of Geier *et al.*, (1992).

1.4.1. Role of non-subunit proteins (assembly factors of bc_1 complex)

Formation and assembly of the OXPHOS enzyme complexes require a multitude of specific nuclearly encoded proteins. For example, in the case of cytochrome *c* oxidase (COX or complex IV) more than 30 genes have been identified, which are required for specific steps in the formation of the enzyme (Pel *et al.*, 1992; Barrientos *et al.*, 2002). These proteins include gene-specific factors for the maturation and translation of *COX* transcripts, enzymes required for the formation and delivery of prosthetic groups, and specific assembly factors (Barrientos *et al.*, 2002). Similar factors have been reported for ATP synthase assembly (Ackerman and Tzagoloff, 1990) and complex III. In the latter case, gene specific translational activators for cytochrome *b* synthesis (Rödel, 1997) as well as three non-subunit proteins, which are important for assembly and/or stability have been detected. Bcs1p is necessary for expression

of functional Rip1p and its assembly (as well as of the 8.5 kDa subunit, Qcr10p) into the bc_1 complex (Nobrega *et al.*, 1992; Cruciat *et al.*, 1999). Cbp3p and Cbp4p have been proposed to function as enzyme-specific chaperones (Wu and Tzagoloff, 1989; Crivellone, 1994). Molecular chaperones mediate the correct folding of target proteins and their assembly into oligomeric structures without being components of the final structures (Ellis and van der Vies, 1991). The proposal that Cbp3p and Cbp4p act as chaperons in complex III formation resulted from the observation that mutations in the respective genes lead to the same phenotype, namely to loss of bc_1 activity accompanied by reduced steady-state levels of Cobp, Rip1p, subunit Qcr7p and subunit Qcr8p. The presence of fully processed cytochrome *b* mRNA and its translation in the mutants suggest that Cbp3p and Cbp4p are not involved in an early post-transcriptional event, but rather in a late stage of complex III assembly (Wu and Tzagoloff, 1989; Crivellone, 1994).

1.5. Cbp3 and Cbp4, assembly factors specific for bc_1 complex

Cbp3p and Cbp4p homologues have been identified in various eukaryotic organisms. Both proteins are members of larger protein family, which plays role in the assembly and/or stability of supramolecular enzymes required for electron transport and ATP synthesis (e.g. Atp10p, Atp11p, Atp12p, Cox10p, Cox11p, Sco1p).

Several roles have been proposed for Cbp3p and Cbp4p during enzyme assembly (Crivellone, 1994). These proteins may interact with and stabilize a selected group of subunits during enzyme assembly. The association of subunits into a partially assembled complex could provide the binding sites of selected groups of complex III subunits for interaction with other subunits of the enzyme. It is also possible that Cbp3p and Cbp4p protect protease labile subunits from proteolysis until they assemble. These proteins can mask the interactive sites of newly folded subunits, until the correct partner is found. This would prevent unwanted interactions with other proteins belonging to other mitochondrial membrane enzymes.

Both proteins are localised to the mitochondrial membrane. Cbp3p was completely extracted into the supernatant following treatment with 0,1 M sodium carbonate (pH=11,7) (Wu and Tzagoloff, 1989). This concentration of sodium carbonate does not solubilize integral membrane proteins suggesting that Cbp3p is not an integral membrane protein. Upon treatment of mitochondria with 0,1 % deoxycholate Cbp3p was detected in the membrane fraction (Crivellone, 1994). This concentration of deoxycholate is sufficient to disrupt and

release all matrix proteins of the organelle suggesting that Cbp3p is peripherally associated with the membrane.

Cbp4p was not extracted into the supernatant following treatment with 0,1 M sodium carbonate (pH=11,5) suggesting that Cbp4p is tightly associated with the membrane (Crivellone, 1994). Cbp4p was detected after the treatment of mitochondria with proteinase K demonstrating that Cbp4p is not found on the outer surface of the mitochondria (Crivellone, 1994).

Cbp4p is a protein with a molecular weight of 20, 313 daltons. The N-terminus exhibits a cluster of basic and hydroxylated residues, a common feature of proteins targeted to mitochondria (Hartl *et al.*, 1987; Pfanner *et al.*, 1987). The functional domain(s) of the protein are in the N-terminus and/or middle regions since the C-terminal 24 residues of Cbp4p are not essential for its biological activity (Crivellone, 1994). Swissprot computational analysis (Letunic *et al.*, 2004) predicts one transmembrane domain between amino acid (aa) residues 28-50 and a coiled coil region (124-162 aa). The coiled coil represents a novel functional motif in chaperones (e.g. in the AAA + ATPases). They form mechanically rigid structures, can undergo dynamic rearrangements by refolding into an alternate structure (e.g. membrane fusion proteins) and are encountered as oligomerization domains (leucine zippers). Their ability to expose extended surfaces with amphiphilic properties enables them to act as scaffolds for protein assemblies. Their exact properties depend on a combination of core (hydrophobic) and surface (hydrophilic) residue characteristics (Lupas, 1996a; Lupas, 1996b).

Cbp3p has a molecular weight of 38 kDa and does not possess any homology to Cbp4p. The N-terminal 38 residues of Cbp3p are rich in basic aa, which are generally known to be important in mitochondrial import signals. However, deletion mutants of residues Δ12-96 were respiratory competent, indicating that these residues are not essential for Cbp3p function, stability and mitochondrial import (Shi *et al.*, 2001). Three regions essential for Cbp3p activity were identified, region 1 (Cys124 through Ala140) and region 3 (Gly223 through Asp229) were required for Cbp3p function, while region 2 (Leu167 - Pro175) was necessary for protein stability. Prediction programs predicted one transmembrane domain (148-174 aa) for Cbp3p that overlaps with region 2, which has been shown to be important for protein stability (Shi *et al*., 2001). This region also overlaps Ubiq_cyt_C_chap domain (145-303) predicted by PFAM program (Letunic *et al.*, 2004). No signal peptide cleavage site in both Cbp3p and Cbp4p was predicted.

Shi *et al*., (2001) noted that Cbp3p is physically associated with Cbp4p, however experimental data for this interaction have not yet been presented. In this respect it is

surprising that deletion of *CBP3* neither alters the steady state level nor the membrane association of Cbp4p (Crivellone, 1994). Possibly mutations in *CBP3* and *CBP4* affect the same step of complex III assembly. This idea is supported by the observation that the phenotype of a double mutant resembles that of the single mutants (Crivellone, 1994).

1.6. BN-PAGE electrophoresis as a new tool to study multi-protein complexes and their assembly

In the recent post-genome era a big challenge is to assign a function to each protein and describe its relationship to other proteins in the cell. Many proteins assemble into multisubunit complexes to fulfill their function and the mitochondrial OXPHOS complexes are not an exception. To investigate respiratory chain complexes was a difficult task as they are embedded in the mitochondrial inner membrane: too little detergent and the complexes would be not released from the membrane, too much and the complexes would dissociate. Electrophoresis was also problematical because of high salt concentration used in standard purification protocols perturb electrophoresis and additionally, proteins (complexes) need a charge to have mobility to move in electric field. The charge usually comes from a chemical additive such as SDS, but as SDS is a strong detergent, it causes not only dissociation of protein complexes, but also complete denaturation.

Schägger and von Jagow (1991) elegantly solved these problems by including Serva Blue G (Coomassie Blue G250) in the extraction and electrophoresis buffers. Coomassie Blue is present during the electrophoresis and binds specifically to the proteins introducing the charge similar to SDS without disturbing protein-protein interactions. Protein complexes are solubilized with non-ionic detergents like n-dodecylmaltoside, Triton X-100 or digitonin, which do not dissociate protein complexes. Extraction of protein complexes was aided by inclusion of the zwitterionic salt aminocaprionic acid, which is uncharged at pH 7 and therefore does not effect electrophoresis. The technique was named Blue Native polyacrylamide gel electrophoresis (BN-PAGE) owing to the color of the crucial compound Serva Blue G.

The separating principle of BN-PAGE is not the charge/mass ratio, but the decreasing pore size of the discontinuous polyacrylamide gradient gel, which reduces the protein migration velocity according to the mass of the complex and can completely stop protein mobility at the mass-specific pore-size limit. BN-PAGE resolves protein complexes approximately between 100 and 1000 kDa. After the BN-PAGE individual bands are visible and correspond to different protein complexes. This facilitates excision of selected bands and recovery of native

proteins by electroelution. Individual subunits within these protein complexes can easily be analyzed in a second gel dimension in the presence of SDS.

Since its inception, Blue Native electrophoresis has been adapted to a large number of applications that build on the basic principles. The method was standardized for separation of complexes between 100-1000 kDa using gradient of 5-13 % acrylamide. Other gradient gels may be preferred to focus on a specific complex or subcomplex. For example, a 10-20 % gradient gel was employed to resolve protein complexes between 20-200 kDa (Nijtmans *et al.*, 1998). Agarose gels are more appropriate for the analysis of complexes higher than 1 MDa (Henderson *et al.*, 2000).

Complexes separated by BN-PAGE are still biochemically active as demonstrated in the original article (Schagger and von Jagow, 1991). The enzyme activity of complex III after extraction from BN-gel was comparable with chromatographically isolated complex III after activation with phospholipids. Specific staining methods based on histochemical assays were developed to visualize the enzyme activity in the gel (Zerbetto *et al.*, 1997). In-gel activity measurements are available for complex I, II, IV and V. Special attention is paid to apply the BN-PAGE in investigation the dynamics of complex assembly. The dynamics of protein synthesis and complex assembly can be addressed using BN/SDS-polyacrylamide 2D electrophoresis in combination with pulse chase labeling of proteins in cultured cells (Nijtmans *et al.*, 2002b).

Recent development in biology is high-throughput mass spectrometry (MS) (Mann *et al.*, 2001). This technique is very sensitive and allows a rapid identification of a spot on the gel. Combination of MS together with BN-PAGE either with or without a second dimension SDS-PAGE represents a very powerful tool to study protein complexes and their composition.

Although Blue Native electrophoresis was developed in 1991 it was only few years ago before other groups discovered many benefits of the method. These days BN-PAGE is employed in many laboratories in studying membrane-bound complexes. It is expected that the use of BN-PAGE will increase as it can be set up easily and has a wide range of applications. Additionally, the use of BN-PAGE is not restricted only to study the membrane-bound complexes, but can be applied in a proteomics approach for identification and analysis of multi-protein complexes required their separation under native conditions as for example used to identify protein complexes from whole cellular lysates (Camacho-Carvajal *et al.*, 2004).

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Instruments

LI-COR DNA sequencer (model 4000/4200)	MWG-BIOTECH
French Press	SLM-AMINCO
Dounce homogeniser	BRAUN

2.1.2. Materials

Acrylamide/Bisacrylamide	SIGMA
Antimycin A	SIGMA
BenchMark™ Prestained	INVITROGEN
Calmodulin Affinity Resin	STRATAGENE
Coenzyme Q_{10}	SIGMA
CombiZym DNA Polymerase	InViTek
Complete EDTA-free (Protease inhibitor cocktail)	ROCHE
Coomassie® Brilliant Blue G250	MERCK
Cytochrome *c*	SIGMA
Digitonin	SIGMA
Dithiothreitol	SIGMA
dNTPs	GIBCO-BRL/INVITROGEN
DSP	PIERCE
ECL^{plus}-System™	AMERSHAM PHARMACIA BIOTECH
Gel Blotting Paper	SCHLEICHER & SCHUELL
Herring Sperm DNA	INVITROGEN
Immobilon™-P PVDF-Membrane	MILLIPORE
λ-DNA *Eco*RI/*Hind*III	GIBCO-BRL
Maltoside	SIGMA
PMSF	SIGMA
Primers	MWG-BIOTECH
Protein A-Agarose	SANTA CRUZ BIOTECHNOLOGY

Proteinase K	ROTH
Rabbit IgG Agaraose	SIGMA
Restriction Enzymes	GIBCO-BRL
Rotiphorese® Gel 30	ROTH
Seakem® LE Agarose	CAMBREX
Taq DNA Polymerase	INVITROGEN
T4-DNA-Ligase	PROMEGA
TEMED	GIBCO-BRL
Triton X-100	ROCHE
Tween 20	ROTH
X-ray films	AMERSHAM PHARMACIA BIOTECH
Zymolyase 20T	ICN

2.1.3. Kits

DC-Protein Assay	BIO-RAD
NucleoSpin® Extract	MACHEREY-NAGEL
NucleoSpin® Plasmid Quick Pure	MACHEREY-NAGEL
Thermo-Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP	AMERSHAM PHARMACIA BIOTECH
Wizard® SV Gel and PCR Clean-Up System	PROMEGA

2.1.4. Antibodies

	Dilution in 1x TBS-T with 5 % (w/v) skimmed milk powder
Rabbit-Anti-yAco1p (kindly provided by R. Lill, Marburg)	1:2000
Rabbit-Anti-yCyt1p (Zollner *et al.*, 1992)	1:1000
Rabbit-Anti-yCytb2 (Krummeck, 1992)	1:2000
Mouse-Anti-yCox3p (MOLECULAR PROBES)	1:1000
Mouse-Anti-Pgk1p (MOLECULAR PROBES)	1:1000
Mouse-Anti-Porin (MOLECULAR PROBES)	1:1000
Mouse-Anti-EGFP (ROCHE)	1:1000

Mouse-Anti-HA (ROCHE)	1:1000
Mouse-Anti-CMYC (ROCHE)	1:400
Rabbit-Anti-yRip1p (kindly provided by R. Lill, Marburg)	1:2000
Rabbit-Anti-TAP (OPEN BIOSYSTEMS)	1:2000
Rabbit-Anti-goat IgG-HRP (DAKO A/S)	1:2000
Rabbit-Anti-ySco1p (Buchwald *et al.*, 1991)	1:3000
Sheep-Anti-mouse IgG-HRP (AMERSHAM PHARMACIA BIOTECH)	1:5000
Donkey-Anti-rabbit IgG-HRP (AMERSHAM PHARMACIA BIOTECH)	1:5000

2.1.5. Strains

2.1.5.1. *Escherichia coli* (*E.coli*)

Strain	Genotype	Reference
DH5α	ϕ80d*lac*ZΔM15, *rec*A, *end*A1, *gyr*A96, *thi*-1, *hsd*R17(r_K^-, m_K^-), *sup*E44, *rel*A1, *deo*R, Δ(*lac*ZYA-*arg*F)U169	Hanahan (1983)
XL10 Gold	Tet^r Δ(*mcr*A)183 Δ(*mcrCB-hsdSMR-mrr*)173 *end*A1, *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, *lac* Hte [F´*pro*AB *lac*I^q ZΔM15 *Tn*10(Tet^r) Amy Cam^r]^a	STRATAGENE

2.1.5.2. *Saccharomyces cerevisae* (*S. cerevisae*)

Strain	Genotype	Reference
BY4741	*MAT a*; *his*3Δ1; *leu*2Δ0; *met*15Δ0; *ura*3Δ0	Euroscarf accession Y00000
***cbp3*Δ**	*MAT a*; *his*3Δ1; *leu*2Δ0; *met*15Δ0; *ura*3Δ0; *cbp*3::kanMX4	Euroscarf accession Y01077
***cbp3*Δ**	*MAT α*; *his*3Δ1; *leu*2Δ0; *lys2*Δ0; *ura*3Δ0; *cbp*3::kanMX4	Euroscarf accession Y11077
***cbp3*Δ–COR1-TAP**	*MAT a*; *his*3Δ1; *leu*2Δ0; *met*15Δ0; *ura*3Δ0; *cbp*3::kanMX4, *COR1::* (*COR1*-TAP-*URA*3-KL)	this work

***cbp4*Δ**	*MAT a*; *his*3Δ1; *leu*2Δ0; *met*15Δ0; *ura*3Δ0; *cbp*4::kanMX4	Euroscarf accession Y04804
***cbp4*Δ**	*MAT α*; *his*3Δ1; *leu*2Δ0; *lys2*Δ0; *ura*3Δ0; *cbp*4::kanMX4	Euroscarf accession Y14804
***cbp4*Δ–COR1-TAP**	*MAT α*; *his*3Δ1; *leu*2Δ0; *lys2*Δ0; *ura*3Δ0; *cbp*4::kanMX4, *COR1::* (*COR1*-TAP-*URA*3-KL)	this work
***bcs1*Δ**	*MAT a*; *his*3Δ1; *leu*2Δ0; *met*15Δ0; *ura*3Δ0; *bcs1*::kanMX4	Euroscarf accession Y04211
***cyt1*Δ**	*MAT a*; *his*3Δ1; *leu*2Δ0; *lys2*Δ0; *ura*3Δ0; *cyt1*::kanMX4	Euroscarf accession Y11841
10000M	*MAT a*; *ura*3-52; *leu*2Δ1; *trp*1Δ63; *his*3Δ200; *GAL*2	Euroscarf accession 10000M
***crd1*Δ**	*MAT a*; *his*3Δ1; *leu*2Δ0; *met*15Δ0; *ura*3Δ0; YDL142c::kanMX4	Euroscarf accession Y03840
***taz1*Δ**	*MAT a*; *his*3Δ1; *leu*2Δ0; *met*15Δ0; *ura*3Δ0; YDL140w::kanMX4	Euroscarf accession Y05555
KL14-4A	*MATa, his1, trp2, [rho^0]*	Wolf *et al.* (1973)
IL993-5c	*MATα, ilV5, [rho^0]*	Wolf *et al.* (1973)
***fyv5*Δ**	*MAT a*; *his*3Δ1; *leu*2Δ0; *met*15Δ0; *ura*3Δ0; *fyv5*::kanMX4	Euroscarf accession Y06877
yar069Δ	*MAT a*; *his*3Δ1; *leu*2Δ0; *met*15Δ0; *ura*3Δ0; *yar069*:: *URA*3	this work
ZK1	*MAT a*; *ura*3-52; *leu*2Δ0; *trp1Δ63; his*3Δ200; *GAL*2, *CBP*3*::* (*CBP*3-HA-*Sphis*5$^+$), *CBP*4*::*(*CBP*4-cMyc-*TRP*1)	this work
ZK1-B	*MATα*; *ura*3-52; *leu*2Δ0; *trp1Δ63; his*3Δ200; *GAL*2, *CBP*3*::* (*CBP*3-cMyc-*TRP*1), *CBP*4*::*(*CBP*4-HA-*Sphis*5$^+$)	this work
ZK2	*MAT a*; *his*3Δ1; *leu*2Δ0; *met*15Δ0; *ura*3Δ0, *CBP*3*::* (*CBP*3-TAP-*URA*3-KL), *CBP*4*::*(*CBP*4-cMyc-*HIS*3)	this work
ZK3	*MAT a*; *ura*3-52; *leu*2Δ0; *trp1Δ63; his*3Δ200; *GAL*2, *CBP*3*::* (*CBP*3-HA-*Sphis*5$^+$), *CBP*4*::*(*CBP*4-cMyc-*TRP*1), *COR*1*::* (*COR*1-TAP-*URA*3-KL)	this work
ZK4	*MAT a/α*; *ura*3-52/*ura*3-52; *leu*2Δ0/*leu*2Δ0; *trp1Δ63/trp1Δ63; his*3Δ200/*his*3Δ200; *GAL*2/*GAL*2, *CBP*4*::* (*CBP*4-cMyc-*TRP*1)/*CBP*4*::* (*CBP*4-HA-*URA3*)	this work

ZK5	*MAT a/α*; *ura*3-52/*ura*3-52; *leu*2Δ0/*leu*2Δ0; *trp1Δ63/trp1Δ63; his*3Δ200/*his*3Δ200; *GAL2*/*GAL2*, *CBP3::* (*CBP*3-cMyc-*TRP*1)/*CBP3::* (*CBP*3-HA-*URA3*)	this work
ZK5-B	*MAT a/α*; *his*3Δ1/*HIS*3; *leu*2Δ0/*leu*2Δ0; *met*15Δ0/*MET*15; *TRP*1/*trp1Δ63*; *ura*3Δ0/*URA*3, *GAL2*/*GAL2*, *CBP*3::(*CBP*3-TAP-*URA*3-KL)/(*CBP*3-cMyc-*TRP*1)	this work
QCR7-TAP	*MAT a his*3Δ1; *leu*2Δ0; *met*15Δ0; *ura*3Δ0, *QCR7::* (*QCR7*-TAP-tag-*HIS3MX6*)	Open Biosystems YSC1178-7501236
QCR8-TAP	*MAT a his*3Δ1; *leu*2Δ0; *met*15Δ0; *ura*3Δ0, *QCR8::* (*QCR8*-TAP-tag-*HIS3MX6*)	Open Biosystems YSC1178-7500107

2.1.5.3. *Schizosaccharomyces pombe* (*S. pombe*)

Strain	Genotype	Reference
KO 105	h^{-S} *ura4-D18*	K. Ostremann, Dresden

2.1.6. Media

LB

1,0 % (w/v) Tryptone
0,5 % (w/v)Yeast extract
0,5 % (w/v) NaCl
2,0 % (w/v) Agar (for LB-Plates)

YP-

1 % (w/v)Yeast extract
2 % (w/v) Peptone
2 % (w/v) Glucose, Raffinose, Galactose

or

3 % (w/v) Glycerin
2,0 % (w/v) Agar (for YP-Plates)

WO

1,7 g/l Yeast nitrogen base
5,0 g/l Ammonium sulphate
2,0 % (w/v)Glucose
2,0 % (w/v) Agar (for MM-Plates)
Amino acids / Nucleotide (see Table 2)

Amino acid/ nucleotide	Final concentration
L-Histidine	60 mg/l
L-Leucin	80 mg/l
L-Lysin	30 mg/l
L-Methionine	20 mg/l
Uracil	30 mg/l
L-Tryptophane	80 mg/l

Table 2. Amino acids and nucleotide used for minimal medium. According to the auxotrophic markers of used strains, the above listed amino acids and nucleotides were added to minimal medium in the respective final concentration.

