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Site-directed Mutagenesis of Five Conserved Residues of Subunit I of the Cytochrome cbb_3 Oxidase in *Rhodobacter capsulatus*

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Cytochrome cbb_3 oxidase is a member of the heme-copper oxidase superfamily that catalyses the reduction of molecular oxygen to the water and conserves the liberated energy in the form of a proton gradient. Comparison of the amino acid sequences of subunit I from different classes of heme-copper oxidases showed that transmembrane helix VIII and the loop between transmembrane helices IX and X contain five highly conserved polar residues; Ser333, Ser340, Thr350, Asn390 and Thr394. To determine the relationship between these conserved amino acids and the activity and assembly of the cbb_3 oxidase in *Rhodobacter capsulatus*, each of these five conserved amino acids was substituted for alanine by site-directed mutagenesis. The effects of these mutations on catalytic activity were determined using a NADI plate assay and by measurements of the rate of oxygen consumption. The consequence of these mutations for the structural integrity of the cbb_3 oxidase was determined by SDS-PAGE analysis of chromatophore membranes followed by TMBZ staining. The results indicate that the Asn390Ala mutation led to a complete loss of enzyme activity and that the Ser333Ala mutation decreased the activity significantly. The remaining mutants cause a partial loss of catalytic activity. All of the mutant enzymes, except Asn390Ala, were apparently correctly assembled and stable in the membrane of the *R. capsulatus*.

Keywords: Cytochrome cbb_3-type oxidase activity, Respiration and site-directed mutagenesis, *Rhodobacter capsulatus*

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Introduction

Members of the superfamily of heme-copper oxidases (HCOs) terminate the aerobic respiratory chains of most eukaryotes and prokaryotes (Garcia-Horsman *et al*., 1994; Gray *et al*., 1994). These enzymes catalyze the reduction of molecular oxygen to water, coupling the redox free energy of the reaction to proton translocation across the membrane (Malatesta *et al*., 1995; Schultz and Chan, 2001).

The superfamily is quite diverse in terms of electron donors, subunit composition and heme types. Recent work on the evolutionary relationships of the heme-copper superfamily based on the sequence alignments and structural information and proton indicates three very distinct families (Pereira *et al*., 2001). Type A oxidases (aa_3-type oxidases) are most closely related to the mitochondrial oxidases. Type B oxidases (ba_3-like oxidases) are grouped around the cytochrome ba_3 oxidase. Type C oxidases are all cytochrome cbb_3-type oxidases, typified by the FixN enzyme from *Bradyrhizobium japonicum* (Preisig *et al*., 1993). The cbb_3-type oxidases are found only in some of the proteobacteria and have been reported not only to have a very high affinity for oxygen (Preisig *et al*., 1996) but also to retain the ability to conserve the energy liberated from the oxygen reduction reaction (de-Gier *et al*., 1996). The type C oxidases have been speculated to be most phylogenetically ancient, and their sequence similarities to the nitric oxide reductases have led to the idea that the respiratory oxidases were derived from enzymes involved in denitrification (Vanderoost *et al*., 1994). Another view is that they are not ancient but have simply evolved independently in proteobacteria to meet their particular needs and are in fact a very modern oxidase (Pereira *et al*., 2001).

The cbb_3-type oxidases are expressed in various proteobacteria under microaerobic conditions (Myllykallio and Liebl, 2000). In the super family of heme-copper oxidases characterized by a unique subunit composition and B-type hemes in the catalytic subunit, subunit I. Cytochrome cbb_3 oxidases are

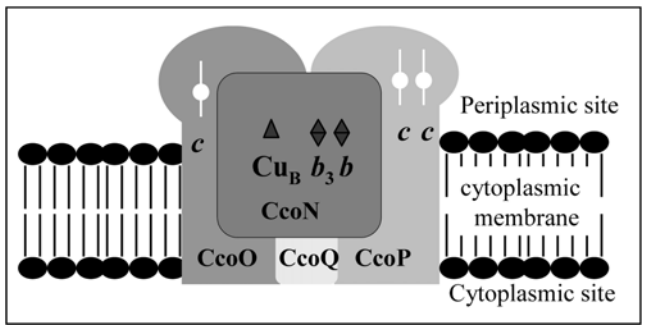

Fig. 1. Organization of the CcoNOQP subunits of cbb_3-type oxidase in *R. capsulatus*. The *CcoNOQP* operon of *R. capsulatus* encodes the four structural proteins that comprise the cytochrome cbb_3 oxidase complex. Although CcoQ is shown as an integral part of the complex there is no evidence for its presence in the cytochrome cbb_3 oxidase purified from *R. capsulatus* which is a three subunit (CcoNOQP) complex.