Lactate Medium

22,0 ml/l Lactate (90 %)
1,0 g/l Glucose
3,0 g/l Yeast extract
1,0 g/l KH_2PO_4
1,0 g/l NH_4Cl
0,5 g/l $CaCl_2$ x H_2O
0,6 g/l $MgCl_2$ x $2H_2O$
0,5 g/l NaCl
8,0 g/l NaOH

Add distilled H_2O to 1 l and set the pH with NaOH to 5,5.

2.1.7. Buffers

TBE	90 mM Tris 90 mM Boric acid 2,5 mM EDTA
TBS	137 mM NaCl 20 mM Tris-HCl (pH 7,4)
TBS-T	1 x TBS 0,1 % (v/v) Tween 20

2.1.7.1. Buffers and Solutions for SDS-PAGE

Running gel (10% or 12%)	375 mM Tris-HCl, pH 8,8 10 % or 12 % (w/v) Acrylamide 0,32 % Bisacrylamide 0,1 % (w/v) SDS 0,1 % (w/v) Ammonium persulphate 0,1 % (v/v) TEMED
Stacking gel	125 mM Tris-HCl, pH 6,8 4 % (w/v) Acrylamide 0,1 % Bisacrylamide 0,1 % (w/v) SDS 0,1 % (w/v) Ammonium persulphate 0,1 % (v/v) TEMED
Running buffer	25 mM Tris 192 mM Glycin 0,1 % (w/v) SDS
6 x loading buffer	300 mM Tris-HCl, pH 6,8 30 % (w/v) Glycerol 10 % (w/v) SDS

	0,1 % (w/v) Bromphenol Blue 600 mM DTT or 5 % β-mercaptoethanol (freshly added)
Coomassie blue staining	45 % (v/v) Methanol 17 % (v/v) Acetic acid 0,1% (w/v) Coomassie brilliant blue G250
Destaining solution	30 % (v/v) Methanol 7 % (v/v) Acetic acid

2.1.7.2. Buffers and Solutions for Western blot

Transfer buffer	192 mM Glycine 25 mM Tris 5,0 % (v/v) Methanol 0,1 % (w/v) SDS
Ponceau S staining	0,5 % (w/v) Ponceau S 1,0 % (v/v) Acetic acid
Blocking solution	TBS-T 5 % (w/v) skimmed milk powder

2.1.7.3. Buffers and Solutions for isolation of yeast mitochondria

MTE	1,8 m M KH_2PO_4, pH 7,3 0,65 M Mannitol 20 mM Tris-HCl, pH 7,1 1 mM EDTA 1 mM PMSF (freshly added)
Buffer A	0,1 M Tris/SO_4, pH 9,4

Buffer B	1,2 M Sorbitol 20 mM KH_2PO_4, pH 7,4
Buffer C	1,2 M Sorbitol
Buffer D	0,65 M Mannitol 10 mM Tris-HCl, pH 7,4

2.1.7.4. Buffers and Solutions for BN-PAGE

Gel buffer	1,5 M Aminocaproic acid 150 mM Bis-Tris
Kathode buffer A	7,5 mM Imidazol 50 mM Tricine 0,02 % (w/v) Serva Blue G-250
Kathode buffer B	7,5 mM Imidazol 50 mM Tricine 0,002 % (w/v) Serva Blue G-250
Anode buffer	25 mM Imidazol
Solubilization solution	50 mM NaCl 5 mM Aminocaproic acid 50 mM Imidazol
Denaturation solution	1 % (w/v) SDS 1 % β-mercaptoethanol

2.1.7.5. Buffers and Solutions for Co-immunoprecipitation

Lysis buffer I	50 mM Tris-HCl, pH 7,6 150 mM NaCl

	1% (w/v) Detergent 2 mM DTT 1 mM EDTA 1 mM PMSF 1 x PI-Mix
Buffer for crosslinking	50 mM HEPES, pH 7,9 150 mM NaCl 0,6 M Sorbitol 1 mM PMSF 1 x PI-Mix
Quenching buffer	1 M Tris-HCl, pH 8,0
Washing buffer after crosslinking	50 mM HEPES, pH 7,9 0,6 M Sorbitol 1 mM PMSF 1 x PI-Mix
Solubilization buffer	50 mM HEPES, pH 7,9 150 mM Potassium acetate 2 mM Magnesium acetate 2 mM ATP 1 mM PMSF 1 x PI-Mix

2.1.7.6. Buffers and Solutions for TAP

IPP150- buffer	10 mM Tris-HCl, pH 8,0 150 mM NaCl 0,1 % (w/v) Detergent (Triton X-100)
TEV-buffer	10 mM Tris-HCl, pH 8,0 150 mM NaCl

	0,1 % (w/v) Detergent (Triton X-100) 0,5 mM EDTA 1 mM DTT
Calmodulin binding buffer	10 mM β-mercaptoethanol 10 mM Tris-HCl, pH 8,0 150 mM NaCl 1 mM Magnesium acetate 1 mM Imidazol 2 mM $CaCl_2$ 0,1 % (w/v) Detergent (Triton X-100)
Calmodulin elution buffer	10 mM β-mercaptoethanol 10 mM Tris-HCl, pH 8,0 150 mM NaCl 1 mM Magnesium acetate 1 mM Imidazol 2 mM EGTA 0,1 % (w/v) Detergent (Triton X-100)

2.1.8. Vectors

Vector	Genetic marker	Reference
p416 ADH	Amp^r, *URA*3, *CEN-ARS, ADH*-promoter	Mumberg *et al*. (1995)
p426 ADH	Amp^r, *URA*3, 2-micron, *ADH*-promoter	Mumberg *et al*. (1995)
p426 GPD	Amp^r, *URA*3, 2-micron, *GPD*-promoter	Mumberg *et al*. (1995)
pBS1539	Amp^r, *URA*3-KL, CoIE1 origin	Rigaut *et al.* (1999)
puZV 108	Amp^r, HA3-HISMX, *lacZ´*	W. Zacharie, Dresden
puZV 89	Amp^r, HA3-*TRP*1-KL *lacZ´*	W. Zacharie, Dresden
puZV 87	Amp^r, MYC9-*TRP*1-KL *lacZ´*	W. Zacharie, Dresden
pGA 2254	Amp^r, MYC9- HISMX, *lacZ´*	W. Zacharie, Dresden
pTG9	Amp^r, GFP-*ura4*$^+$, *nmt1*	K. Ostremann, Dresden

2.1.9. Primers

Primer	Sequence (5´to 3´)	Enzyme site
CBP3-HA-c-myc for	TTA CCA AGC GAG AGA AGT AGG CTG TCA TAT ACA AAC **TCC GGT TCT GCT GCT AG**	
CBP3-HA-c-myc rev	TGT GCG GAT AGT ATC TGA AGA ATA AAT AAG TGT TTT CAT AAG **CCT CGA GGC CAG AAG AC**	
CBP3-HA-myc check for	GCC ACA GGT AAA TTC AAG TTT G	
CBP4-HA-c-myc for	ATA GTC CAG GAT AAG CAG GTT AAA AGC TGG TGG CGC TTC TGG **TCC GGT TCT GCT GCT AG**	
CBP4-HA-c-myc rev	ATG ACA CAG ACG TAT GTT TTA GGA TGC AAA AGT TCA AGC TGC **CCT CGA GGC CAG AAG AC**	
CBP4 ORF for	TAT TAT GGA TCC ATG CAG TGC GCG ATA ACT CC	
CBP4 ORF rev	TAT TAT GAA TTC CTA CCA GAA GCG CCA CCA GC	
CBP3-TAP for	CCT AAA ACC TTA CCA AGC GAG AGA AGT AGG CTG TCA TAT ACA AAC **TCC ATG GAA AAG AGA AG**	
CBP3-TAP rev	TGT GCG GAT AGT ATC TGA AGA ATA AAT AAG TGT TTT CAT AAG **TAC GAC TCA CTA TAG GG**	
CBP3-TAP check	GAG GAA ATG AAA GTT AAT TCT GG	
COR1-TAP for	TTA TTA GAT TAC ATG AGA ATC AGA AGT GAC ATG TCC ATG ATG AGA TGG **TCC ATG GAA AAG AGA AG**	
COR1-TAP rev	CAT TTA TGG TGT GAG TGT GCG AGC GTG CCT GTA TAC ATC TAG CAG CTT **TAC GAC TCA CTA TAG GG**	
COR1-TAP check	AAG AGA TTA TGG GAC	
*URA*3KLfor	GCA GGT GGC TAT TAG	
*URA*3KLrev	ATG GTC TGA TAT CAC	
*HIS*for	CAA GTA GAC ACG GGA ATT GG	
*HIS*rev	TTT CAG CAC GAT GAT GGG TC	
YAR1 for	ACA GTC GGT CGG ATA ATA TCT CCT TCC AAC AGT AGG AGA CGT ACG CTG CAG GTC GAC	
YAR1 rev	AAG ACT CGA CAA TAC AAT TTT CTT ATA CTA AAC GTA GAT ATC GAT GAA TTC GAG CTC G	
YAR check for	GAA CTA CAG AAT GTT ATG CG	
YAR check rev	GCC CAC ACG ATT CCT GTG CAA AG	
*Sc*CBP3 for	TAT TTA *CTG CAG* CCA ACA CTT GAT GAT GTC AGT	PstI

	CAA TAG	
*Sc*CBP3 rev	AAA TAT *ATC GAT* GCT CGT TTA GTT TGT ATA TGA CAG CC	ClaI
*Sc*CBP3-HindIII for	TAT TAT *AAG CTT* CAC TTG ATG ATG TCA GTC AAT AG	HindIII
CMYC rev	TAT TAT *ATC GAT* GTT CAA GTC TTC TTC TGA GAT TAA	ClaI
*Sp*CBP3 for	TAT TAT *TCT AGA* ATG AGT CGC TGC TGT TTA AAT TTG CC	XbaI
*Sp*CBP3 rev	TAT TAT *ATC GAT* TCA AAT ATT TGT TTG TGC ACT TTC TTC AGG	ClaI
*Sp*CBP3-GFP for	TAT TAT *GAG CTC* AAA TGA GTC GCT GCT GTT TAA ATT TG	SacI
*Sp*CBP3-GFP rev	TAT TAT *CTG CAG* AAA TAT TTG TTT GTG CAC TTT CTT C	PstI
*Hs*CBP3 for	TTA TTA *ATC GAT* ATG GCG TTG CTG GTG CGA GTC	ClaI
*Hs*CBP3 rev	TAT ATA *CTC GAG* AGC CCA TCA AAG TCC CTC GTC	XhoI
CP1 for	CAT CTG AAT TCG AAA CCT CAA GAT CCA CGT	
CP1 rev	ACG TGG ATG TTG AGG TTT CGA ATT CAG ATG	
CP2 for	CAC TTC GTC CCA CCT GGG GTG AAG GTC GAA	
CP2 rev	TTC GAC CTT CAC CCC AGG TGG GAC GAA GTG	
C4 for	GAT GTT CAG CAG CGC TTA TTC GAG GAA ATG AAA	
C4 rev	TTT CAT TTC CTC GAA TAA GCG CTG CTG AAC ATC	
C5 for	GAC ATT GAG CTG AGA GGC AGA GTC ATG GGG	
C5 rev	CCC CAT GAC TCT GCC TCT CAG CTC AAT GTC	
EGFP-for *	CTG GTT TAG TGA ACC GTC AG	
EGFP-rev *	ACG CTG AAC TTG TGG CCG TT	
uni-21 *	TGT AAA ACG ACG GCC AGT	
rev-29 *	CAG GAA ACA GCT ATG ACC	

Table 3. Primers used for the plasmids construction, integrations cassettes construction and for sequencing. The enzyme recognition sites are indicated in italic. Primers used for construction of integrations cassettes for HA-, CMYC- and TAP-tagging and sequences of anneling regions are in bold. (*) indicates 5` IRD800 labelled sequencing primers.

2.1.10. Recombinant plasmids

Here are listed recombinant plasmids constructed in this work. The names of the plasmids are indicated on the left and the resulting protein or gene on the right. The numbers in brackets indicate aminoacid positions. (/) indicates the switch between two genes. P = Promoter

Plasmid	**Insert**
ScCBP3-p416*ADH*	*ADH1*-P, *ScCBP3*-ORF
SpCBP3-p416*ADH*	*ADH1*-P, *SpCBP3*-ORF
HsCBP3-p416*ADH*	*ADH1*-P, *HsCBP3*-ORF
SpCBP3-GFP-pTG9	*NMT1*-P, *SpCBP3*-GFP Fusion
CHP1-p416*ADH*	*ADH1*-P, ORF *Sc*Cbp3p(1-58)/*Sp*Cbp3p(59-291)/ *Sc*Cbp3p(292-337), Fig. 9
CHP2-p416*ADH*	*ADH1*-P, ORF *Sc*Cbp3p(1-58)/*Sp*Cbp3p(58-301), Fig. 9
HC1-p416*ADH*	*ADH1*-P, ORF *Sc*Cbp3p(1-144)/*Hs*Cbp3p(145-291)/ *Sc*Cbp3p(292-337), Fig. 10
HC2-p416*ADH*	*ADH1*-P, ORF *Sc*Cbp3p(1-144)/*Hs*Cbp3p(145-246)/ *Sc*Cbp3p(247-337), Fig. 10
HC3-p416*ADH*	*ADH1*-P, ORF *Sc*Cbp3p(1-144)/*Hs*Cbp3p(145-198)/ *Sc*Cbp3p(199-337), Fig. 10
HC4-p416*ADH*	*ADH1*-P, ORF *Sc*Cbp3p(1-198)/*Hs*Cbp3p(199-246)/ *Sc*Cbp3p(247-337), Fig. 10
HC5-p416*ADH*	*ADH1*-P, ORF *Sc*Cbp3p(1-198)/*Hs*Cbp3p(199-291)/ *Sc*Cbp3p(292-337), Fig. 10
CBP3-CMYC-p416*ADH*	*ADH1*-P, *CBP3-CMYC* Fusion
CBP4-CMYC-p416*ADH*	*ADH1*-P, *CBP4-CMYC* Fusion
CBP3-CMYC-p426*ADH*	*ADH1*-P, *CBP3-CMYC* Fusion
CBP4-CMYC-p426*ADH*	*ADH1*-P, *CBP4-CMYC* Fusion
CBP3-CMYC-p426*GPD*	*GPD1*-P, *CBP3-CMYC* Fusion
CBP4-CMYC-p426GPD	*GPD1*-P, *CBP4-CMYC* Fusion

2.2. Methods

2.2.1. Amplification of DNA by Polymerase Chain Reaction (PCR)

Standard PCR

The *CBP3* gene of *S. cerevisiae*, *S. pombe* and human were amplified from genomic DNA by standard PCR. Respective PCR products were subsequently cloned into an appropriate vector as indicated in the result part. The oligonucleotide primers are listed in section 2.1.9. The anneling temperature for PCR reaction was calculated according to the following formula (MWG-BIOTECH):

Ta = 69,4°C + 0,41 x (GC-Percent) – 650/ Primer length - 6°C

The following mix was used :

Components	Final concentration
PCR-Buffer (10x)	1 x
Template (x ng/µl)	1 ng/µl
dNTP Mix (each dNTP 10 mM)	0,2 mM
forward-Primer (100 pmol/µl)	1,5 pmol/µl
reverse-Primer (100 pmol/µl)	1,5 pmol/µl
DNA Polymerase (5 U/µl)	2,5 U
Total volume (H_2O upto:)	100 µl

Following programe was used for DNA amplification:

General PCR

5 min 95°C	Initial denaturation	1x
1 min 94°C 1 min Ta 2 min 72°C	Denaturation Annealing Elongation	20x
5 min 72°C	Final Elongation	1x

Overlap Extension PCR (Pogulis *et al.*, 1996)

By using the overlap extension PCR one can fuse DNA fragments without using the restriction and/or ligation procedure. Overlap PCR was used to construct different chimeras between the *CBP3* gene of *S. cerevisiae* and *S. pombe* or human, respectively. Primers used for overlapping PCR contain about 20 bp overlapping region to the part coming from another sequence and are listed in section 2.1.9 (CP1-2 and C4-5). The respective sub-fragments of the genes were amplified by general PCR. Purified sub-fragments served as templates for amplification of further product consisting of all parts used in the reaction yielding the final fused gene product. The annealing temperature was calculated from the flanking primers not overcoming the annealing temperature of overlapping regions of the sub-fragments. Obtained chimeras were cloned into vector p416ADH.

Following cycles were used:

5 min 95°C	Initial denaturation	1x
1 min 94°C 1 min Ta 2 min 72°C	Denaturation Annealing Elongation	10x
1 min 94°C 1 min Ta + 5°C 2 min 72°C	Denaturation Annealing Elongation	20x
5 min 72°C	Final Elongation	1x

2.2.2. Analysis of DNA

Standard techniques were used for restriction endonuclease analysis of DNA, ligation of DNA fragments, transformations and recovery of plasmid DNA from *E. coli* (Sambrook *et al.*, 1989). Purification of PCR products and plasmids was carried out with the kits listed in section 2.1.3 according to the instructions of the manufacturers.

2.2.3. Sequencing

DNA sequences were determined by the dideoxy chain determination method of Sanger (Sanger *et al.*, 1977) using 5´IRD800-labelled primers and the Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP as described by the manufacturer and

the LI-COR DNA sequencer. Primers for sequencing are listed in Table 3. The annealing temperature of the primers was calculated according the following formula (MWG-BIOTECH):

Ta = 69,4°C + 0,41 x (GC-Percent) – 650/ Primer length + 3°C

2.2.4. Transformation of yeast

Yeast cells were transformed using the LiAc procedure (Gietz *et al.*, 1995)

2.2.5. Isolation of yeast mitochondria

2.2.5.1. Mechanical Isolation of yeast mitochondria (quick method)

Yeast cells were grown to stationary phase in an appropriate medium. Cells were harvested by centrigugation at 3000 x g for 5 minutes. After washing with 1ml ice-chilled water, cells were resuspended in 0,5 ml ice-chilled MTE buffer. Lysis of cells was performed by vigorous vortexing for 5 minutes in the presence of 400 µl glass beads (∅ 0,45 mm). Supernatants were collected to new tubes and glass beads were washed with 0,5 ml ice-cold MTE. The supernatants of the washing step were added to the previous supernatants. Unbroken cells and cell debris were removed by centrigugation at 3000 x g for 3 min at 4°C. Mitochondria were pelleted by centrifugation at 13 000 x g for 20 min at 4°C. Mitochondria were resuspended in the 50-100 µl MTE and stored at – 80°C.

2.2.5.2. Enzymatical Isolation of yeast mitochondria (long method)

Daum *et al.* (1982)

Yeast cells were grown to stationary phase in an appropriate medium. Cells were harvested by centrigugation at 3000 x g for 5 minutes. After washing with 1 ml water, cells were resuspended in WCW/0,5 = …ml buffer A (WCW- wet cell weight). To the cell suspension 1,54 mg DTT per 1 ml of buffer A was added and incubated 10 min by 30°C. The cells after harvesting (3000 x g, RT, 5 min) were washed in ca. 30 ml of buffer C and resuspended in WCW/0,15 = …ml buffer B. 2 mg of Zymolyase 20T per 1g of WCW was added and incubated 30-60 min at 30°C. Control of spheroplasting was performed by measuring the

OD_{600}. 10 µl of cell suspension before Zymolyase 20T treatment were resuspended in 1 ml of watter. Spheroplasting was controlled every 15 min. The following steps were performed on ice (4°C), buffers D and C were ice-chilled. Spheroplasts were harvested (3000 x g, RT, 5 min) and washed once with buffer C. Spheroplasts were resuspended in ca. 10 ml of buffer D, 1 mM PMSF, freshly added. Spheroplasts were 3x homogenised in Dounce-Homogenisator (15 ml, Wheaton, USA) always in 10 ml buffer D. Lysates were collected in the centrifugation tubes. The rest of the cells was separated from the lysate by centrifugation (3000 x g, RT, 5 min.). Supernatants were collected and pellets again pottered. After the last pottering the Dounce-Homogenisator was washed with buffer D. Collected supernatants were centrifuged 10 min, 12000 x g, 4°C. Pellets were washed once with ca. 30 ml buffer D (+1mM PMSF) and again the rest of cell fragments were separated by 3000 x g, 5 min, 4°C centrifugation. Mitochondria were pelleted by 12000 x g, 10 min, 4°C centrifugation step, resuspended in the 100-300 µl buffer D and stored at – 80°C.

2.2.6. Solubilization of mitochondria

In principle, any neutral detergent can be used for the solubilization of biological membranes if the detergent can solubilize the desired protein and keep it in the native state. We preferentially use Triton X-100, digitonin and dodecyl-β-D-maltosid, all of which are stored as 10 % stock solutions. Triton X-100 and dodecyl-β-D-maltosid/protein ratio of 2-3 g/g is required for solubilization of mitochondrial membranes. Using digitonin, the detergent/protein ratio is doubled to 4-6 g/g, respectively. The most suitable detergent/protein ratio is equilibrated for each studied protein complex. 50-500 µg of total protein from isolated mitochondria are resuspended in the lysis buffer and the detergent is added in the required concentration. Solubilization is complete within 10-20 min incubation on ice. The sample is then centrifuged for 20 min at 20 000 x g. Solubilized protein complexes are in the supernatant.

2.2.7. Immunoprecipitation

Immunoprecipitation of mitochondrial proteins was performed with anti-HA and anti-cMyc antibodies. 50 µl protein A-Sepharose beads were incubated with antibodies for 2 h at 4°C on an orbital shaker. 500 µg mitochondria were lysed as described in section 2.7 and the supernatant was incubated with the protein A-Sepharose beads for 4h at 4°C. Beads were

washed 3-4 times with 1 ml lysis buffer. Immunoprecipitates were examined by Western blot analysis.

2.2.8. Western blot analysis

Proteins were separated on 10 % or 12 % polyacrylamide gels in the presence of SDS. Proteins were transferred to a PVDF membrane using the semi-dry method for 1h at RT at 1,5 mA/cm^2. Membranes were blocked at least 1h at RT or over night at 4°C with 5 % (w/v) non-fat dry milk in TBS-T. Membranes were probed with polyclonal or monoclonal antisera for 1h at RT and then washed three times for 10 minutes in TBS-T. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies were used as secondary antibodies. The secondary antibody was added to the membranes for 30 min at RT. Before the antigen-antibody complexes were visualised by enhanced chemiluminiscence (ECL plus), the membranes were washed three times for 10 minutes in TBS-T.

2.2.9. Blue native polyacrylamide gel electrophoresis

For blue native polyacrylamide gel electrophoresis (BN-PAGE; Schagger, 2001), 200 µg mitochondrial protein were sedimented by centrifugation (12.000 x *g*, 10 min, 4°C), resuspended in 20 µl low-salt buffer (50 mM NaCl, 5 mM ɜ-aminocaproic acid, 50 mM imidazol/HCl, pH 7.0, 1 mM PMSF, 1× proteinase inhibitor cocktail; Roche) containing digitonin (Sigma; detergent/protein = 4:1). This detergent has been shown not to disrupt complex III/IV supracomplexes (Schagger, 2002). After incubation on ice for 10 min the insoluble material was removed by centrifugation (20.000 x *g*, 20 min). Immediately after the addition of 4 µl Coomassie-free loading buffer (10% glycerol, 0.01% Ponceau S), the samples were loaded onto polyacrylamide gradient gels as indicated in the text. A high molecular weight gel filtration calibration kit (Amersham Pharmacia Biotech) was used to determine the apparent molecular weights (thyroglobulin monomer/dimer at 669/1.300 kDa, ferritin at 440 kDa, aldolase at 158 kDa, bovine albumin at 66 kDa).

2.2.10. *In-gel* activity assay for complex IV

Solubilized mitochondrial complexes were separated by BN-PAGE under native conditions. The activity of complex IV was visualized in the gel as described by Nijtmans *et al*. (2002).

2.2.11. Determination of the protein concentration

The protein concentration was determined using the DC protein assay as described by the supplier.

2.2.12. Epitope tagging

Fusion of proteins (Cbp3p, Cbp4p, Cor1p) with high affinity tags (e.g. cMyc, HA, Tap) was achieved by homologous recombination of the corresponding integration cassettes into a 3´-region of the chromosomal loci (Knop *et al.*, 1999) of the strains (ZK1-5) as indicated in section 2.1.5.2. Integration cassettes for HA-tagging and cMyc-tagging were PCR amplified from vector puZV108 (containing three tandem repeates of the HA epitope) and puZV87 (containing nine tandem repeats of the cMyc epitope, both a kind gift of W. Zachariae, Dresden). Integration cassettes for Tap-tagging were PCR amplified from vector pBS1539 (Rigaut *et al.*, 1999).

2.2.13. Multicopy suppression analysis

The *CBP3-cMYC* and *CBP4-cMYC* genes were cloned behind the strong *ADH1* and *GPD1* promoters in the multicopy vectors p426ADH and p426GPD, respectively (Humberg *et al*., 1995). The plasmids were transformed into the Δ*cbp*3 and Δ*cbp*4 strains. The transformants were cultured for seven days on YPGly plates.

2.2.14. Thin layer chromatography (TLC)

Isolated mitochondria were purified by gradient centrifugation as described by Meisinger *et al*. (2000) and lipids were extracted as described by Bligh and Dyer (1959). TLC was performed on a silica 60 TLC plate (MERCK) using TEA (triethylamine)/chloroform/ethanol/water (35:35:40:8, v/v/v/v) as the first mobile phase and hexane/ethylacetate (5:1; volume 120 ml) as the second mobile phase. After drying the plate, the spots were visualized by incubation with sulphuric acid.

2.2.15. Complex III activity measurement

Complex III activity was measured with a spectrophotometer by following the increase in reduced cytochrome *c* absorbance at 550 nm. The reaction buffer consisted of 50 mM potassium phosphate (pH 7.4), 0.5 mM EDTA, 0.5 mM KCN, 20 mM succinic acid and 60 μM cytochrome *c*. Isolated mitochondria (30 μg of mitochondrial protein) were incubated for 20 min at 30°C in the reaction buffer lacking cytochrome *c*. Reaction was started by addition of cytochrome *c* and monitored for 5 min. After 4 minutes the reaction was stopped with the complex III inhibitor Antimycin A. The activity is expressed as a first order rate constant (k), $k = \ln (\Delta E_0/\Delta E_1)$, where ΔE_0 is the absorption difference between the start and the end of reaction and ΔE_1 the absorption difference between the start and the time of 1 minute.

2.2.16. Proteinase K assay

Mitochondria were isolated from yeast by the enzymatic method (Daum *et al.*, 1982) and resuspended in 50-100 μl Mannitol-buffer (0.65 M Mannitol, 10 mM Tris/HCl, pH 7.4). Half of the isolated mitochondria were used to prepare mitoplasts. Mitoplating was induced by diluting mitochondria up to 0.1 M Mannitol and incubation for 30 min. on ice. Prepared mitochondria and mitoplasts were treated with increasing concentrations of Proteinase K (0,5-10 μg of PK per μg of mitochondrial protein) for 30-60 min. on ice in presence or absence of 1 % Triton X-100. The amount of mitochondria varied between 20-50 μg depending on stability and abundance of the analysed protein. After the treatment, Proteinase K was inactivated by adding proteinase inhibitors cocktail (PI-Mix) and PMSF (final concentration 1 mM). Treated mitochondria and mitoplasts were spinned down (12 000 x g, 10 min.) and resuspended in 6x Laemmli buffer (final concentration 1x), boiled for 5 min. and loaded on the SDS-PAA gel.

2.2.17. Heat shock

Yeast strain (wt) expressing Cbp4p was cultured on YPD and on YPG at 30°C. Upon reaching the OD_{600} = 0,5 (exponential growth phase) the cells were shifted to higher temperatures, 37°C and 42°C respectively for 30 min. After the heat shock, 2 ml of culture was spinned down, washed with watter and boiled in 50 μl Laemli buffer without the

coomassie blue. Protein concentration was meassured as decsribed in section 2.12. Equal protein concentration was used for SDS-PAGE.

2.2.18. PET test

PET test is used to complete the complementation analysis of respiratory defective strains with mutations in genomic DNA. It verify, if respiratory deficient phenotype is not caused by the loss of mitochondrial DNA. PET-tester strains are *rho*^{0} (do not contain mitochondrial DNA), but contain intact genomic DNA. Haploid strains are crossed with PET tester strains of respective MAT locus type (e.g. KL14-4A (=*MATa*) or IL999-5c (=*MAT*α)) and incubated overnight at 30° C on YPD. Respective diploids are then transferred on selective YPG media. If the mtDNA of transformants is intact, the diploids are able to grow on YPG media.

3. RESULTS

3.1. Protein Homologues of Cbp3p and Cbp4p

The assembly pathways of respiratory chain complexes seem to be evolutionary conserved. Both, Cbp3p and Cbp4p are members of larger protein families, which play a role in the assembly and/or stability of supramolecular enzymes required for electron transport and ATP synthesis. To identify related proteins, the protein sequence of Cbp3p and Cbp4p was used as the query for PSI-BLAST analysis against the UniRef90 protein dataset (http://www.pir.uniprot.org/database/DBDescription.shtml) and UniProt (http://www.pir.uniprot.org). Cbp3p and Cbp4p homologues were identified in various organisms beginning from eubacteria (*Pasteruella multocida*, *Clostridium teteni*, *Bacillus cereus*), other yeast species (*Candida glabrata, Kluyveromyces lactis, Yarrowia lipolytica*), to plants (*Oryza sativa, Ababidobsis thaliana*) and higher eukaryots (*Drosophila melanogaster, Mus musculus*, human).

BLAST-P analysis (Altschul *et al.*, 1997) of Cbp4p revealed a similarity to many proteins that act as stress response proteins, e.g. yeast mitochondrial Hsp78 protein (21% identity, 42% similarity) or ClpB of the bacterium *Brucella melitensis* (39% identity, 52% similarity of C-terminus, 125-162 aa). Hsp78p, a Clp homologue within mitochondria, seems to be involved in the turnover of unassembled mitochondrial proteins and can substitute for chaperone functions of mt-Hsp70p (Schmitt *et al.*, 1995). ClpB is part of a stress-induced multi-chaperone system involved in the recovery of the cell from heat-induced damage in cooperation with dnaK, dnaJ and grpE. It acts prior to dnaK in the processing of protein aggregates (Ekaza *et al.*, 2001). mRNA expression of *CBP4* is increased 2-3 fold upon heat shock of 15-30 min. (Gasch *et al.*, 2000). Additionally, expression of *CBP4* mRNA is increased 1,6 fold upon overexpression of Msn4p, a zinc-finger transcription factor that binds to the CCCCT sequence, also called “stress response element” (Gasch *et al*., 2000). Therefore I analysed the promoter region of *CBP4* for the presence of stress elements. The identification of heat-shock elements (AGAAN and CCCCT) in the promoter region of *CBP4* suggested that expression of Cbp4p may be induced upon the heat-shock. Expression of Cbp4p-cMyc was monitored at mild heat shock temperature (37°C) and at high temperature (42°C) as described in Materials and Methods (section 2.2.17). Expression of Cbp4p-cMyc was slightly increased upon heat shock when cells were growing in YPD medium. Expression of Cbp4p-cMyc in cells growing on glycerol at all temperatures was increased compared to the cells

growing on glucose. Cbp4p-cMyc expression during heat shock in cells growing on glycerol was constant (up to 42°C), no further induction of expression was observed (Fig. 6).

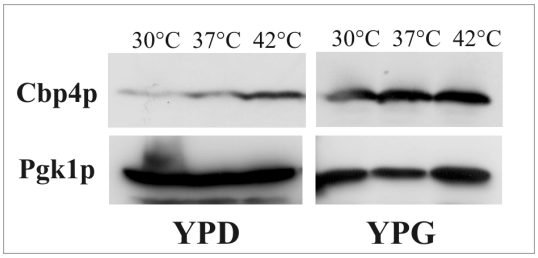

Figure 6. Expression of Cbp4p upon heat shock. Expression of Cbp4p was monitored upon 30 min. heat shock in cells growing on glucose (YPD) or glycerol (YPG). Expression of Cbp4p was slightly increased in the cells growing on YPD at 42°C. Cbp4p expression was elevated in all cells growing on YPG; no further increase in expression mediated by heat shock was observed. Detection with Pgk1p was used to demonstrate an equal protein loading on the SDS gel.

BLAST-P analysis (Altschul *et al.*, 1997) of Cbp3p identified homologues in *S. pombe* and human. Significant similarity of Cbp3p to basic FGF repressed zinc-binding proteins of *Xenopus laevis*, mouse and human (30 - 33 % identity, 48 – 51 % similarity) was revealed. The latter three zinc-binding proteins belong to the *CBP3* family and have two (mouse) or three (human) isoforms (Vetter and Wurst, 2001; Strausberg *et al.*, 2002). The function of the proteins and the biological processes, they are involved in, are not known. Cbp3p also shares 22 % identity and 45 % similarity with mouse heat shock 70 kDa protein 4 (Hsp74p). However, neither global mRNA expression micro-arrays analysis, nor monitoring of Cbp3p expression (this work) have shown an increase in expression upon heat shock. Accumulation of low molecular weight immunoreactive proteins derived from Cbp3p hint at the increased turn-over at higher temperatures (data not shown).

3.2. Functional analysis of baker's yeast Cbp3p and its homologues of *S.pombe* and human

Cbp3p of *Saccharomyces cerevisiae* (*Sc*Cbp3p) is a member of an evolutionary highly conserved protein family. While some data are available on *Sc*Cbp3p, almost nothing is known about its homologues in *Schizosaccharomyces pombe* (*Sp*Cbp3p) and *Homo sapiens* (*Hs*Cbp3p). The budding yeast *S. cerevisiae* and the fission yeast *S. pombe* are as different from each other as either is from mammals, but many *S. pombe* proteins turned out to be more similar to their mammalian homologues than they are to their *S. cerevisiae* counterparts

(Sipiczki, 1995). As Cbp3p is involved in mitochondrial complex III assembly, the identification of new mutations with clinical phenotypes can be expected. Therefore, it is important to show that these three proteins are orthologues to ensure that knowledge obtained from studies on *Sc*Cbp3p can be applied for its homologues in other organisms. It was shown before that *S. cerevisiae* and human Cbp3p are imported into mitochondria (Wu and Tzagoloff, 1989; V. Petruzzella -personal communication). However, two different cDNA clones for *Hs-CBP*3 were identified. Based on the sequencing data, the two isoforms of human Cbp3 (short form and the long form) show differences of the N-termini of the translation products, presumably due to alternative splicing of the primary transcript. The long form of human Cbp3 protein shows mitochondrial localization. By contrast, the short form of h-CBP3 failed to be imported into mitochondria (V. Petruzzella, personal communication). Mitochondrial localization of *Sp*Cbp3p was assumed on the basis of homology with *Sc*Cbp3 protein, but never experimentally verified. A fusion protein consisting of full length *Sp*Cbp3p and GFP was constructed and expressed in *S. pombe* wild type strain (section 2.1.5.3). By Western blot analysis I was able to confirm the localization of *Sp*Cbp3p in mitochondria (data not shown). In the second step the functionality of full length *Sp*Cbp3 and *Hs*Cbp3p proteins in the *S. cerevisiae Δcbp*3 strain was monitored. I have to note, that cDNA of the short form of h-*CBP*3 (the one that failed to be imported into human mitochondria) was used to construct recombinant plasmid expressing h-CBP3 that we here call as full length *Hs*Cbp3p. *Hs*Cbp3p lack ~ 50 amino acids of the N-terminus compared to *Sc*Cbp3p as shown in Fig. 8. The *CBP3* genes from all three organisms were cloned into single copy vector p416ADH and transformed into *S. cerevisiae Δcbp*3 strain. Complementation analysis was performed at three different temperatures (23°, 30°, 37°C) on glycerol, a non-fermentable carbon source, where only respiratory competent cells can grow. As expected, Δ*cbp*3 transformants expressing *Sc*Cbp3p were growing as wild type cells and *Hs*Cbp3p was not able to complement the Δ*cbp*3 mutation in *S. cerevisiae*. Transformants carrying *Sp*Cbp3p showed a faint growth at 30°C, whereas no growth at 23° and 37°C was observed (Fig. 7). We conclude, that *Sp*Cbp3p is partially able to complement the Δ*cbp*3 mutation in a temperature sensitive way.

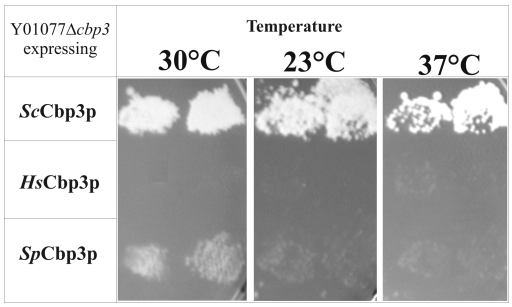

Figure 7. Complementation analysis. Full length *CBP3* genes of *S. cerevisiae* (Sc), *S. pombe* (Sp) and human (Hs) were cloned into the single copy vector p416ADH and transformed to *S. cerevisiae cbp3*Δ strain. Restoration of respiratory growth was tested on YPGlycerol medium at 30°, 23° and 37°C. Two different clones from each construct are shown.

As complementation analysis of the human full length protein did not reveal its functional homology to *Sc*Cbp3p, synthetic chimeric proteins between the *Sc*Cbp3p and the *Hs*Cbp3p were constructed. In addition, two synthetic chimeric proteins between *Sc*Cbp3p and *Sp*Cbp3p were constructed to identify functional domains. Construction of chimeras was based on the sequence homology comparison between all three proteins (Fig. 8). As can be seen, the central part up to the C-terminus (150-280 aa) is most conserved. To ensure correct import of the human protein into yeast mitochondria, the N-terminal end (1-58 aa or 1-144 aa) of *S. cerevisiae* protein was present in the chimeras. Two chimeras, CHP1 and CHP2 were constructed carrying the N-terminus of *Sc*Cbp3p and differing at the C-terminus. CHP1 contains the C-trminus of *Sc*Cbp3p (292-337 aa), while CHP2 contains C-terminus of *Sp*Cbp3 (section 2.1.10.). The transformants carrying *Sc/Sp* chimeras (CHP1, CHP2) were respiratory competent at 30°C, slightly cold sensitive (23°C) and heat sensitive (no growth at 37°C) (Fig. 9). Five different *Sc*/*Hs* chimeras (Fig. 10 or see section 2.1.10.) were constructed, that were analyzed in the same way as the full length protein. Chimeras HC1 and HC2 were not able to complement the Δ*cbp*3 mutation. Three of five chimeras, representing 48.2% of the human protein were able to complement the Δ*cbp*3 mutation. The respective transformants expressing chimeric proteins HC3, HC4, HC5 are respiratory competent at 30°C, slightly cold sensitive (23°C) and heat sensitive (no growth at 37°C) (Fig. 10).

The 3 chimeras (HC3, HC4, HC5) represent a yeast model that can be used to test mutations of human *CBP3* for their pathogenicity. Although ubiquinol-cytochrome *c* reductase deficiencies are rare, pathogenic mutations in the gene encoding mitochondrial cytochrome *b* have been described (Valnot *et al.*, 1999). Mutations in nuclearly-encoded subunits of complex III have not been reported so far. However, de Lonlay *et al.* (2001) reported complex

III deficiency in patients with tubulopathy, encephalopathy and liver failure caused by a mutation in complex III assembly protein - Bcs1p and Visapää *et al*. (2002) reported a new point mutation in *BCS1L* in patients with GRACILE syndrom. No *cbp3* mutations were reported up to now, but the identification of new mutations with clinical phenotypes can be expected.