predicted to contain four subunits, although in most preparations reported to date, there is only firm evidence for the presence of CcoNOQP (Fig. 1). There are four subunits in the cbb_3-type oxidases: the catalytic subunit CcoN, which is homologous to subunit I of all heme-copper oxidases; the probable immediate electron donor ccoO (Zufferey *et al*., 1996), which is a membrane-anchored monoheme protein; CcoP, a membrane-anchored diheme protein; and CcoQ, comprised of a single transmembrane helix (Toledo-Cuevas *et al*., 1998). Both subunits O and P have been suggested to be part of the electron transfer chain, while the role of subunit Q is unknown (Zufferey *et al*., 1996; Koch *et al*., 1998 and Pitcher *et al*., 2002). Only subunit I is common to all HCOs and membership of the superfamily is defined by this subunit which contains the catalytic centre, the heme-copper binuclear site, and its immediate electron donor, the low-spin heme. Like subunit I in other heme-copper oxidase, CcoN, has at least 12 transmembrane helices and contains the active site, a high-spin heme (heme b_3) magnetically coupled to an adjacent copper ion known as Cu_B to form a binuclear center (Babcock and Wikstrom, 1992). A second heme (heme b), which serves to transfer electrons to the active site, is also contained within subunit I.

High resolution X-ray structures of the type A cytochrome *c* oxidase from bovine heart mitochondria (Yoshikawa *et al*., 1998 and Tsukihara *et al*., 1996), *Paracoccus denitrificans* (Iwata *et al*., 1995 and Ostermeier *et al*., 1997) and *Rhodobacter sphaeroides* (Svensson-Ek *et al*., 2002) confirmed the organization of the metal cofactors. These structural studies of heme-copper oxidases also identified two pathways, known as the D-channel and the K-channel, capable of conducting protons from the negative face of the membrane, towards the dinuclear centre (de Gier *et al*., 1996). The specific roles of these two channels have been elucidated by site-directed mutagenesis. The K-channel contains a conserved lysine (K362 in the *R. sphaeroides* cytochrome *c* oxidase) and appears to be used to transfer protons required for the (re-)reduction of the dinuclear center during turnover (Adelroth *et al*., 1998). The D-channel is named after a conserved aspartate residue (D132) at its entrance which changing to either to alanine (D132A) or the corresponding amide (D132N) by site-directed mutagenesis results in a loss of activity (Mills *et al*., 2000).

Sequence analysis shows not only that cbb_3 oxidases are the most distant members of the HCO superfamily (Castresana *et al*., 1994) and that apart from the six invariant histidines ligating the metal atoms that very few residues are conserved (Zufferey *et al*., 1998). Two non-canonical histidine residues which are conserved almost all members of the super family are required for activity or assembly of the cbb_3-type oxidases from *B. japonicum* (Zufferey *at al.* 1998), *R. sphaeroides* (Oh, 2006) and *R. capsulatus* (Öztürk and Mandaci, 2006). Particularly striking is the fact that none of the residues that contribute to the D-channel in type A HCOs are present in all cbb_3 oxidases although elements of the K-channel remain (Pereira *et al*., 2001). This is potentially very significant because in the type A oxidases the D-channel is required to conduct at least six of the eight protons required in the reaction, while blocking the K-channel hinders the reductive phase of the catalytic cycle in the well-studied aa_3-type oxidases (Konstantinov *et al*., 1997; Bloch *et al*., 2004).

Three of these conserved residues threonine or serine residues located in helix VIII and are represented in subunit I of the cbb_3-type oxidase from *R. capsulatus* by Ser333, Ser340 and Thr350 (Figs. 2 and 3). The serine residue at position 340 (which is homologous to threonine residue at position 351 of the aa_3-type oxidase from *P. denitrificans*) is proposed to contribute to an alternative K-channel (Pereira *et al*., 2001). In the *R. capsulatus* cbb_3-type oxidase Asn390 and Thr396 are located in a loop connecting helices IX and X at the periplasmic face of the inner membrane (Figs. 2 and 3). In all other oxidases Asn390 is represented either by an aspartate or an asparagine residue, whilst Thr396 is either a serine or a threonine residue. Despite such a high level of conservation of these amino acids, the significance of these residues (e.g., Thr350 and Thr396) for the proper functioning of heme-copper oxidase has not been looked into in detail yet (Pereira *et al*., 2001).