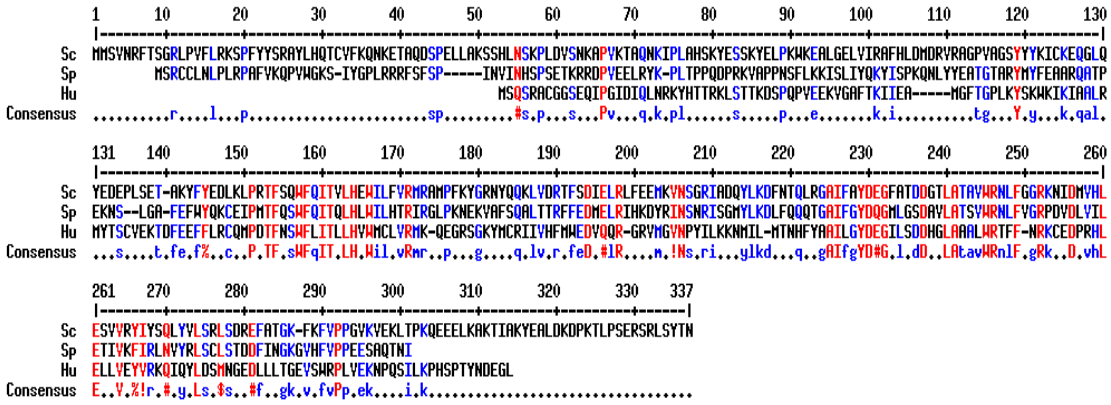

Figure 8. Sequence homology comparison. Protein sequences derived from the *CBP3* genes of *S. cerevisiae* (Sc), *S. pombe* (Sp) and human (Hs) were aligned. Identical or highly conserved positions are marked in red, amino acids which are identical in two of sequences are marked in blue.

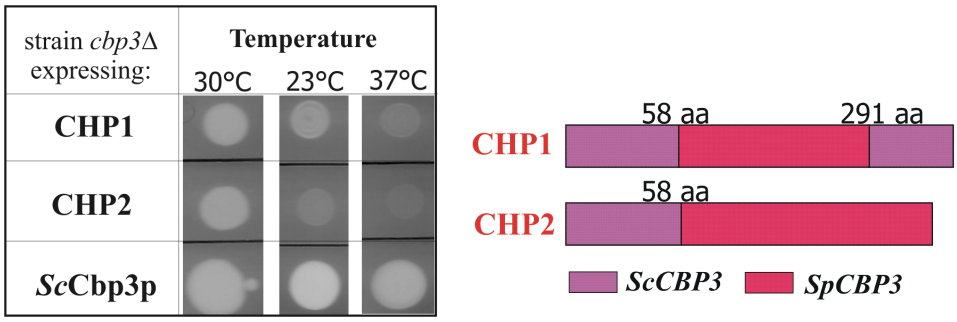

Figure 9. Complementation analysis of *Sc/Sp* chimeric proteins. Two chimeras were constructed by overlapping PCR. Numbers on the top of the schemes correspond to amino acid positions at which the sequence was switched based on Fig. 8. Chimeras were cloned into single copy vector p414ADH and transformed to *S. cerevisiae cbp3*Δ strain. Restoration of respiratory growth was tested on YPGlycerol medium at 30°, 23° and 37°C.

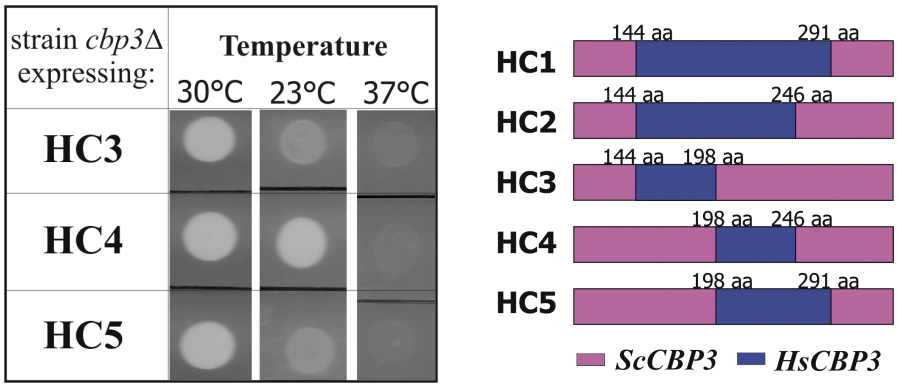

Figure 10. Complementation analysis of *Sc/Hs* chimeric proteins. Five different chimeras were constructed by overlapping PCR. Numbers on the top of schemes correspond to amino acid positions at which the sequence was switched based on Fig. 8. Chimeras were cloned into single copy vector p414ADH and transformed to *S. cerevisiae cbp3*Δ strain. Restoration of respiratory growth was tested on YPGlycerol medium at 30°, 23° and 37°C. Only transformants expressing HC3, HC4, and HC5 are shown. Transformants expressing HC1 or HC2 remained respiratory deficient.

PET test analysis (see section 2.2.18.) confirmed that mtDNA was intact in all transformants and the respiratory deficient phenotype was caused by the mutation in genomic DNA, which can be rescued by the plasmid-born genes.

3.3. Topology of Cbp3p and Cbp4p

By carbonate extraction Cbp3p was shown to be peripherally associated with the membrane (Wu and Tzagoloff, 1989), whereas Cbp4p appears to be anchored in the mitochondrial membrane, but not exposed on the outer membrane (Crivellone, 1994). The software used to predict the transmembrane domains (Persson and Argos, 1994) indicated one transmembrane domain in both Cbp3p and Cbp4p. To further characterize the localization (outer membrane, intermembrane space, inner membrane) and topology (spatial orientation of N- and C-terminus) mitochondria and mitoplasts (outer membrane is disrupted) were treated with proteinase K (PK) in the absence or presence of detergent. Strain ZK1 expressing Cbp3p-HA and Cbp4-cMyc grew on lactate medium to early stationary phase and thereafter mitochondria were isolated. Mitochondria and mitoplasts were treated with proteinase K as described in Materials and Methods (Fig. 11). Cbp3p is completely protected against PK in mitochondria and mitoplasts suggesting its location on the matrix side of inner mitochondrial membrane. Cbp4p is protected against PK degradation in mitochondria, but sensitive to increasing concentrations of PK in mitoplasts. Detection with antibody against Aconitase (Aco1p), a

protein located in the mitochondrial matrix, shows that mitochondria and mitoplasts are not disturbed and matrix proteins are protected against the degradation. Sco1p, a mitochondrial IM protein that contains one tramsmembrane domain and whose C-terminus is exposed to the IMS (Buchwald *et al.*, 1991) is protected against proteinase K in mitochondria, but sensitive to degradation with increasing concentration of PK in mitoplasts confirming that mitoplasts have been formed. Based on the prediction of one transmembrane domain of Cbp4p, its presence in pellet fraction after carbonate extraction and the PK assay shown here, it could be concluded that Cbp4p, like Sco1p, is an inner mitochondrial membrane protein with the C-terminus exposed to intermembrane space.

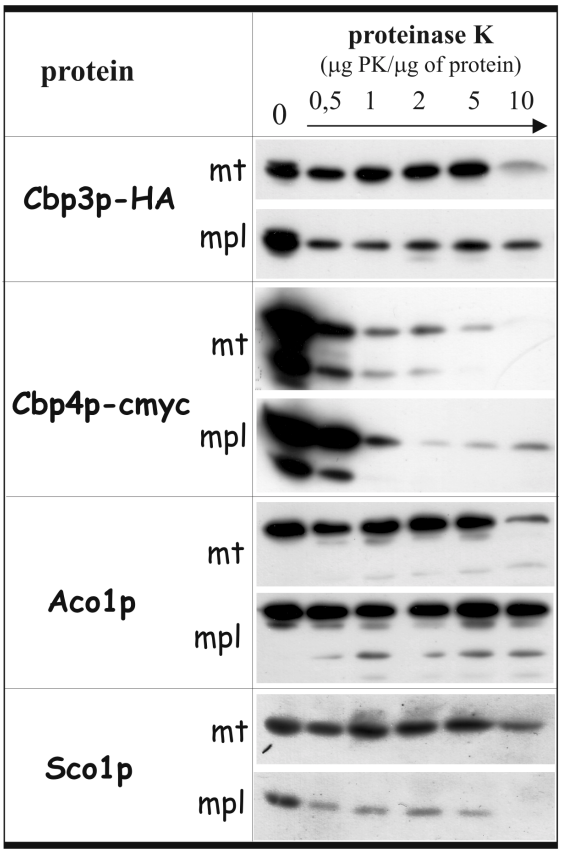

Figure 11. Proteinase K assay. Mitochondria were isolated from strain ZK1 expressing Cbp3p-HA and Cbp4p-cMyc proteins. Mitochondria (mt) and mitoplasts (mpl) were treated with increasing concentration of PK in the absence of detergent. Proteins were analysed by SDS-PAGE and Western blot analysis was performed with antibodies directed against the indicated proteins.

It has been reported that glutamate residues in nuclearly encoded mitochondrial inner membrane proteins occur ten times more often on the IMS side than on the matrix side, suggesting a role of thess aa residues for protein topology (Gavel and von Heijne, 1992). The fact that the c-myc epitope contains three glutamate residues may be a factor influencing

Cbp4p-cMyc topology. Therefore Cbp4p was tagged with HA epitope, which lacks glutamate residues and the PK assay was repeated. Cbp4p-HA is protected in mitochondria, but sensitive to increasing PK concentration in mitoplasts (data not shown) confirming the IMS orientation of the C-terminus of Cbp4p.

3.4. Cbp3p and Cbp4p interact and are present in common high molecular weight complexes.

To test whether Cbp3p and Cbp4p interact in a common structure as mentioned by Shi *et al.* (2001) we constructed strain ZK1-B in which Cbp3p and Cbp4p are replaced by the tagged versions Cbp3p-cMyc and Cbp4p-HA (see section 2.1.5.2.). This strain is viable and shows no difference in growth on lactate medium compared to the wild type strain (data not shown). Functionality of the tagged proteins is also documented by the finding that the combined complex II-III activity is comparable to that of the wild type strain (Table 4).

Strain	Rate constant (k)
BY 4741 (wild type)	0,728
ZK1-B	0,75

Table 4. Complex II-III activity. The activity was measured by monitoring the increase in reduction of cytochrome *c*. It is expressed as a first order rate constant (k), for details see Materials and Methods. *cbp3*Δ and *cbp4*Δ mutant strains exhibit no detectable activity.

Mitochondrial proteins of strain ZK1-B were separated by BN-PAGE and a subsequent second dimension SDS-gel, blotted and analyzed with antibodies against HA, cMyc, Cyt1p and Cox3p (Fig. 12). Detection with the Cyt1p antibodies shows that complex III is assembled in a dimeric complex of about 700 kDa and in additional complexes of higher molecular weight. Anti-Cox3p antibodies detect the complex IV dimer (~ 400 kDa) and in addition high molecular weight complexes (≥ 669 kDa), presumably corresponding to complex III/IV supracomplexes. These results indicate that formation of complex III and its assembly into supracomplexes is not impaired by the tagged versions of Cbp3p and Cbp4p. Cbp3p and Cbp4p are both organized in complexes of high molecular weight: the majority of the proteins is detected as an continuous streak ranging from > 60 kDa up to > 669 kDa. The distribution of Cbp3p and Cbp4p is very similar and may indicate their presence in common

complexes. In the case of Cbp4p-HA two additional bands of about 60 and 80 kDa are detected (Fig. 12), whose identities are not clear. Possibly they result from protein interactions, which persisted the denaturing conditions of the electrophoresis.

Interestingly a small portion of both Cbp3p and Cbp4p can be detected in the molecular weight range of 669 kDa and higher (Fig. 12, lanes A and B) that overlaps with the signals of complex III/IV supracomplexes. This offers the possibility that Cbp3p and Cbp4p may not only be involved in complex III formation, but also in its assembly to supracomplexes.

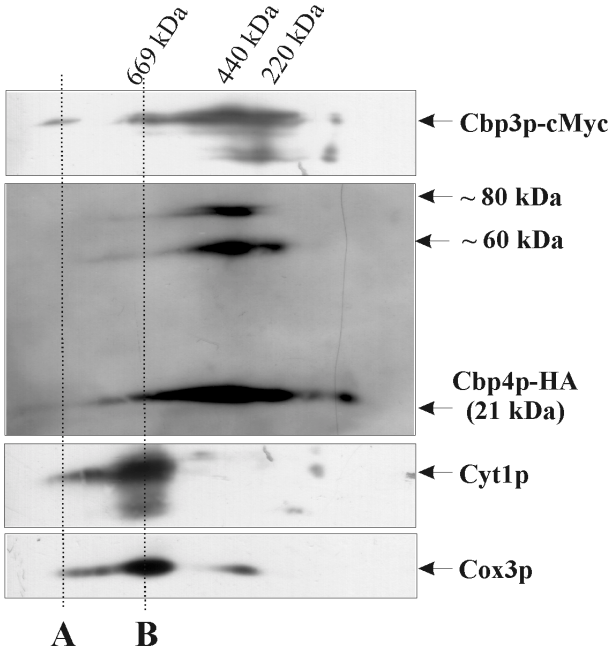

Figure 12. Organisation of Cbp3p and Cbp4p. Mitochondrial protein complexes of strain ZK1-B were separated by BN-PAGE in a 3-12 % gradient gel and subsequently in the second dimension on a 10 % SDS-gel. Antibodies directed against cytochrome c_1, Cox3p, HA (Cbp4p) and cMyc (Cbp3p) were used for detection. (A, B-supracomplexes of complex III and IV, for details see text) Sizes of marker proteins are indicated.

Physical association of Cbp3p and Cbp4p *in vivo* was verified by Co-IP. Sepharose-bound anti-HA and anti-cMyc antibodies were used to precipitate Cbp4p-HA and Cbp3p-cMyc, respectively, from mitochondrial lysate of strain ZK1-B. Both antibodies are highly specific for their respective tags and show no cross-reaction with other tags (data not shown). The precipitates were separated by SDS-PAGE, blotted and detected with antibodies against cMyc (Fig. 13, upper part) or HA (Fig. 13, lower part). In both cases we were able to detect both proteins concomitantly in the precipitate. Therefore the Co-IP experiments confirm the interaction of Cbp3p and Cbp4p in a stable complex.

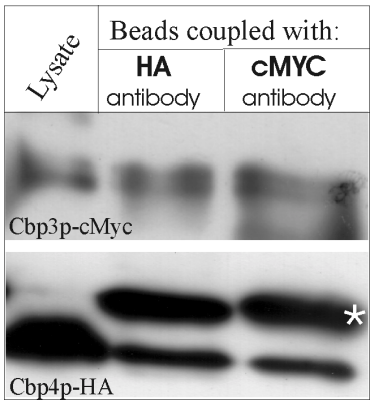

Figure 13. Co-immunoprecipitation of Cbp3p-cMyc and Cbp4p-HA. Mitochondrial lysate from strain ZK1-B expressing Cbp3p-cMyc and Cbp4p-HA was incubated with sepharose-bound antibodies against HA or cMyc. 1/50 of the lysate and the complete precipitates were subjected to SDS-PAGE and detected with cMyc-antibody (anti cMyc; upper part) or with HA-antibody (anti HA; lower part). The upper band (*), which is detected with the HA-antibody, corresponds to the light chain of antibody which is recognized by the secondary antibodies.

Both, Cbp3p and Cbp4p were identified in different high molecular weight complexes in the range between 60 kDa to 700 kDa. Additionally, in the second dimension of BN-PAGE a shift of both proteins to higher molecular weight, probably resulting from less sufficient denaturing conditions is observed (Fig. 11). We have shown by coimmunoprecipitation that Cbp3p and Cbp4p interacts, but detection of signals of both proteins at a different molecular weight than that corresponding to Cbp3p/Cbp4p dimer suggest that these proteins may also form homodimers. To test this hypothesis a diploid strain (ZK5) expressing Cbp3p-HA and Cbp3p-cMyc and a diploid strain (ZK4) expressing Cbp4p-HA and Cbp4p-cMyc were constructed. Digitonin-solubilized proteins/complexes of mitochondria isolated from strains ZK4 and ZK5 were separated by BN-PAGE in the first dimension and by SDS-PAGE in the second dimenion. Cbp4p-HA or -cMyc are detected in the same positions as illustrated in Fig. 13 by dotted-lines. This suggests that both tagged versions of Cbp4p are assembled into the same complexes or interact with the same proteins. Based on BN-PAGE we can not confirm or exclude the formation of Cbp4p homodimers. Therefore coimmunoprecipitation (co-IP) was performed. The co-IP was performed in the absence and presence of the cross-linker, DSP. Cbp4p-HA was precipitated with beads coupled with anti-HA antibody and Cbp4p-cMyc was precipitated with the beads coupled with anti-cMyc antibody. When precipitated with anti-cMyc antibody only very week signal of Cbp4p-HA was detected. Similarly, when precipitated with anti-HA antibody, the signal for Cbp4p-cMyc was very weak suggesting no formation of Cbp4p homodimers. Interestingly, cytochrome c_1 (Cyt1p) was precipitated with both Cbp4p-HA or Cbp4p-cMyc in comparable concentration suggesting an interaction of

Cbp4p with Cyt1p (Fig. 15). Detection with Cox3p revealed only a very weak signal, except upon precipitation with Cbp4p-cMyc in the presence of cross-linker, DSP.

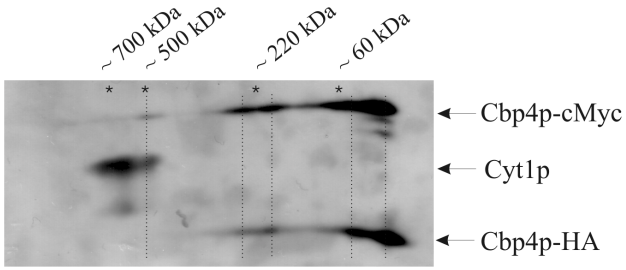

Figure 14. Western blot analysis of strain ZK4. Mitochondrial protein complexes of Cbp4p were monitored. Solubilized complexes were separated by BN-PAGE in a 5-13 % gradient gel and subsequently in the second dimension on a 10 % SDS-gel. Antibodies directed against HA (Cbp4p), cMyc (Cbp4p) and cytochrome c_1 were used for detection and an overlay of all three proteins is shown. Both Cbp4p are detected in the complexes of the same molecular weight. Sizes of marker proteins are indicated with *.

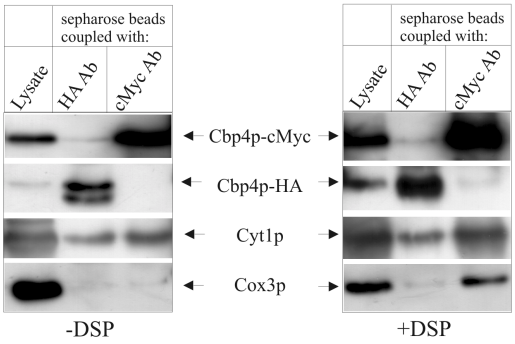

Figure 15. Co-immunoprecipitation of Cbp4p-cMyc and Cbp4p-HA. Co-immunoprecipitation was performed with lysates from crosslinked (+DSP) or non-crosslinked (-DSP) mitochondria of strain ZK4 expressing Cbp4p-cMyc and Cbp4p-HA. Solubilized mitochondrial proteins were incubated with sepharose-bound antibodies against HA or cMyc. 1/50 of the lysate and the complete precipitates were subjected to SDS-PAGE and detected with antibodies against cMyc-tag, HA-tag, Cyt1p and Cox3p.

The diploid strain ZK5 was used to study formation of Cbp3p homodimers (Fig 16). As expected, the organization of Cbp3p is comparable with Fig. 12. We have shown previously, that Cbp3p that is HA tagged is not fully functional, but still is able to assemble into some complexes as can be seen in Fig. 16. However, Cbp3p-cMyc tagged is functional and assembles into higher molecular weight complexes, similar to Cbp4p. The signal of Cbp3p-cMyc can be followed up to molecular weights corresponding to supracomplexes. Portion of Cbp3p (similarly to Cbp4p) overlaps with Cyt1p corresponding to assembled bc_1 complex and its supracomplexes, highlighted by dotted-lines in Fig. 16. Second dimension analysis after

BN-PAGE (Fig. 16) reveals that Cbp3p-HA can be detected at the molecular weight corresponding to the monomeric form (42 kDa) as well as in the molecular weight of ~90 kDa. Cbp3-cMyc tagged is preferentially detected in the molecular weight of ~90 kDa and only a very weak signal is detected at the molecular weight corresponding to monomeric form (52 kDa). As shown in the overlay, Cbp3p-HA and Cbp3p-cMyc completely overlap in the position of ~90 kDa, strongly suggesting, that Cbp3p can make homodimers that persist the denaturing condition of SDS-PAGE. We were not able to confirm that hypothesis by coimmunoprecipitation, because Cbp3p-HA is not fully functional. Cbp3p-HA can not bind Cbp4p and is not able to complement deletion of *CBP3* (data not shown). Surprisingly, while the interaction of Cbp3p-HA with Cbp4p or itself could not be observed, the interaction of Cbp3p-HA or Cbp3p-cMyc with Cyt1p was detected by co-IP (data not shown).