This work represents the first attempt to critically evaluate the roles of these five residues in the type C (cbb_3-type) oxidases. Each residue was substituted with alanine by site-directed mutagenesis and the phenotype of the mutants determined in membranes of *R. capsulatus*. We demonstrate that substitution of the asparagine residue at position 390 with alanine leads to either to an assembly defect, or instability in the cbb_3-type oxidase in the membrane which results in almost complete loss of oxidase activity. Another mutant, S333A, is correctly assembled in the membrane but its catalytic activity is significantly reduced.

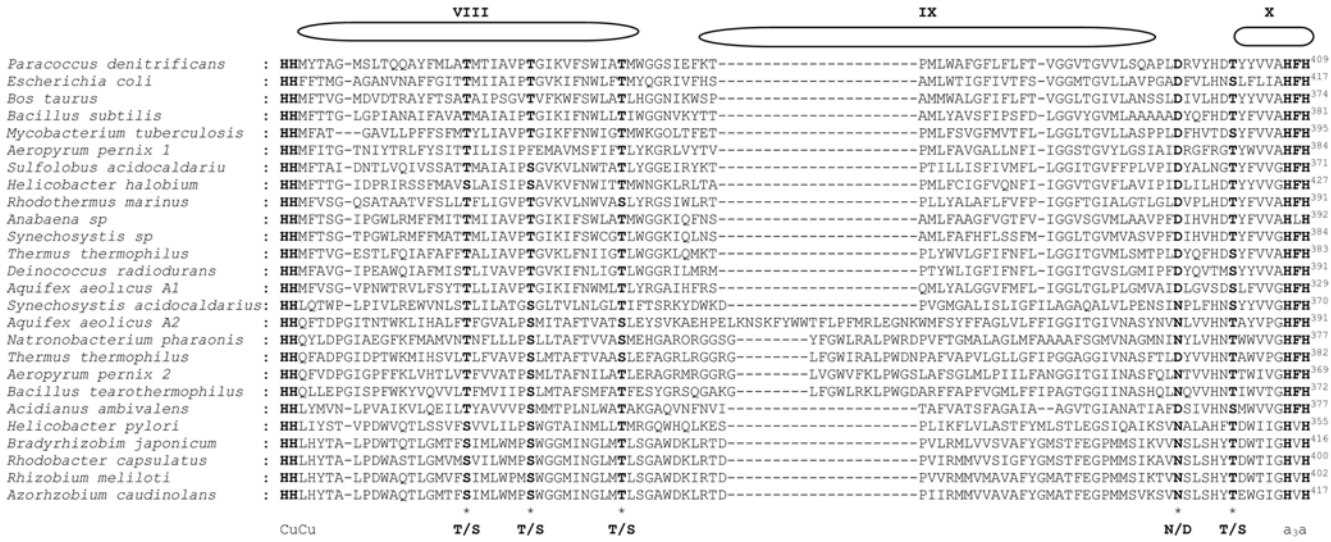

Fig. 2. A part of the amino acid sequence alignment of subunit I of heme-copper oxidase. *P., Paracoccus* (P082837), *E., Escherichia* (P18400), Bo., Bos (P00396), B., *Bacillus* (*subtilis caa*$_3$, P24010, *stearothermophilus ba*$_3$, 082837), M., *Mycobacterium* (O53290), *A., Aeropyrum* (1,Q9YDX6, 2, BAA80883), *S., Sulfolobus* (SoxM, p39481, SoxB, S21042), *R., Rhodothermus* (CACO8532), *A., Anabaena* (*Anabaena sp*. Strain PCC 7937, Z 98264), *Sy., Synechosystis* (*Synecosystis sp*. Strain PCC 6803, Q06473/P73261), *T., Thermus* (*caa*$_3$, P98005, *ba*$_3$, CAB06339), *D., Deinococcus* (G75251), *Aq., Aquifex* (A1, C70488, A2, E70488), *N., Natronobacterium* (CAA71525), *A., Acidianus* (CAA69980), *H., Helicobacter* (AAD0571), *Br., Bradyrhizobim* (Q0373), *Rhd., Rhodobacter* (AAC46108), *Rh, Rhizobium* (A39988), *Az., Azorhzobium* (CAA52429). Strictly and almost strictly conserved amino acid residues are shaded in black. The histidine ligands to low- and high-spin hemes and to Cu_B are indicated by *a*, a_3 and Cu, respectively. It was modified from Pereira *et al.*, (2001).

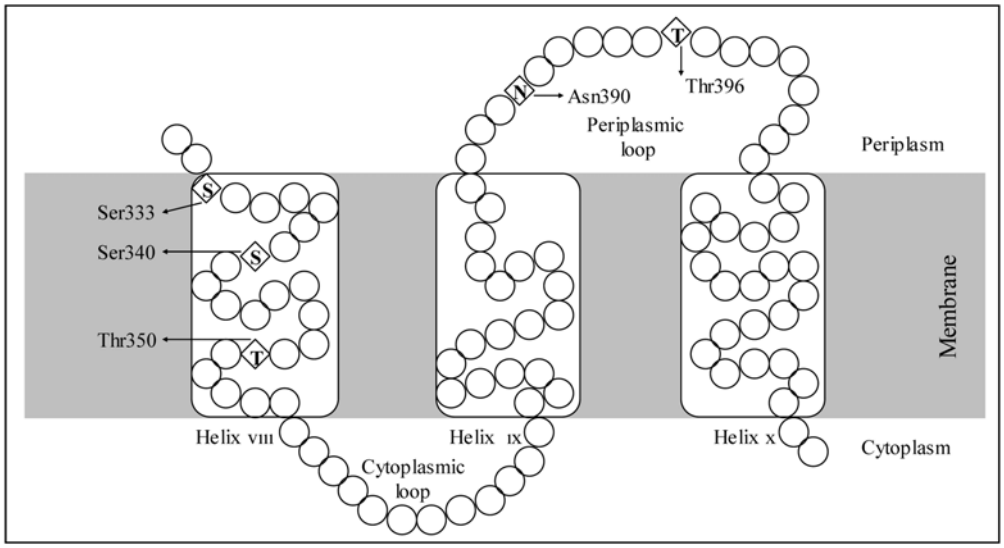

Fig. 3. Topological model of a part of the subunit I (ccoN) of cbb_3-type oxidase in *R. capsulatus*. Circles represent amino acids in helix and loop. Squares, ◇, represent conserved and mutated amino acids present in subunit of all heme-copper oxidase. S: Serine. T: Threonine, N: Asparagine. This figure was drawn manually by using TMHMM Server V.2.0 (http://www.cbs.dtu.dk/services/TMHMM).