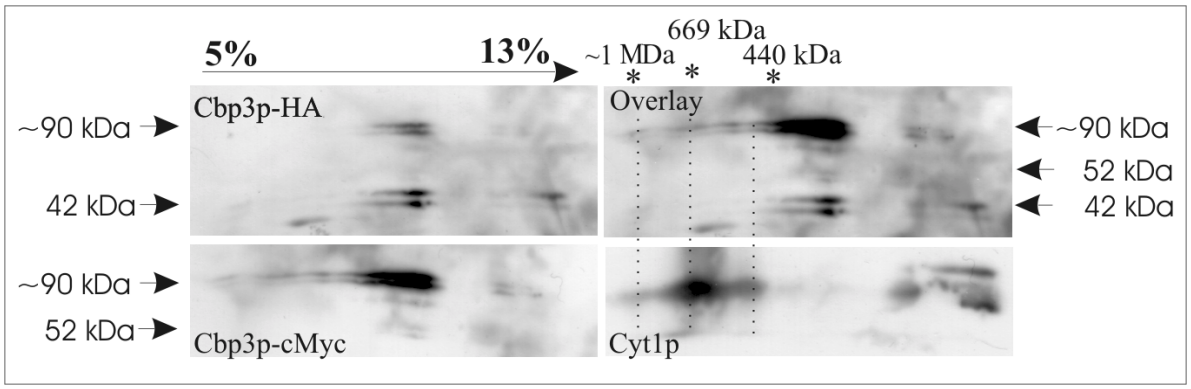

Figure 16. Western blot analysis of strain ZK5. Mitochondrial protein complexes of Cbp3p were monitored. Solubilized complexes were separated by BN-PAGE in a 5-13 % gradient gel and subsequently in the second dimension on a 10 % SDS-gel. Antibodies directed against HA (Cbp3p-HA), cMyc (Cbp3p-cMyc) and cytochrome c_1 (Cyt1p) were used for detection. Both variants of Cbp3p are detected in different concentration in complexes of similar molecular weight (overlay) comigrating with Cyt1p complexes (dotted lines). Sizes of approximate protein positions in the SDS PAGE are indicatedby arrows. (*) indicates sizes of protein complexes in the first dimension (BN-PAGE).

To test the possibility that Cbp3p forms a homodimer, diploid yeast strain (ZK5-B) expressing functional Cbp3p-Tap and Cbp3-cMyc proteins was constructed. Cbp3p-cMyc was identified by BN-PAGE in 3 different positions. Similarly to the situation in Fig. 16, the first position corresponded to a monomeric form (52 kDa). The two additional signals were identified at positions ~70 kDa and ~100 kDa (data not shown). The signal at the position ~100 kDa could correspond to Cbp3p-cMyc dimer. The signal detected at the position of ~70 kDa may come from interaction of Cbp3p-cMyc with some other proteins (e. g. with Cbp4, as assumed from the approximate molecular weight of Cbp3p/Cbp4p dimer). Detection of

Cbp3p-Tap revealed only a very weak signal at position ~100 kDa overlapping with signal of Cbp3p-cMyc. To test the possibility of Cbp3p homodimer formation, TAP-purification was performed. Cbp3p-cMyc was purified in the first step with IgG beads. After the TEV cleavage the supernatant was incubated with calmodulin beads, but Cbp3p-cMyc remained within the unbound material and its further purification was not observed (data not shown). Based on these results we can not confirm, as well as not exclude the formation of Cbp3p homodimers.

3.4.1. Interacting proteins of Cbp3p

Two putative Cbp3p-interacting proteins, Yar069p and Fyv5p, have been identified in a high-throughput 2-hybrid analysis (Ito *et al.*, 2001). Both proteins have unknown function and intracellular localization. In order to elucidate the function of these proteins, the respective *S. cerevisiae* null mutant Δ*YAR*069 was constructed. *FYV*5 null mutant strain was obtained from EUROSCARF. The phenotype of both mutants was compared with the phenotype of the respiratory defective *cbp*3Δ strain on media containing glycerol as the only non-fermentable carbon source. Both null mutants were respiratory competent but slightly temperature sensitive when growing on 23° and 37°C. The respiratory competent phenotypes of the null mutant strains of the two candidate proteins, the Fyv5p and Yar069p indicate that these proteins are not essential for the respiratory chain assembly. PSORT prediction (http://psort.ims.u-tokyo.ac.jp/), Target P V 1.0 (Emanuelsson *et al.*, 2000) and Signal P V 1.1 (Nielsen *et al.*, 1997) analysis does not predict their mitochondrial localization. Two-hybrid analysis often indicate false positive interactions with proteins exhibiting hydrophobic transmembrane segments. Both Fyv5p and Yar069p contain putative hydrophobic transmembrane segments, Fyv5p has five and Yar069p three. Combined with the fact, that deletion of the respective genes does not result in a respiratory deficient phenotype (see above), this observation raises the possibility that interaction of these proteins with Cbp3p may be artificial. Because of the above mentioned reasons we decided not continue in further investigation of these gene products.

3.5. Overexpression of *CBP*3 and *CBP*4 in *cbp3*Δ and/or *cbp4*Δ strain, respectively

The phenotype of a *cbp3*Δ/*cbp4*Δ double mutant resembles that of the respective single mutants. This, together with the notion of Shi *et al.* (2001) that Cbp3p might be physically associated with Cbp4p, hints at the possibility that the two proteins cooperate in the same step

of complex III assembly. To address this question we tested the effect of overexpression of *CBP3* in the *cbp4* null mutant, and *vice versa*, on respiratory growth. To this end *CBP3-cMYC* and *CBP4-cMYC* were overexpressed from multi copy plasmids (Fig. 17) in the *cbp3*Δ and *cbp4*Δ strains. Both tagged genes are able to substitute for the authentic genes. Interestingly, upon high level expression in both cases protein variants with reduced electrophoretic mobility accumulate. In both cases these proteins might represent precursor forms with aminoterminal cleavable mitochondrial targeting sequences. However, we cannot exclude that the altered electrophoretic mobility of the proteins results from unknown modifications. The *cbp3*Δ and *cbp4*Δ transformants were cultured on glycerol as a non-fermentable carbon source (Fig. 17). Even after 10 days at 30 °C no growth of the *cbp3*Δ strain expressing Cbp4p-cMyc was observed. Clearly Cbp4p is not able to substitute the function of Cbp3p. In the case of the *cbp4*Δ strain we observe a very faint growth in the untransformed strain as well as in transformants bearing *CBP3-cMYC* on the single copy vector p416ADH. Interestingly upon high level expression of Cbp3p from the multicopy plasmids p426ADH or p426GPD growth of the transformants is slightly improved compared to the untransformed mutant strain (Fig. 18). These findings may be explained by a partial compensation of Cbp4p function by a high concentration of Cbp3p.

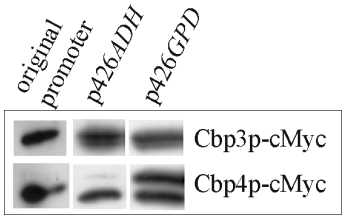

Figure 17. High level expression of Cbp3p and Cbp4p. Expression of Cbp3p-cMyc and Cbp4p-cMyc under the control of different promoters was monitored. High level expression results in the accumulation of a Cbp4p-cMyc form with a reduced electrophoretic mobility possibly reflecting a precursor form.

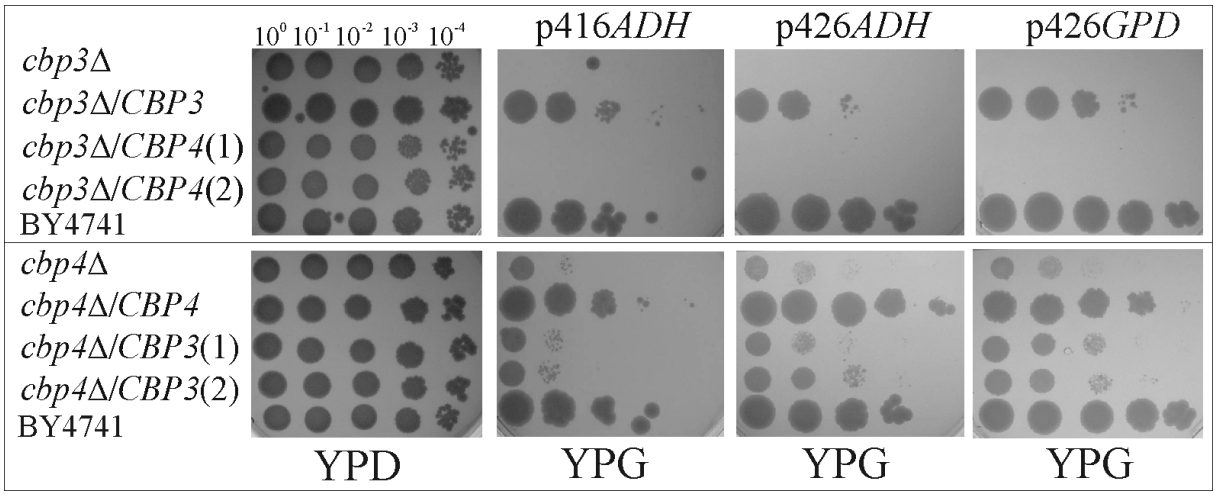

Figure 18. Effect of overexpression of Cbp3p-cMyc and Cbp4p-cMyc in the indicated strains on respiratory growth. Strains were grown in minimal media at 30 °C. An equal number of cells of OD_{600} = 0,5 was diluted as indicated and dropped on the respective plates. Plates were cultured at 30 °C for a maximum of 10 days. Two different *cbp4*Δ/*CBP3* transformants are shown and indicated as (1) and (2).

3.6. Western blot analysis of *cbp3* and *cbp4* deletion strains

To reveal differences between the deletion strains, the molecular organization of complex III was analyzed by BN-PAGE after solubilization with digitonin. Mitochondrial proteins of the *cbp3*Δ and *cbp4*Δ strains were separated and compared to a wild type and a *bcs1*Δ mutant strain. The latter strain accumulates a dimeric bc_1 precomplex of 500 kDa lacking Rip1p and Qcr10p (Cruciat *et al.*, 1999). Western blot analysis of the BN-PAGE gel was performed with Cyt1p antibodies (Fig. 19). The *bcs1*Δ mutant strain accumulates the expected bc_1 precomplex of ~ 500 kDa, whereas in the wild type a complex of > 700 kDa can be detected. This band probably corresponds to a supracomplex, consisting of complex III and IV. In the *cbp4*Δ mutant the intensity of the > 700 kDa complex is significantly reduced and accompanied by the appearance of a number of low molecular weight bands with a strong accumulation in the range of < 150 kDa. In the *cbp3*Δ mutant only trace amounts of the high molecular complexes can be seen, instead some weak bands of lower molecular weight are detected. These results are in line with the proposal that Cbp3p and Cbp4p are required for assembly of complex III.

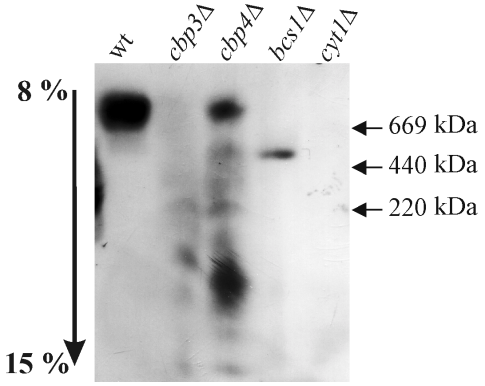

Figure 19. BN-PAGE analysis of strains deficient in complex III assembly. Mitochondrial fractions of wild type (wt) and the indicated mutant strains were separated by BN-PAGE on a 8-15 % native gradient gel and detected with antibodies directed against cytochrome c_1.

Complex III assembly of the two mutant strains was further characterized by combining the BN-PAGE with a second dimension gel in the presence of SDS (Fig. 20). Complex III subcomplexes were characterized with antibodies against Cyt1p, whereas an antibody against Cox3p was used to identify supracomplexes of complex III and IV. Complex V assembly was tested with the Atp2p antibody.

No differences between the various strains were observed upon detection with the Atp2p antibody, indicating that complex V assembly is not disturbed in the *cbp3*Δ and *cbp4*Δ mutants. Detection with Cyt1p antibodies confirms the results of the BN-PAGE: in wild type cells Cyt1p is almost exclusively organized in high molecular weight complexes (> 650 kDa) (designated “S”), whereas in the *cbp3*Δ strain Cyt1p is detected in various smaller complexes ranging from 100 to ~ 500 kDa. The *cbp4*Δ strain reveals a similar pattern of complexes in the lower molecular weight range, but in addition a high molecular weight complex of > 700 kDa can be detected.

Western blot analysis with the Cox3p antibody identifies a complex of about 400 kDa in all strains (Fig. 20), which represents the complex IV dimer (IV_{DIM}). Obviously, COX-assembly is not affected in any of the mutant strains. In wild type cells one or even more additional complexes of higher molecular weight can be detected, which are probably supracomplexes of complex III and IV as reported by Cruciat *et al.* (2000). These supracomplexes are completely absent in the *cbp3*Δ mutant, whereas in the *cbp4*Δ mutant a faint band corresponding to a complex of about 700 kDa (designated “Y”) is seen. This complex could correspond to a complex III/IV supracomplex. The identified supracomplexes were tested for COX-activity

by an *in-gel* activity test (Fig. 21). Whereas the wild type complexes exhibit a high activity, the > 700 kDa complex detected in the *cbp4*Δ strain yields only a very weak signal.

The complex of about 500 kDa, which can be seen in the *cbp3*Δ mutant (designated "X") could reflect a dimeric bc_1 precomplex that is probably non-functional as can be deduced from the respiratory defective phenotype of the mutant.

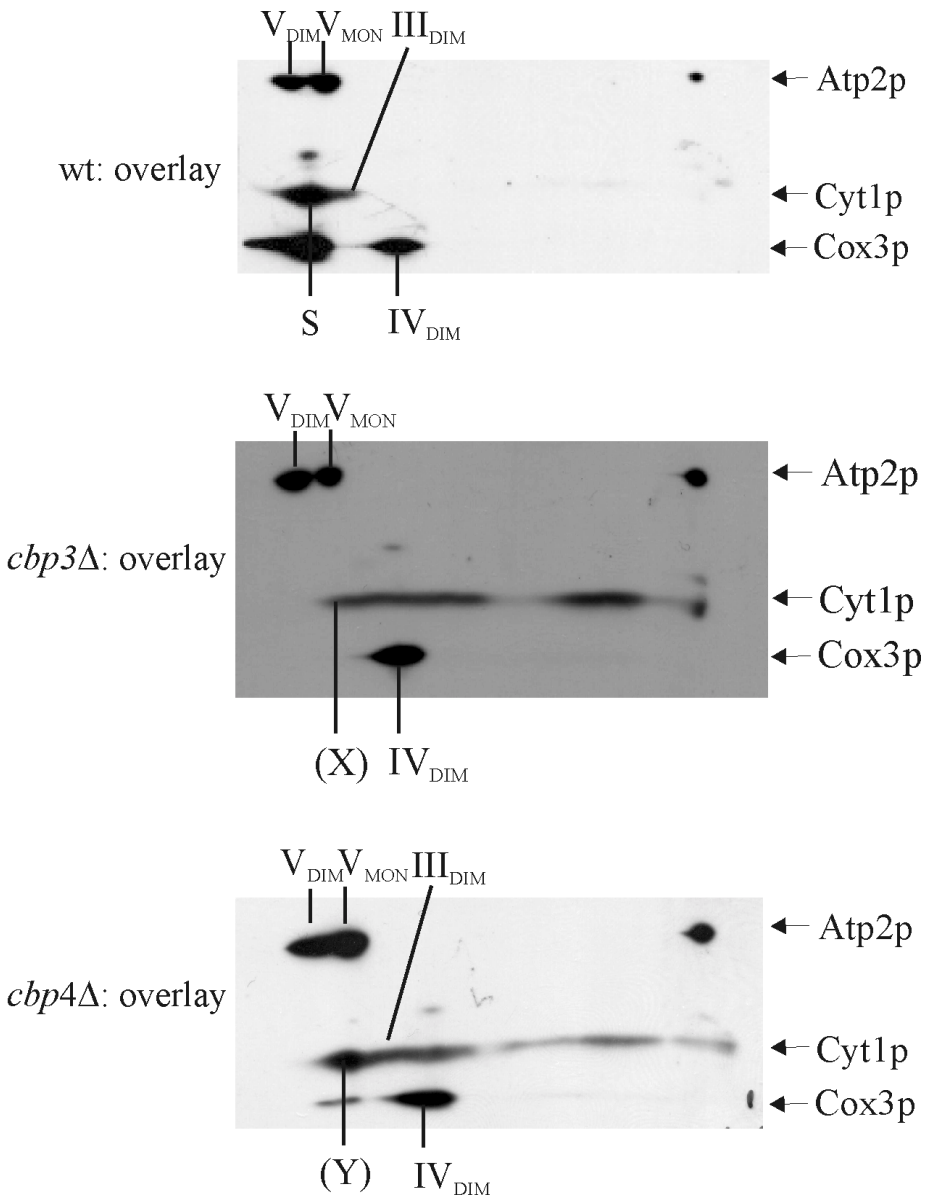

Figure 20. Western blot analysis of respiratory chain complexes. Mitochondrial fractions of wild type (wt) and the indicated mutant strains were separated by BN-PAGE in a 8-15 % gradient gel and in the second dimension on a 10 % SDS-gel. Antibodies directed against cytochrome c_1, Cox3p and Atp2p were used for subsequent detection. Overlays of the resulting films are shown. (V_{DIM}- dimer of complex V, V_{MON}- monomer of complex V, IV_{DIM}- dimer of complex IV, III_{DIM}- dimer of complex III, S- supracomplex of III_{DIM} in combination with IV_{MON} or IV_{DIM}, X-complex III precomplex, Y- supracomplex of complex III and IV, details see text)

Together these results show that deletion of *CBP3* or *CBP4* result in a disturbance of complex III assembly which is associated – especially in the *cbp3*Δ mutant - with the accumulation of similar low molecular weight complexes. At the same time BN-PAGE revealed a clear difference between *cbp3*Δ and *cbp4*Δ mutant strains in the form of a putative COX-positive supracomplex which is exclusively detected in the *cbp4*Δ mutant.

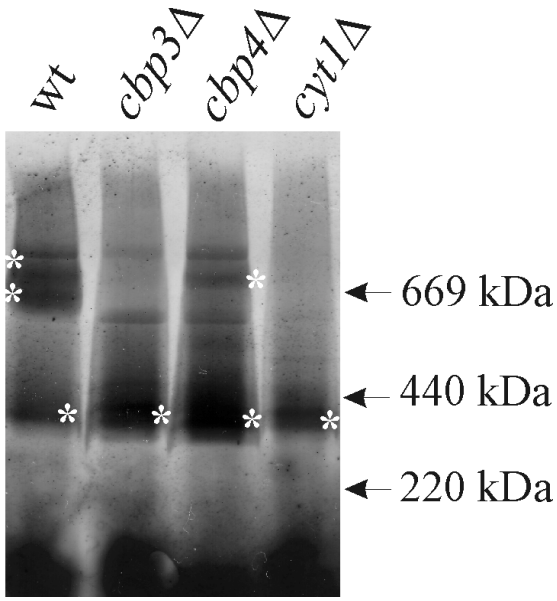

Figure 21. *In-gel* activity of cytochrome *c* oxidase. Mitochondria from the indicated strains were solubilized with digitonin on ice and loaded onto a 5–13 % native gradient gel. *In-gel* activity assay for COX was performed as described in Materials and Methods. Protein bands exhibiting COX activity are marked by stars. Other visible bands result from Coomassie blue staining.

3.7. Analysis of complex III assembly

The process of bc_1 complex assembly is not fully understood. As outlined in the introduction, probably three sub-complexes are formed before the enzyme is fully assembled.

cbp3 and *cbp4* null mutant strains accumulate different intermediate-sized bc_1-complexes, as outlined above. Because of the lack of antibodies recognising subunits of complex III we were not able to determine the protein composition of these complexes. Therefore at least one subunit from each of the proposed bc_1-subcomplexes was tagged with a high-affinity epitope and expressed from its natural chromosomal position in wild type, *cbp4*- and *cbp3*- null mutant strains (see section 2.1.5.2). Although it is clear that Cbp3p and Cbp4p are assembly factors of the bc_1 complex, it remains to be elucidated in which step of the bc_1 complex assembly they act. Two methods have been chosen to study the assembly of the bc_1 complex,

the Blue Native PAGE (BN-PAGE) and Tandem affinity Purification methods (TAP). Mitochondria isolated from respective strains were solubilized with digitonin, complexes analysed by BN-PAGE and protein interaction were studied with TAP method.

3.7.1. BN-PAGE analysis

According to the model of bc_1 comlex assembly (see introduction) three subcomplexes are assembled into a functional enzyme. I have already analysed the relationship of Cbp3p and Cbp4p with one of these subcomplexes, the cytc_1-subcomplex. BN-PAGE analysis of strain ZK1B (Fig. 12) revealed that the signals of Cbp3p and Cbp4p are overlapping with the signal for Cyt1p; co-IP analysis showed that both assembly factors interact with Cyt1p (Fig. 15). The strains expressing Cor1p-Tap were prepared to analyse the relationship of the two assembly factors to Cor1p, a subunit of the core subcomplex. Proteins from digitonin-solubilized mitochondria of wt-, ZK3, *cbp3*- and *cbp4*- null mutant strains expressing Cor1p-Tap were separated under native conditions (BN-PAGE), followed by separation under denaturing conditions (SDS-PAGE) in the second dimension. In wild type strain, the Cor1p-Tap was exclusively located in the 3 different complexes in the range of ~700 - 1MDa corresponding to different supracomplexes (Fig. 22A). This means that the TAP-tag Cor1p is functional and does not interfere with complex III assembly. In strain ZK3 we see the same organization of Cor1p except for an additional signal in the lower molecular weight range that could represent assembly intermediate(s). As mentioned earlier, in the *Δcbp4* strain we were able to detect a high molecular weight complex with anti-Cyt1p antibody, corresponding to the bc_1-supracomplex. A complex of the same molecular weight was detected also with Cor1p-Tap antibody (Fig. 22B). Surprising results were obtained with the *Δcbp3* strain expressing Tap-tagged Cor1p. I was able to detect Cor1p-Tap in the SDS-PAGE, but never in the BN-PAGE suggesting that Cor1p is present in the mitochondria, but not assembled into a complex. Interestingly, detection with the Cyt1p antibody in all strains except for the Δ*cbp3*-Cor1p-Tap strain reveals 3 main spots in the lower molecular weight range that probably represent subcomplexes of bc_1 complex. Cyt1p was also detected in a high molecular weight region where it colocalizes with the Cor1p-Tap respresenting assembled (supra)-complexes. In the Δ*cbp4* strain the signals in the lower molecular weight were increased, probably due to accumulation of pre-assembled complexes or due to degradation of non-assembled enzyme. As shown earlier (Fig. 20) in the Δ*cbp3* strain, the bc_1 complex is not assembled and different pre-assembled or degraded complexes in the low molecular weight range are accumulating.

(Fig. 22B). These data also suggest that the effect of *CBP3* deletion on the bc_1 complex assembly is more severe than the deletion of *CBP4*.

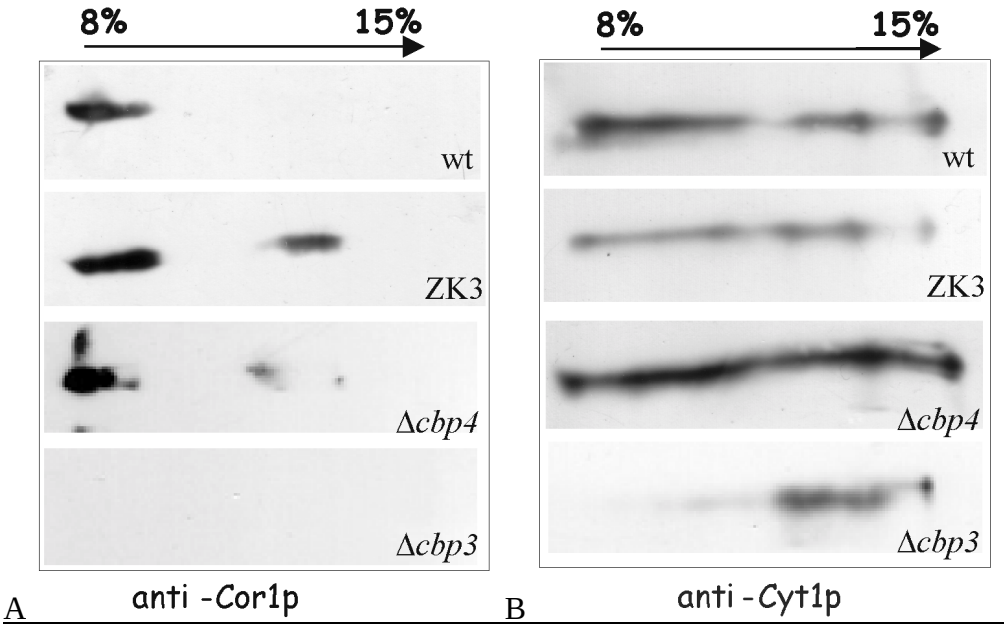

Figure 22. Assembly of Cor1p and Cyt1p into bc_1-complex. Assembly of Cor1p-subcomplexes and Cyt1p-subcomplexes were monitored in the wild type (wt), ZK3, Δ*cbp3* and Δ*cbp4* strains. Solubilized mitochondrial complexes were separated by BN-PAGE in a 8-15 % gradient gel and subsequently in the second dimension on a 10 % SDS-gel. Antibodies directed against TAP-tag (A, Cor1p-Tap) and cytochrome c_1 (B, Cyt1p) were used for detection. The results suggest that deletion of *CBP3*, but not *CBP4* has a negative effect on Cor1p stability and/or assembly.

Organization of Cbp3p and Cbp4p compared to Cor1p and Cyt1p is shown in Fig. 23. Cor1p is assembled into 3 distinct supracomplexes and into additional low molecular weight complexes. However, distribution of Cbp3p and Cbp4p is different compared to Cor1p, but presence of both assembly factors in the complexes containing Cor1p is detected.

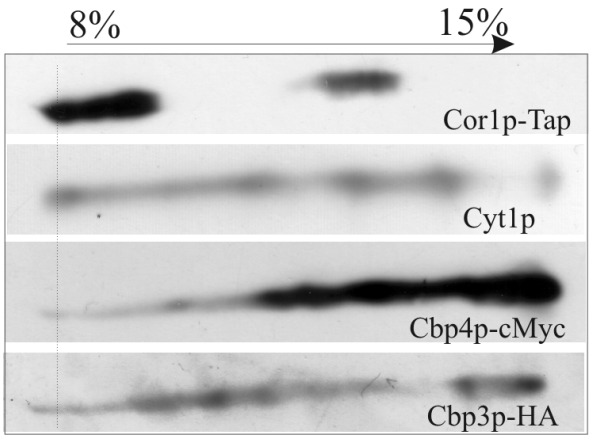

Figure 23. Organization of Cbp3p-HA and Cbp4p-cMyc compared to Cor1p and Cyt1p. Solubilized mitochondrial complexes of strain ZK3 were separated by BN-PAGE in a 8-15 % gradient gel and subsequently in the second dimension on a 10 % SDS-gel. Antibodies directed against TAP-tag (Cor1p-Tap), cytochrome c_1 (Cyt1p), cMyc (Cbp4p-cMyc) and HA (Cbp3-HA) were used for detection. Cbp3p-HA and Cbp4p-cMyc are present in complexes of similar molecular weight, some overlapping with positions of Cor1p and Cyt1p. Signals of both assembly factors can be followed to molecular weight corresponding to supracomplexes (shown by dotted-line).

Qcr7p and Qcr8p are subunits of the third proposed subcomplex that we call cyt*b*-subcomplex. These two proteins were TAP-tagged (Ghaemmaghami *et al.*, 2003) in the wild type strain (see section 2.1.5.2). Unfortunately, Δ*cbp3* and Δ*cbp4* strains expressing HA- or cMyc-tagged version of Qcr7p or Qcr8p could not be obtained. Wild type strains expressing TAP-tagged versions of Qcr7p and Qcr8p are viable and grow on non-fermentable carbon source. BN-PAGE analysis of strains expressing Qcr7p-Tap or Qcr8p-Tap reveals that the Tap-tag does not affect the assembly of cyt*b*-subcomplex. Detection of Rip1p and Cyt1p manifests that the bc_1 complex is assembled. Formation of supracomplexes was checked with detection of Cox3p. Cox3p is assembled in complex IV dimer and in the supracomplexes consisting of complex III/IV (Fig. 24 and 25). This suggests that formation of supracomplexes is not disturbed. Organization of Cbp3p-cMyc and Cbp4p-HA was not influenced by TAP-tagged Qcr7p and Qcr8p (Fig. 26). Both assembly factors were detected in the same positions as shown earlier (Fig. 12 and Fig. 23). Both assembly factors are present in complexes of different molecular weight, including positions corresponding to high molecular weight complexes.

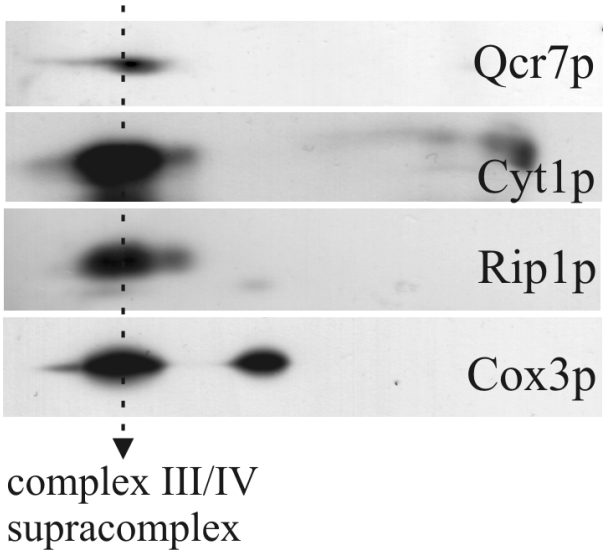

Figure 24. Assembly of Qcr7p and Cyt1p into complex III. Assembly of Qcr7p and Cyt1p into complexes was monitored in the wild type strain expressing Tap-tagged Qcr7p. Solubilized mitochondrial complexes were separated by BN-PAGE in a 8-15 % gradient gel and subsequently in the second dimension on a 10 % SDS-gel. Antibodies directed against TAP-tag (Qcr7p-Tap) and cytochrome c_1 (Cyt1p), Rieske Fe-S (Rip1p) and Cox3p were used for detection.

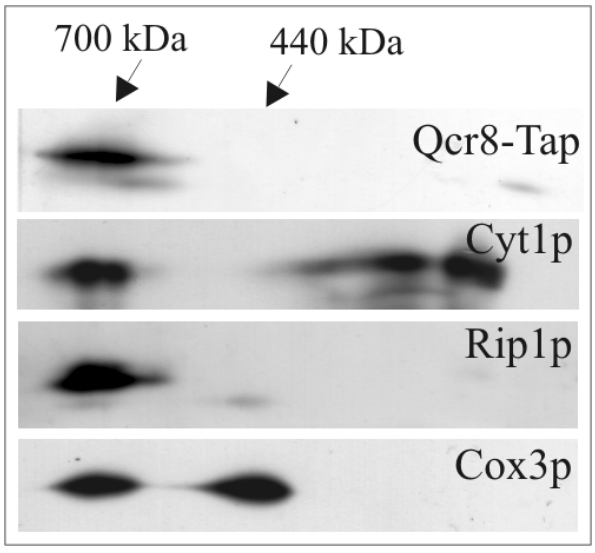

Figure 25. Assembly of Qcr8p and Cyt1p into complex III. Assembly of Qcr8p and Cyt1p into complexes was monitored in the wild type strain expressing Tap-tagged Qcr8p. Solubilized mitochondrial complexes were separated by BN-PAGE on a 8-15 % gradient gel and subsequently in the second dimension on a 10 % SDS-gel. Antibodies directed against TAP-tag (Qcr8p-Tap) and cytochrome c_1 (Cyt1p), Rieske Fe-S (Rip1p) and Cox3p were used for detection.

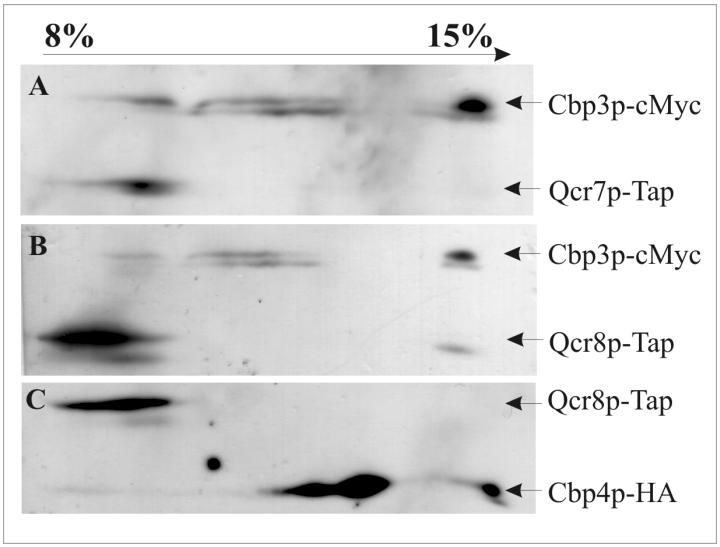

Figure 26. Neither Qcr7p-Tap nor Qcr8p-Tap affects the organization of Cbp3p or Cbp4p. Solubilized mitochondrial complexes of three different strains (A, B, C) were separated by BN-PAGE on a 8-15 % gradient gel and subsequently in the second dimension on a 10 % SDS-gel. Antibodies directed against TAP-tag (Qcr8p-Tap or Qcr7p-Tap), cMyc (Cbp3p-cMyc) and HA (Cbp4p-HA) were used for detection. (all strains are wild type strains; strains A and B express Qcr7p-Tap and Qcr8p-Tap, respectively, from gDNA and Cbp3p-cMyc from a plasmid-born gene; strain C expresses both Qcr8p-Tap and Cbp4p-HA from the gDNA, g = genomic).

3.7.2. TAP purification

By using the TAP purification method, interactions of Cbp3p and Cbp4p proteins with the subunits of bc_1 complex were studied. Interaction of both, Cbp3p and Cbp4p with Cyt1p was observed by co-IP (Fig. 15). The strains ZK3 (Cor1-Tap, Cbp3-HA, Cbp4-cMyc), Δ*cbp4*-Cor1-Tap and Δ*cbp3*-Cor1-Tap were used to study the interactions of Cor1p with other subunits of the bc_1 complex and to analyse the relationship of the two assembly factors to Cor1p.

Cytochrome c_1 was precipitated in the final eluate via Cor1p-Tap in the strains ZK3 and Δ*cbp4*-Cor1-Tap, but increased degradation of Cyt1p was observed in the latter strain. In the Δ*cbp3*-Cor1-Tap strain a strong degradation of Cor1p-Tap and no interaction with cytochrome c_1 was observed. The interaction between Cor1p and Cbp4p-cMyc could not be detected. Cbp3p-HA was not detected in the final eluate, but in the eluate from calmodulin beads (CaB). In the further purification with Immunoglobuline G (IgG) beads the signal of Cbp3p-HA was no longer detectable (data not shown).

In the next step, the interactions of Qcr7p-Tap and Qcr8p-Tap with Cbp3p-cMyc and Cbp4p-HA assembly factors was analyzed. I was able to precipitate cytochrome c_1 with both Qcr7p-Tap and Qcr8p-Tap. Interaction of Cbp3p-cMyc with Qcr7p-Tap or Qcr8p-Tap was not

detected. Cbp4p-HA was purified with Qcr8p-Tap to IgG beads, but the signal in the further purification was very weak and not detectable in the final eluate. The interactions of bc_1 complex subunits and of both assembly factors are summarized in the Table 5.

TAP purification with TAP-tagged:	**Purified proteins:**		
	Cyt1p	**Cbp3p**	**Cbp4p**
Cor1p-Tap in wt	✓	✓	×
Cor1p-Tap in *Δcbp3*	×		
Cor1p-Tap in *Δcbp4*	✓		
Qcr7p-Tap in wt	✓	×	×
Qcr8p-Tap in wt	✓	×	✓

Table 5. Summary of TAP-purification. Subunits of bc_1 complex, namely Cor1p, Qcr7p and Qcr8p were TAP-tagged and used for purification. Interactions of these subunits with Cyt1p and with assembly factors Cbp3p and Cbp4p were monitored. Purification of the protein with the respective TAP-tagged subunit is shown by ✓. No detected interaction is indicated by ×.

3.8. Cbp3p and Cbp4p are presumably not involved in mitochondrial lipid metabolism

Phospholipids are essential for the activity of several membrane proteins (Dowhan, 1997), including complex III (Schägger *et al*., 1990). A characteristic lipid of the inner mitochondrial membrane is cardiolipin, which has been shown essential for complex III activity (Gomez and Robinson, 1999). Cardiolipin is associated with Cobp and is required for the formation of complexes III/IV supracomplexes (Zhang *et al.*, 2002). A role of other phospholipids such as phosphatidylethanolamin (PE) or phosphatidylinositol (PI) in stabilization of bc_1 complex has been proposed (Lange *et al.*, 2001). As supracomplex formation seems to be affected in *cbp3*Δ and *cbp4*Δ mutant strains (see Fig. 20 and 21), we analysed the lipid composition of the deletion strains and compared it with that of the respective transformants bearing the wild type genes on a plasmid, and of the wild type strain. To exclude secondary effects due to respiratory deficiency, we also included the respiratory deficient *cyt1*Δ strain in the analysis. Mitochondrial preparations of all strains were used for lipid extraction and the lipids were separated by TLC as described in Material and Methods. As shown in Fig. 27, the concentrations of CL and of the other lipids do not differ significantly between the strains.

To exclude the possibility that Cbp3p or Cbp4p act in cardiolipin biosynthesis we analysed complex III and IV assembly of *crd1*Δ and *taz1*Δ mutants by BN-PAGE. *CRD1* is a structural gene encoding cardiolipin synthase. The *crd1*Δ strain possesses no detectable CL and exhibits a growth defect in galactose and in non-fermentable carbon sources (Zhong *et al.*, 2004). The *TAZ1* gene, which encodes a putative acyltransferase, is required for the normal phospholipid content of mitochondrial membranes. Similar to human tafazzin, which is implicated in Barth syndrome (Gu *et al.*, 2004), Taz1p may remodel the acyl groups of cardiolipin in the inner mitochondrial membrane. If Cbp3p or Cbp4p would be involved in CL biosynthesis or its remodeling, *cbp3*Δ and *cbp4*Δ strains should exhibit a phenotype similar to that of *crd1*Δ and *taz1*Δ mutants. BN-PAGE analysis revealed that both the *crd1*Δ and *taz1*Δ mutant strain exhibit a fully assembled complex III (detected with Cyt1p antibody) (Fig. 28). Detection with Cox3p antibody confirmed that CL is required for supracomplex formation as complex IV is present only in the dimeric form (~ 400 kDa) in *crd1*Δ (no CL is present) compared to *taz1*Δ mutant (CL is present), where complex III/IV supracomplexes are formed (Fig. 28). The pronounced impairment of complex III formation and the presence of CL in *cbp3*Δ strain, however, argues against an involvement of Cbp3p in CL synthesis or remodeling. In the case of *cbp4*Δ mutant strain, where we can observe supracomplex formation as in the *taz1Δ* mutant, an involvement of Cbp4p in CL remodeling cannot be completely excluded. However, the clear effect of *CBP4* deletion on complex III formation as well as the association of Cbp4p with Cbp3p favours the idea that it acts as a specific complex III assembly factor.

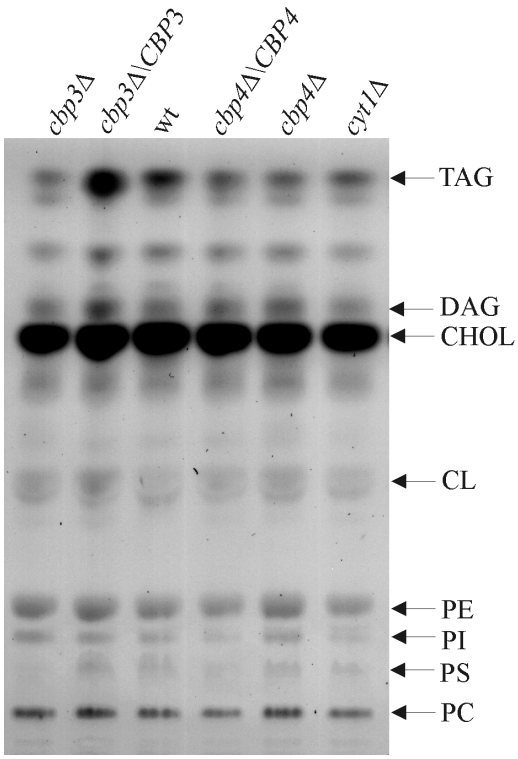

Figure 27. Lipid composition. Lipids were extracted from mitochondria of the indicated strains and analysed by Thin Layer Chromatography (TLC) as described in Material and Methods. (PC-phosphatidylcholin, PS-phosphatidylserin, PI-phosphatidylinositol, PE-phosphatidylethanolamin, CL-cardiolipin, CHOL-cholesterol, DAG-diacylglycerol, TAG-triacylglycerol).

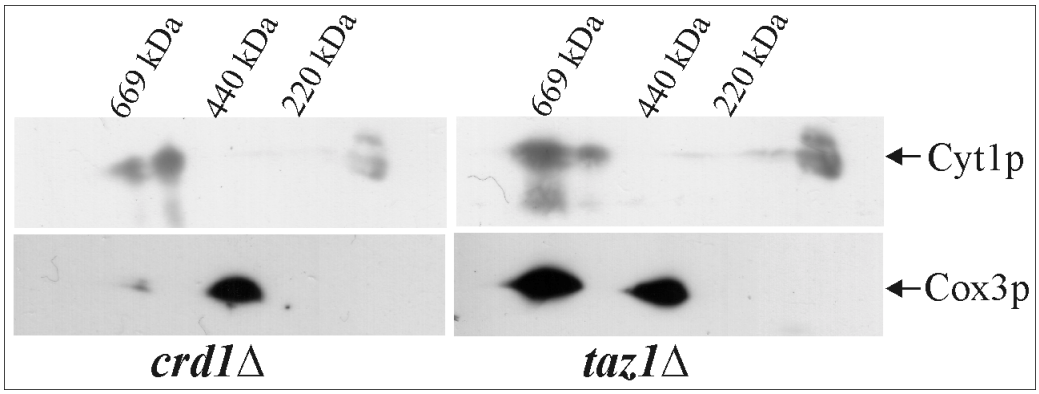

Figure 28. Western blot analysis of respiratory chain complexes in *crd1*Δ and *taz1*Δ strains. Mitochondrial fractions of the indicated mutant strains were separated by BN-PAGE in a 8-15 % gradient gel and in the second dimension on a 10 % SDS-gel. Antibodies directed against cytochrome c_1 and Cox3p were used for detection.

4. DISCUSSION

Cbp3p of *Saccharomyces cerevisiae* (*Sc*Cbp3p) is a member of an evolutionary conserved protein family with homologues identified in many organisms. In this study we have confirmed that the Cbp3p proteins of *S. cerevisiae, S. pombe* and human are functional homologues. We demonstrated that full length *Sp*Cbp3 and chimeras of human and *S. cerevisiae* proteins are able to complement the deletion of the *CBP3* gene in baker´s yeast. The full length *Hs*Cbp3p was not complementing the *CBP3* deletion in spite of the high sequence homology. However, it was expected because the human protein lacking more than 50 amino acid residues in the N-terminal part as can be seen from sequence comparison (Fig. 8) was used. This short form of h-CBP3 failed to be imported even to human mitochondria (V. Petruzzella, personal communication), suggesting that its import to the yeast mitochondria is as well disturbed. Therefore chimeric human/*S. cerevisiae* proteins were constructed that contain the N-terminal part derived from yeast, the central most conserved region from the human protein and C-terminal part (that is lacking in human protein) also derived from yeast. Surprisingly, chimeras HC1 and HC2 containing a large central part of human gene were not complementing the *CBP3* deletion in *S. cerevisiae* suggesting that the most conserved regions are not sufficient for the protein function. Therefore three other chimeras were constructed where the central conserved part was divided into two halves. All three chimeras (HC3, HC4, HC5) were complementing the *CBP3* deletion in *S. cerevisiae*. Why can the central region when in total not complement for *Sc*Cbp3p function, but can when divided into two parts? Thorough inspection of protein sequence comparison revealed significant differences between yeast (both *S. cerevisiae* and *S. pombe*) and human proteins in the regions between amino acid residues 170-194 and 207-224. While *S. cerevisiae* and *S. pombe* share significant homology, human protein differs almost in all amino acids within these regions. This fact may explain why even a full length *Sp*Cbp3p can partially complement *cbp3* deletion in *S. cerevisiae*, but not human full length Cbp3p and chimeras HC1 and HC2, which lack the mentioned regions. The importance of residues in these regions is also supported by study of Shi *et al.* (2001) where the authors showed that the mutation of Gly223 influenced Cbp3p functional activity and affected assembly of the 14 kDa subunit (Qcr7p) of bc_1 complex. Additionally, they showed that mutations in the region between Leu167 to Pro175 altered the association of Cbp3p with the mitochondrial membrane resulting in enhanced protein turnover. All the mentioned residues are not present in human homologue and therefore only chimeras containing at least one half of central region from *S. cerevisiae* and the second from human

(HC3, HC4, HC5) can complement for the *Sc*Cbp3p function, albeit only in a temperature sensitive way. Shi *et al.* (2001) showed that regions Lys124-Ala140, Leu167-Pro175 and Gly223-Asp229 are important for Cbp3p function or stability. Our studies confirm that these regions belonging to the most conserved part seem to be important for the function of *S. pombe* and human genes and extend functionally important regions of *Sc*Cbp3p to residues 170-194 and 207-224.

4.1. Organization of Cbp3p and Cbp4p

Both Cbp3p and Cbp4p are mitochondrial proteins associated with or anchored in the membrane, respectively. In this study we have treated mitochondria and mitoplasts with proteinase K in order to determine the location and topology of both proteins. PK analysis shows that Cbp3p C-terminus is protected against degradation in mitochondria. Slight decrease of the signal in mitoplasts after addition of PK is probably due to disrupted mitoplasts when a small portion of Cbp3p became accessible to proteinase K. Importantly, the signal intensity of Cbp3p remains stable with further increase of PK concentration suggesting the C-terminus being located on the matrix side of inner mitochondrial membrane. Based on a) the fact that upon treatment with PK we see no shift of Cbp3p during separation on SDS-PAGE, b) the results of alkaline extraction suggesting its membrane association (Shi *et al.* 2001) and c) the principle of protein import to mitochondria we assume that also the N-terminus of Cbp3p faces the matrix side of mitochondria.

A different behavior is detected for Cbp4p. Cbp4p seems to be extremely sensitive to proteinase K and we can see its partial degradation already in PK-treated mitochondria, suggesting disturbances of the outer mitochondrial membrane (Cbp3p is also degraded in mitochondria treated with the highest PK concentration). Additionally, other experiment in which mitochondria were treated with high concentration of PK (5 μg/μg) for one hour and sample was taken every 10 min. (data not shown) confirmed that Cbp4p is protected in mitochondria, as shown by Crivellone (1994). Alkaline extraction shows that Cbp4p seems to be integral membrane protein (Crivellone, 1994) with the C-terminus facing the inner membrane space as has been shown in this study by PK assay.

Even though these data show different topologies of Cbp3 and Cbp4p we have shown by Co-IP (Fig. 13) that Cbp3p and Cbp4p interact. Presently we don´t know whether this interaction is direct or mediated by so far unknown components, *e.g.* subunits of precomplexes, which could also explain the presence of both proteins in common high molecular weight

complexes. This possibility is supported by a similar distribution of both Cbp3p and Cbp4p with Cyt1p (Fig. 12) and the fact that Cyt1p was precipitated with both Cbp3p and Cbp4p (Fig. 13). Alternatively, these high molecular weight complexes may correspond to heteromeric structures as reported for prohibitins (Back *et al.*, 2002; Nijtmans *et al.*, 2002a). To test this hypothesis the homomer formation of Cbp3p and Cbp4p, respectively was analysed by BN-PAGE and Co-IP in the diploid strains ZK4 and ZK5. Cbp4p-HA and -cMyc were concomitantly expressed in ZK4 strain and detected at the same positions in BN-PAGE. This observation suggests that both tagged versions of Cbp4p are functional and either assembled into the same complexes or interact with the same proteins. To confirm or exclude the formation of Cbp4p homodimers a co-IP was performed. The results of co-IP give no indication for Cbp4p to form homodimers, because Cbp4p-HA could not be purified via antibodies directed to Cbp4p-cMyc and *vice versa*. Importantly, co-IP analysis showed strong interaction of Cbp4p with Cyt1p that was already indicated by BN-PAGE where Cbp4p and Cyt1p had similar distribution. This fact can also explain the presence of a very weak signal of Cbp4p-HA precipitated with Cbp4p-cMyc antibodies and *vice versa*. All latter data support the interpretation that Cbp4p does not form homodimers. Surprisingly, Cox3p a subunit of complex IV that assembles with bc_1 complex to a supracomplex was precipitated with Cbp4p if cross-linker was present. This suggests that Cbp4p could be cross-linked to the supracomplex consisting of complex III and complex IV suggesting a role of Cbp4p not only in complex III assembly but also in formation of supracomplexes. The exact role of Cbp4p in supracomplex formation is not clear because tiny amounts of supracomplex in Δ*cbp4* strain is observed (Fig. 20), however complex IV is preferentially detected in the dimeric form. Therefore the presence or the interaction of both Cbp3p and Cbp4p may be essential for supracomplex formation. The observation of a high molecular weight structure ("A" in Fig. 12 and Fig. 16), which yields signals for Cbp3p may hint at the possibility that the presence of these two proteins may be important for supracomplex formation.

BN-PAGE analysis of ZK5 strain suggests that Cbp3p may form homodimers (Fig. 16), but this possibility could not be tested, because of the disturbed functionality of HA tagged Cbp3p. The co-IP analysis of mitochondrial lysate of ZK5 strain did not reveal any interaction between Cbp3p molecules. Therefore, another approach, the TAP method was used to prove formation of Cbp3p homodimers. Cbp3p was Tap-tagged and diploid strain (ZK5-B) expressing Cbp3p-Tap and Cbp3-cMyc was constracted. BN-PAGE analysis of mitochondrial complexes of strain ZK5-B again confirmed the presence of Cbp3p in high molecular weight complexes in the first dimension and a shift in molecular weight of Cbp3p in the second

dimension. Signals of both Tap- and cMyc-tagged Cbp3p overlapped at the position of ~100 kDa, which could correspond to molecular weight of a Cbp3-cMyc/Cbp3-Tap dimer. However, it is difficult to confirm the formation of Cbp3p homodimers, even though Cbp3p-cMyc was purified with Cbp3p-Tap in the first step of TAP- purification. If Cbp3p would form homodimers, then three different forms of dimers are expected in BN-PAGE, as summarized in Table 6. The differences in molecular weight between the respective putative dimers are about 8 and 16 kDa. These differences may be too small, to be resolved in 10% SDS-PAGE used in the second dimension. Additionally, only a very weak signal of Tap-tagged Cbp3p was obtained, hinting at the possibility, that the epitope for anti-Tap antibody is partially hidden. Therefore, we can not conclude that Cbp3p is able to form dimers, but we can not exclude this possibility. A convenient method to prove protein dimerization would be FPLC (fast protein liquid chromatography) that was also used to study the role of oligomerization of subunit III of complex IV (Finel and Wikstrom, 1986). An alternative approach to study Cbp3p dimerization would be to combine BN-PAGE for separation of Cbp3p complexes and mass spectrometry (e. g. MALDI-TOFF) for identification of proteins present in the analysed complex. Such an approach has been used to study prohibitin oligomerization (Back *et al*., 2002).

Dimer	**Molecular weight**
Cbp3p-**Tap**/ Cbp3p-**Tap**	~116 kDa
Cbp3p-**Tap**/ Cbp3p-**cMyc**	~108 kDa
Cbp3p-**cMyc**/ Cbp3p-**cMyc**	~100 kDa

Table 6 Putative dimers formation. Diploid strain ZK5-B expressing two differently tagged Cbp3 proteins, Cbp3p-Tap and Cbp3p-cMyc was used to study Cbp3p dimerization. In case of dimerization three possible dimers are expected to be formed with respective molecular weights.

Surprisingly, the interaction of Cbp3p-HA or -cMyc tagged with Cyt1p was detected (data not shown). Is the interaction observed for Cbp3p and Cbp4p direct or mediated via Cyt1p if both proteins interact with Cyt1p? If the interaction of Cbp3p and Cbp4p would be mediated via Cyt1p then Cbp4p would be precipitated also with Cbp3p-HA that is still able to bind to Cyt1p. Probably the HA-tag influences the interaction of Cbp3p with Cbp4p and this causes its partial non-functionality. All these data suggest that interaction of Cbp3p with Cbp4p is rather direct or mediated through other subunits of bc_1 complex than Cyt1p.

Interestingly, two protein variants of Cbp3p appear in the Western blot analysis of second dimension of BN-PAGE. We have discussed earlier that one of the two variants of Cbp3p with different electrophoretic mobility may represent a precursor form. However, this interpretation does not seem to be probable, because both forms are assembled into complexes of high molecular weight. The second form could raise from translation initiated on an additional AUG codon. Therefore, I have analysed the sequence of *ScCBP3* and additional ATG codons were found downstream of the first ATG codon. However, initiation of translation on these additional codons seems to be not probable, because of the presence of not only one, but the two ATG codons at the 5`end of the *CBP*3 coding region. Interestigly, two different cDNA clones for *Hs-CBP*3 were identified. Based on the sequencing data, the two isoforms of human Cbp3 (short form and the long form) show differences of the N-termini of the translation products, presumably due to alternative splicing of the primary transcript. The long form of human Cbp3 protein shows mitochondrial localization. By contrast, the short form of h-CBP3 failed to be imported into mitochondria (V. Petruzzella, personal communication). It is not probable that yeast Cbp3p exists in the two different splicing forms, because yeast *CBP*3 does not contain any introns and no reports of alternative splicing in yeast was published up to now. The evaluation of this possibility is a challenge for further investigation.

4.2. Characterization of deletion strains and effect of Cbp3p and Cbp4p overexpression

The respiratory deficient *cbp3*Δ and *cbp4*Δ strains lack ubiquinol-cytochrome *c* reductase activity and show reduced steady-state levels of some complex III subunits including Cobp and Rip1p. Based on these findings it was proposed that Cbp3p and Cbp4p act as chaperones in the formation of complex III (Wu and Tzagoloff, 1989; Crivellone, 1994).

In this study we provide experimental evidence that Cbp3p and Cbp4p are indeed complex III assembly factors. We used BN-PAGE to characterise the mitochondrial respiratory complexes in both mutants. Complex III assembly was followed with antibodies against Cyt1p, the subunit which seems to be least influenced by the absence of other subunits of the complex (Zara *et al.*, 2004). Unlike in wild type cells, in *cbp3*Δ and *cbp4*Δ mutant strains Cyt1p is not detected in distinct spots, but rather in the form of long drawn-out bands covering a wide molecular weight range up to about 700 kDa (Fig. 20). Although this size corresponds to the monomeric and dimeric form of the bc_1 complex, complex II/III activity was undetectable. Therefore these complexes must represent either unproperly assembled complex III or aggregates of accumulated subunits or pre-complexes. The failure to detect Cyt1p in distinct

spots may hint at disturbances in the process of complex III assembly leading to the accumulation of various intermediate-sized complexes which are prone for degradation. The similar distribution of Cyt1p in both mutants strengthens the idea that the two proteins are engaged in the same step(s) in the formation of complex III. However, there is one striking difference between the two mutants: in the *cbp4*Δ mutant a high molecular weight complex ("Y" in Fig. 20) can be detected, which is absent in the *cbp3*Δ mutant. Detection of a complex with identical size by Cox3p antibody strongly suggests that it corresponds to a complex III/IV supracomplex. This complex possesses no detectable ubiquinol-cytochrome *c* reductase activity (Table 4), but a substantial COX-activity in the *in-gel* assay (Fig. 21). According to these data Cbp3p is required for supracomplex formation, whereas the role of Cbp4p in the association of complex III and IV is less clear. In line with this conclusion Cbp3p can be detected in high molecular weight complexes ("A" and "B" in Fig. 12), which are likely to represent complex III/IV supracomplexes. However, Cbp4p is also detectable in "B", albeit at a low concentration, suggesting that it is also associated with some forms of supracomplexes.

What is the molecular basis of the observed complex III assembly deficiency in the *cbp3*Δ and *cbp4*Δ mutants? Inspection of the data provided by Wu and Tzagoloff (1989) show that the *cbp3*Δ mutant has reduced steady state concentrations of Qcr7p ("core 4"), Qcr8p ("core 5"), Rip1p and Cobp, whereby the levels of Qcr7p and Cobp are more severly reduced compared to Qcr8p. Analysis of complex III subunits in the *cbp4*Δ mutant by Crivellone (1994) revealed a similar pattern except that the concentration of Qcr7p is significantly higher than that of Qcr8p. Therefore in both mutants formation of the subcomplex consisting of Qcr7p, Qcr8p and Cobp is impaired. Possibly Cbp3p is important for the incorporation of Qcr7p, and Cbp4p for the assembly of Qcr8p into the Cobp subcomplex. The membrane-embedded Cobp subcomplex can be regarded as the key structure of complex III assembly. The crystal structure of the yeast bc_1 complex (Lange and Hunte, 2002) revealed that Qcr7p is localised between Cyt1p and Cor1p, which are both components of the other two known subcomplexes of complex III. Qcr7p plays not only an important role in the stability of the Cobp subcomplex (Lee *et al.*, 2001), but is also important for the assembly of the other subcomplexes. The higher steady state concentration of Qcr7p in the *cbp4*Δ mutant compared to the *cbp3*Δ mutant may explain why in the *cbp4*Δ mutant Cyt1p is present in a higher concentration in complexes corresponding in size to assembled complex III (Fig. 20). Cruciat *et al*. (2000) reported that the presence of complex III is required for the formation of supracomplexes. As formation of a supracomplex was also observed in a *qcr6*Δ mutant, which exhibits a 50 % reduction in complex III activity (Schmitt and Trumpower, 1990), the

presence of a wild type complex III seems not to be an essential prerequisite for supracomplex formation. In the case of the *cbp4*Δ mutant, an enzymatically inactive complex of the size of the wild type complex III is formed, suggesting that either the subunit composition or the three-dimensional structure is altered. Obviously this complex can be assembled into a supracomplex. The observation that the *cbp3*Δ mutant seems not to be able to form a supracomplex may reflect differences in the concentration or in the composition of a comparable inactive complex.

The finding that respiratory growth of a *cbp4*Δ strain overexpressing Cbp3p from a multicopy plasmid under the control of a strong promoter (p426GPD) is slightly improved may be explained by a stabilizing effect of Cbp3p on Qcr7p, which - as discussed above - seems to be a key component of Cobp subcomplex formation. Perhaps the availability of a higher concentration of Qcr7p allows the formation of the Cobp subcomplex even in the absence of Cbp4p.

4.3. Cbp3p and Cbp4p are presumably not involved in mitochondrial lipid metabolism

The effect of *cbp3*Δ and *cbp4*Δ mutations on complex III formation may also be explained by participation of Cbp3p and Cbp4p in mitochondrial lipid metabolism. A correct lipid environment is important for the stability and function of respiratory enzymes (Dowhan *et al*., 1997). Formation of supracomplexes between complexes III and IV has been shown to be dependent on cardiolipin, a characteristic lipid of the inner mitochondrial membrane (Zhang *et al.,* 2002). Disturbances in the lipid composition of the mitochondrial membrane due to the mutations in the *CBP3* and *CBP4* gene could therefore result in the observed impairment of complex III and supracomplex assembly. Our data according to which the mitochondrial lipid composition of *cbp3*Δ and *cbp4*Δ strains shows no apparent differences to the wild type, argue against a role of the two proteins in lipid metabolism. This conclusion is further supported by our studies on mutants with an impaired cardiolipin synthesis (*crd1*Δ) or remodeling (*taz1*Δ). As expected the *crd1*Δ mutant strain is unable to form supracomplexes, in this respect resembling the *cbp3*Δ mutant. However, both the *crd1*Δ and the *taz1*Δ mutant are not affected in complex III assembly (Fig. 28). This result and the results of BN-PAGE analysis of *cbp3*Δ and *cbp4*Δ strains strongly favor the idea that Cbp3p and Cbp4p are assembly factors specific for bc_1 complex.

4.4. Role of Cbp3p and Cbp4p

BN-PAGE analysis of *cbp3*Δ and *cbp4*Δ strains revealed the differences between these deletion strains, but the role of the two assembly factors remains unclear. Because of the lack of antibodies recognising subunits of complex III further insights into bc_1 complex assembly and into the role of the two assembly factors in this process were studied in the strains, in which the subunits from different bc_1 subcomplexes were tagged. In the first step the relationship of Cbp3p and Cbp4p with Cor1p was analysed. The essentiality of Cor1p for complex III assembly and activity is supported by the phenotype of *cor1* mutants having difficulties in converting an apocytochrome *b* to mature cytochrome *b* (Tzagoloff *et al.*, 1986; Gatti and Tzagoloff, 1990). BN-PAGE analysis showed that Cor1p-Tap is functional, transported to mitochondria and assembled into the bc_1 complex. One assembly intermediate of Cor1p was detected in the wt strain where only Cor1p was Tap-tagged and two different intermediates were detected in the ZK3 strain expressing Cbp3p-HA that is partialy non-functional and bc_1-assembly seems to be less efficient. In contrast to Cor1p-subcomplex assembly, the Cyt1p was detected in few subcomplexes in all strains. Both, Cbp3p and Cbp4p are present in many complexes as observed also earlier, they have a similar distribution as Cyt1p, and their signal can be followed up to > 1MDa where it overlaps with the signal for Cor1p-Tap. These data also support the role of Cbp3p and Cbp4p in the supracomplex formation as discussed above. To study the role of both proteins in the bc_1 complex assembly the effect of their deletion on Cor1p-Tap was followed. As expected, the bc_1-complex in *Δcbp4* strain is assembled and Cor1p-Tap could also be detected in the range of supracomplexes. Surprisingly, almost no degradation products of Cor1p are observed, but strong degradation of Cyt1p is observed and the bc_1 complex seems to be non-functional. A different situation is seen in *Δcbp3* strain. Cor1p-Tap is detected in mitochondria on SDS-PAGE (data not shown) but no signal is observed in BN-PAGE, suggesting that Cor1p-Tap is expressed, transported to mitochondria, but obviously not assembled into a complex (Fig. 22). Detection with anti-Cyt1p revealed signals preferentially in the low molecular weight range, suggesting that the c_1-subcomplex is formed and also assembled with some other proteins, but not with Cor1p. This fact represents another significant difference between *cbp3Δ* and *cbp4Δ* strains, suggesting that the decrease of apocytochrome *b* in the respective null mutants may have different reasons, in case of *cbp3Δ* strain the failure of core-subcomplex assembly (Tzagoloff *et al.* 1986). These data hint at the possibility that Cbp3p plays a role in the assembly of the core-subcomplex with the c_1-subcomplex that is probably pre-assembled with

the cyt *b*-subcomplex. These data agree with the assembly model in the existence of three subcomplexes, as proposed earlier (Geier *et al*. 1992), but change the order in which the subcomplexes assemble into a functional enzyme. Here we propose that the cyt*b*-subcomplex is first assembled with c_1-subcomplex and the core-subcomplex is added in the last step (Fig. 29).

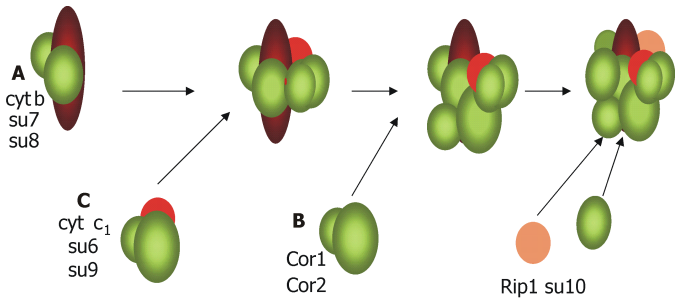

Figure 29. New model for complex III assembly. Three subcomplexes A, B, C are pre-assembled and then associate in this order: A + C = AC + B = ABC. Our data suggest that the assembly of core-subcomplex (B) takes place after cyt*b*- and cyt1-subcomplexes are assembled thus modifying the model of Geier *et al*. (1992).

BN-PAGE analysis suggested that Cbp3p could play a role in the assembly of Cor1p into bc_1 complex. The interactions of Cor1p were monitored by TAP purification method. As expected Cyt1p was purified with Cor1p-Tap in the wild type strain. No purification of Cbp4-cMyc was observed. Cbp3p-HA was detected in the eluate after purifying with CaB-beads, but lost in the further purification with IgG-beads. This result strongly suggests that Cbp3p may interact with Cor1p, but the stability of this interaction is weaked by the influence of HA-tag and/or by the conditions used during the TAP purification procedure. The role of Cbp3p in Cor1p assembly is supported also by the study of stability and assembly of Cor1p in the Δ*cbp3* strain. In this strain Cor1p is not assembled into a complex and undergoes a degradation. This is a striking evidence for Cbp3p acting in Cor1p stability and assembly, as Cor1p belongs to the most stable proteins in the deletion mutants of all structural components of the bc_1 complex (Crivellone *et al.*, 1988). The experiment, where *Δcbp3* strain was transformed with a plasmid expressing Cbp3p-cMyc to confirm the restoration of wild type phenotype is running, but was not completed at the time of writing this thesis. I hope we will be able to confirm the interaction of Cbp3p-cMyc with Cor1p and to demonstrate the role of Cbp3p in Cor1p assembly. Additionally, no interaction of Cor1p with Cyt1p is detected in the *Δcbp3* strain. These data suggest a role of Cbp3p in the step of core-subcomplex insertion into a pre-assembled bc_1 intermediate complex and are in agreement with the data of Wu and Tzagoloff (1989) that Cbp3p functions as chaperone in the late stage of bc_1 complex

assembly. Interaction of Cbp4p with Cor1p was not observed. However, based on these results, the very similar phenotype of both *cbp3*Δ and *cbp4*Δ strains with decreased concentrations of cytochrome *b*, Qcr7p, Qcr8p (Shi *et al.*, 2001; Crivellone, 1994) is surprising but as already suggested may have different causes. Therefore assembly of Qcr7p and Qcr8p was studied. BN-PAGE analysis of strains expressing Qcr7p-Tap or Qcr8p-Tap displays no difficulties in cyt*b*-subcomplex assembly. Both proteins were detected in the high molecular weight range (Fig. 24 and 25). Detection with Rip1p and Cyt1p manifests that the bc_1 complex is assembled, and Qcr7p and Qcr8p are present in the assembled complex as well. The fact, that *cbp3*Δ and *cbp4*Δ transformants expressing Qcr7p-HA or Qcr8p-HA could not be obtained, suggests strong degradation of Qcr7p-HA or Qcr8p-HA in the *cbp3*Δ and *cbp4*Δ strains. Based on the steady state concentraion of bc_1 complex subunits in the *cbp3*Δ and *cbp4*Δ strains (Wu and Tzagoloff, 1989; Crivellone, 1994) we suggested that possibly Cbp3p is important for the incorporation of Qcr7p, and Cbp4p for the assembly of Qcr8p into the Cobp subcomplex (Kronekova and Rodel, 2005). However, we were not able to detect an interaction of Cbp3p with Qcr7p, but our data suggest the interaction of Cbp4p with Qcr8p, but not with Qcr7p. This latter result is in contradiction to Shi *et al*. (2001) who noted the interaction of Cbp4p with both, Qcr7p and Qcr8p.

The main task of this thesis was finding the answer on this question: “What is the role of both Cbp3p and Cbp4p in the bc_1 complex assembly?” Crivellone (1994) mentioned a third model for the role of the assembly factors. She proposed that Cbp3p and Cbp4p can act as scaffolding proteins during the assembly process by stabilizing associations between subunits in partially assembled multipolypeptide complexes. It is conceivable that the assembly proteins may take on more than one of these possible roles. Our data support this hypothesis and suggest multiple roles of these assembly factors in stabilizing protein inteactions between subunits during the assembly into the complex and additonally extend their roles also in supracomplex formation.

Based on all data I propose the hypothesis that Cbp3p and Cbp4p form a “scaffold” for the assembly of all three putative sub-complexes, may act independently in the first steps of bc_1 complex assembly (e. g. the formation of sub-complexes) and interact together to assist the final assembly of sub-complexes into an enzyme.

The role of Cbp4p is not clear, but we have shown that Cbp4p interacts with Qcr8p and Shi *et al*. (2001) mentioned the interaction of Cbp4p with both, Qcr7p and Qcr8p. This result suggests a role of Cbp4p in assembly of the cyt*b*-subcomplex. Crystal structure of the bc_1 complex indicates that cytochrome *b* is the organizing component of the bc_1 complex,

providing eight transmembrane helices (Hunte *et al*., 2000). Zara *et al*. (2004) showed that the presence of cyt*b*-subcomplex is essential for the stability of other structural subunits (as Cor1p, Qcr7p and Qcr8p) and probably also for bc_1 complex assembly. Therefore, deletion of *CBP*4 can destabilize apocytochrome *b* and further influence the degradation of structural subunits, Qcr7p and Qcr8p, thus making the phenotype of *cbp4* null mutant similar to the phenotype of *cbp3* null mutant. We have shown the interaction of Cbp4p with Cyt1p, a subunit of the second so called c_1-subcomplex. Probably, once a cyt*b*-subcomplex is assembled, Cbp4p may interact together with Cbp3p (that also interacts with Cyt1p) and catalyse the assembly of c_1-subcomplex. Additionally, Cbp3p seems to have a further role in the stability and assembly of Cor1p. Cbp3p interacts with Cor1p as shown by TAP purification and the assembly of Cor1p in Δ*cbp*3 strain is disturbed. As Cbp3p interacts with Cyt1p as well, this suggests its essential role in the assembly of the core- and cyt1-subcomplex. Presently we do not know, if core- and cyt1-subcomplexes pre-assemble and then are added to cyt*b*-subcomplex or not. However, in the *cbp4*Δ strain core- and cyt1-subcomplexes seem to be assembled into a bc_1-complex-like structure, as detection of Cyt1p revealed a complex corresponding in size to the bc_1-complex. Therefore, Cbp4p does not seem to be necessary for assembly of core- and cyt1- subcomplexes. Is then Cbp3p involved in Qcr7p stability and/or assembly into a cyt*b*-subcomplex? What could be the reason for the similar phenotype of *cbp3* and *cbp4* null mutants? We did not show the interaction of Cbp3p with Qcr7p, but a role of Cbp3p in Qcr7p stability and assembly is not excluded. Qcr7p is located between Cyt1p and Cor1p as can be seen from the crystal structure of the bc_1 complex (Hunte, 2001). If Qcr7p is not assembled Cor1p could possibly not be inserted. However, if Cbp3p is not present, the core-subcomplex could not be assembled and Qcr7p together with Qcr8p and Cobp that are extremely sensitive to proteases may become more accesible and degraded. Thus the phenotype of *cbp3* null mutant would be very similar to *cbp4* null mutant. Therefore we can not exclude the function of Cbp3p in Qcr7p stability and assembly. However, topology of Cbp3p and Cbp4p in respect to the topology of bc_1-complex subunits also support the proposed model of Cbp3p and Cbp4p function and is summarised in Fig. 30. PK assay localised the N- and C-terminal end of Cbp3p to the matrix side of inner mitochondrial membrane and Cbp3p was shown to be associated with the membrane (Crivellone, 1994). This puts Cbp3p in the position where the core-subcomplex interacts with cyt1-subcomplex. Close to this position is also the position of Qcr7p. These results support the hypothesis that Cbp3p may act as scaffolding protein for stepwise assembly of all three bc_1 sub-complexes. A striking difference between *cbp3*Δ and *cbp4*Δ strains is the detection of

one CIII/IV supracomplex in the *cbp4*Δ strain (Fig. 20). Although this supports the role of Cbp3p also in the supracomplex formation, the role of Cbp4p in this process is not excluded and on the other hand even supported, because formation of complete wild type supracomplexes is inhibited in the *cbp4*Δ strain. The topology of Cbp4p as shown by PK assay (Fig. 11) is different compared to Cbp3p. Cbp4p seems to be a transmembrane protein with the N-terminal end located at the matrix side and the C-terminus located in the intermembrane space. This enables it to function as chaperone for cyt*b*-subcomplex assembly as cytochrome *b* is very hydrophobic and Qcr8p is located on the IMS side of the inner mitochondrial membrane. Additionally, a coiled coil region was predicted in the Cbp4p. The ability of the coiled coil domains to expose extended surfaces with amphiphilic properties enables them to act as scaffolds for proteins and protein complexes assemblies. This make Cbp4p a suitable candidate of a scaffolding protein for bc_1 complex assembly. Our results suggest, that Cbp4p is not only essential for bc_1 complex assembly, but also for supracomplex formation. A role of Cbp3p in supracomplex formation was also suggested by (Cruciat *et al*. 2000). What are then the assembled bc_1 complex-like structures detected with anti Cyt1p antibody in the *cbp3*Δ and *cbp4*Δ strains? Zara *et al*. (2004) showed that the presence of cytochrome *b* is essential for the stability of other structural subunits and probably also for bc_1 complex assembly. But concentration of apocytochrome *b* in *cbp3*Δ and *cbp4*Δ strains is reduced. Our data suggest that also in the absence or the presence of cytochrome *b* in low concentrations, the assembly of bc_1-complex-like structure can occur as observed in *cbp4*Δ strain. Our data suggest that even partially assembled complex III, composed at least of cyt1- and core-subcomplex (in *cbp4*Δ strain) is sufficient for its assembly with complex IV. If these bc_1-complex-like structures present in the *cbp4*Δ and *cbp3*Δ strains contain any residual apocytochrome *b* remains to be elucidated. This result also supports the idea that Cbp3p and Cbp4p may act independently in bc_1 complex assembly and participate together in the supracomplex formation.

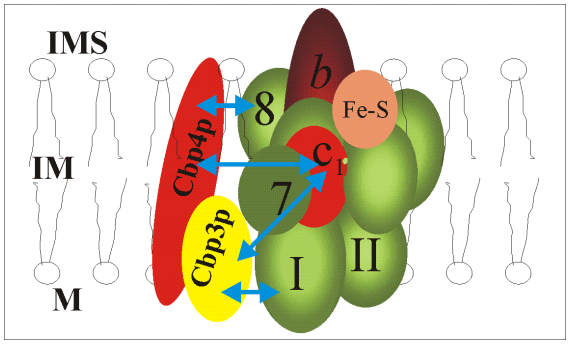

Figure 30. Suggested roles of Cbp3p and Cbp4p in complex III assembly. Schematical drawing of Cbp3p and Cbp4p topology in the inner mitochondrial membrane in relationship to subunits of complex III. Interactions of both assembly factors with complex III subunits identified in this study are shown by blue arrows.

What are the potential interaction sites of complex III and IV supracomplexes? Deletion analyses of subunits of the genes of complex III and IV revealed that Rip1p, Qcr6p, Qcr9p, Qcr10p, Cox8p, Cox12p, Cox13p and cytochrome *c* are not critical for formation of respiratory chain supracomplexes and that the lack of Qcr6p even enhanced the assembly. The most likely candidates for a direct link of complexes III and IV seem to be the central subunits: cytochromes *b* and c_1 of complex III and Cox1p, Cox2p and Cox3p of complex IV (Pfeiffer *et al.*, 2003). Cox3p was purified by co-IP with antibody directed to Cbp4p-cMyc (Fig. 15) supporting the role of Cbp4p in supracomplex formation. We were not able to purify Cox3p with Cbp3p, but Cyt1p was purified. Cytochrome *b* and Cyt1p together with Qcr7p of complex III are the subunits which are tightly bound to cardiolipin (Lange *et al.*, 2001), on which the formation of supracomplexes between complexes III and IV has been shown to be dependent (Zhang *et al*., 2002).

Therefore, I propose that Cbp3p and Cbp4p act as scaffolding proteins that fullfil their individual roles in bc_1 complex assembly and then unite the power for successful maturation of the enzyme. The interaction of Cbp4p with Cbp3p may participate the formation of the supracomplexes, while Cbp4p seems to interact with Cox3p – a protein essential for supracomplex formation.

In this thesis the role of Cbp3p and Cbp4p in complex III assembly and their relationship to subunits of complex III was analysed into detail. However, what is the relationship of Cbp3p and Cbp4p with other proteins essential for complex III assembly and biosynthesis, e.g. Bcs1p, Abc1p and cytochrome *b* translational activators? The answer for this question can be a task for future experiments. However, assembly of Cobp to the membrane is co-translational and association of Cbs2p - one of Cobp translational activators - with mitoribosomes was

shown by Krause-Buchholz *et al*. (2004). A possible assotiation of Cbp3p and Cbp4p with translational machinary is provided by BLAST-P analysis. Sequence similarities between Cbp4p and human RRBP1 protein are obtained. Human RRBP1 acts as ribosome receptor and mediates the interaction of the ribosome with endoplasmatic reticulum (Langley *et al.*, 1998). Cbp3p shared high sequence similarity to basic FGF repressed zinc binding proteins (see results part, section 3.1.). Zinc binding proteins are mostly DNA-binding proteins and a retrovirus-like zinc domain is essential for translational repression of the bacteriophage T4 gene 32 (Shamoo *et al.*, 1991). The presence of fully processed cytochrome *b* mRNA and its translation in the *cbp3* and *cbp4* null mutants suggest that Cbp3p and Cbp4p are not involved in an early post-transcriptional event, but rather in a late stage of complex III assembly (Wu and Tzagoloff, 1989; Crivellone, 1994). We have demonstrated possible roles of Cbp3p and Cbp4p in the assembly of three subcomplexes. Bcs1p acts as an ATP-dependent chaperone, maintaining the precomplex in a competent state for the subsequent assembly of the Rieske Fe-S and Qcr10p proteins (Cruciat *et al*., 1999). Cbp3p and Cbp4p are present in the complexes of high molecular weight as shown by BN-PAGE. Crystal structure of complex III of the bovine heart mitochondria (Zhang *et al.*, 1998) indicates that the N-termini of Rieske FeS interacts with Cor1 protein. It is therefore tempting to predict the presence of Bcs1p together with Cbp3p and Cbp4p in one complex forming a so-called “assemblosome”.

Assembly of the bc_1 complex is very complex process complicated by the presence of very hydrophobic proteins. Although recent data do not provide any evidence for Cbp3p and Cbp4p to be involved in translational machinery, the assotiation of translational and assembly machinery seems to be inevitable and opens new fields for further investigations.

5. SUMMARY

Ubiquinol-cytochrome *c* reductase (complex III) is a central component of the respiratory chain of the inner mitochondrial membrane. It transfers electrons from reduced ubiquinone to ferricytochrome *c*. Correctly assembled and functional complex III is an essential prerequisite for oxidative energy metabolism. Complex III deficiency has been reported to be associated with several neurodegenerative diseases. Formation and assembly of complex III requires a multitude of specific nuclearly encoded proteins. For example, gene specific translational activators for cytochrome *b* synthesis as well as three non-subunit proteins, which are important for assembly and/or stability have been detected. The role of Bcs1p in assembly of Rieske FeS protein and Qcr10p into complex III has been clasified recently. The role of the two putative chaperones, Cbp3p and Cbp4p, is not known.

Moreover, the process of complex III assembly is not fully understood. A model of the bc_1 complex assembly was proposed recently, in which the existence of three distinct subcomplexes is predicted. However, it is not known in which order the subunits interact to form the putative subcomplexes and in which sequence the subcomplexes are assembled into a functional enzyme. An excellent model to study the mitochondrial biogenesis is baker´s yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*). In contrast to higher eukaryotes, *S. cerevisiae* can survive in the absence of the most prominent mitochondrial function, the oxidative phosphorylation. In *S. cerevisiae*, ubiquinol-cytochrome *c* reductase is composed of 10 non-identical subunits with defined catalytic centers consisting of cytochrome *b*, cytochrome c_1 and the Rieske FeS protein. In *cbp3*Δ and *cbp4*Δ strains no complex III activity is detected and levels of 3 subunits (cytochrome *b*, Qcr7p and Qcr8p) are reduced.

In spite of the similar phenotype of *cbp3*Δ and *cbp4*Δ strains, that suggests the role of both proteins in the same step of complex III assembly, we were able for the first time to demonstrate differences on the molecular level between both deletion mutants. We show by BN-PAGE that *cbp3*Δ and *cbp4*Δ mutants are disturbed in complex III assembly and accumulate intermediate-sized forms of the complex. Moreover deletion of *CBP3* interferes with the formation of complex III/IV supracomplexes. Our studies show that Cbp3p and Cbp4p interact and are present in high molecular weight complexes, some of which might represent intermediates of complex III assembly. Overexpression of Cbp4p cannot substitute for the function of Cbp3p, but high level expression of Cbp3p can partially compensate for the lack of Cbp4p. Because lipids play an important role for complex III assembly and stability, we analysed the mitochondrial lipid composition of *cbp3*Δ and *cbp4*Δ mutants. Our

data show that mitochondria of both mutants exhibit a wild type-like lipid composition, that favors the idea that Cbp3p and Cbp4p are specific assembly factors for complex III rather than components of the mitochondrial lipid metabolism.

By complementation studies we have shown that Cbp3 proteins of *S. cerevisiae*, *S. pombe* and human are (partially) functional homologues. A yeast model based on chimeric constructs of *S. cerevisiae* and human proteins was constructed, which allows to test the pathogenicity of human mutations.

To define the role/s of Cbp3p and Cbp4p in the assembly pathway of complex III, interactions of selected subunits with both assembly factors were analysed by TAP- or co-immunoprecipitation. Based on the results of Cbp3p and Cbp4p topologies, BN-PAGE analysis of null mutant strains and interaction studies a model for complex III assembly and the roles of Cbp3p and Cbp4p in this process are proposed. I present a hypothesis, according to which Cbp3p and Cbp4p form a “scaffold” for the assembly of all three putative sub-complexes, may act independently in the first steps of bc_1 complex assembly (e. g. the formation of sub-complexes) and interact together to assist the final assembly of sub-complexes into a mature enzyme.

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