Materials and Method

Bacterial strains and their growth conditions. The bacterial strains and plasmids used and any properties relevant to this study are listed in Table 1. All *Escherichia coli* strains were grown in Luria-Bertini broth (LB) medium supplemented with appropriate antibiotics (ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; tetracycline, 12.5 μg/ml) for plasmid selection and maintenance as previously described (Daldal *et al*., 1986; Jenney and Daldal, 1993). *R. capsulatus* strains were grown on Sistrom's minimal medium A (Med A) (Sistrom *et al*., 1960) or MPYE enriched growth medium (Daldal *et al*., 1986) supplemented with appropriate antibiotics (both supplemented with tetracycline, 2.5 μg/ml; kanamycin, 10 μg/ml; or ampicillin, 10 μg/ml as needed) (Daldal *et al*., 1986) at 35°C under aerobic conditions in the dark on plates or liquid cultures (shaken at 150 rpm).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmids	Genotype	Phenotype	Reference
Strains			
E. coli			
XL1-Blue	rec A end A 1 gyr A986 thi-1hsdr17supE44 rel A1 lac [F' proAB lacIq M15 Tn 10(Tetr)]		Stratagene
XL1-Blue/pMOZI		Amp^r	This work
XL1-Blue/pMOZIS333A		Amp^r	This work
XL1-Blue/pMOZIS340A		Amp^r	This work
XL1-Blue/pMOZIT350A		Amp^r	This work
XL1-Blue/pMOZIN390A		Amp^r	This work
XL1-Blue/pMOZIT396A		Amp^r	This work
HB101	F-proA2 hsdS20 (Rb-, mB-) recA13 ara14 lac Y1 xy15 galK2 rpsL20 supE44 rpsL20 supE44 proA2 mtl-1		[Sambrook *et al.*1989] New England BioLabs
HB101/pACYC177	F-ara-14leu fhuA2 (gpt-proA) 62 lac Y1glnV44 galK2 rspL20 xyl-5 mtl-1 $(mcrC\text{-}mrr)_{HB101}$	Amp^r, Kan^r	
HB101/pMOZI		Amp^r	This work
HB101/pMOZII		Amp^r	This work
HB101/pMOZIIS333A		Amp^r	This work
HB101/pMOZIIS340A		Amp^r	This work
HB101/pMOZIIT350A		Amp^r	This work
HB101/pMOZIIN390A		Amp^r	This work
HB101/pMOZIIT396A		Amp^r	This work
HB101/pOX15		Tet^r	[Koch *et al.,* 1998]
R. capsulatus			
MT1131	*crtD121 Rifr*	Wild type	[Daldal *et al.,* 1986]
GK32	Δ(*ccoNO*::kan)	Kan^r	[Koch *et al.,* 1998]
GK32/pOX15S333A		Tet^r	This work
GK32/pOX15S340A		Tet^r	This work
GK32/pOX15T350A		Tet^r	This work
GK32/pOX15N390A		Tet^r	This work
GK32/pOX15T396A		Tet^r	This work
Plasmids			
pBluescript	SKII+	Amp^r	Stratagene
pMOZI	Bluescript SK+ with a 2.8-kb *Xho*I insert	Amp^r	This work
pMOZIS333A	Substitution of serine 333 by alanine in pMOZI	Amp^r	This work
pMOZIS340A	Substitution of serine 340 by alanine in pMOZI	Amp^r	This work
pMOZIT350A	Substitution of threonine 350 by alanine in pMOZI	Amp^r	This work
pMOZIN390A	Substitution of asparagine 390 by alanine in pMOZI	Amp^r	This work
pMOZIT396A	Substitution of threonine 396 by alanine in pMOZI	Amp^r	This work
pACYC177		Amp^r, Kan^r	New England BioLabs
pMOZII	pACYC177 with a 2.8-kb *Xho*I insert	Amp^r	This work
pMOZIIS333A	pMOZII replaced 0.6-kb *BsaA*I fragment of pMOZIS333A	Amp^r	This work
pMOZIIS340A	pMOZII replaced 0.6-kb *BsaA*I fragment of pMOZIS340A	Amp^r	This work
pMOZIIT350A	pMOZII replaced 0.6-kb *BsaA*I fragment of pMOZIT350A	Amp^r	This work
pMOZIIN390A	pMOZII replaced 0.6-kb *BsaA*I fragment of pMOZIN390A	Amp^r	This work
pMOZIIT396A	pMOZII replaced 0.6-kb *BsaA*I fragment of pMOZIT396A	Amp^r	This work
pOX15	pRK404 with 4.7-kb *ccoNOQP* operon	Tet^r	[Sambrook *et al.*, 1989]
pOX15S333A	pOX15 replaced 2.8-kb *Xho*I fragment of pMOZIIS333A	Tet^r	This work
pOX15S340A	pOX15 replaced 2.8-kb *Xho*I fragment of pMOZIIS340A	Tet^r	This work
pOX15T350A	pOX15 replaced 2.8-kb *Xho*I fragment of pMOZIIT350A	Tet^r	This work
pOX15N390A	pOX15 replaced 2.8-kb *Xho*I fragment of pMOZIIN390A	Tet^r	This work
pOX15T396A	pOX15 replaced 2.8-kb *Xho*I fragment of pMOZIIT396A	Tet^r	This work
pRK2013		Kan^r, helper	[Ditta *et al.*, 1985]

Recombinant DNA work. The 2.8 kb fragment that contains *ccoNO'* part of the structural operon (*CcoNOQP*) that encodes the cytochrome cbb_3-type oxidase in *R. capsulatus* was cloned into both pBluescript II SK+ and pACYC177 (New England BioLabs). The new constructs were named pMOZI, (5.7 kb) and pMOZII (6.8 kb) respectively. The plasmids were maintained in XL1-Blue

Table 2. Oligonucleotides used for site-directed mutagenesiss; F and R denote forward and reverse primers, respectively. The highlighted bases correspond to the genetic codes for amino acids to be mutated

Primers	Sequence of the primers
S333AF	5'-CTG GGC ATG GTG ATG **GCC** GTG ATC CTG TGG ATG-3'
S333AR	5'-CAT CCA CAG GAT CAC **GGC** CAT CAC CAT GCC CAG-3'
S340AF	5'-G ATC CTG TGG ATG CCG **GCC** TGG GGC GGC ATG-3'
S340AR	5'-C ATG CCG CCC CAG GCC **GGC** ATC CAC AGG ATC 3'
T350AF	5'-G ATC AAC GGC CTG ATG **GCG** CTT TCG GGC GCC TGG G-3'
T350AR	5'-C CCA GGC GCC CGA AAG **CGC** CAT CAG GCC GTT GAT C-3'
N390AF	5'-G TCG ATC AAG GCG GTC **GCC** TCG CTC TCG CAC TAC-3'
N390AR	5'-G TAG TGC GAG AGC GAG **GCG** ACC GCC TTG ATC GAC-3'
T396AF	5'-CG CTC TCG CAC TAC **GCG** GAC TGG ACC ATC GG-3'
T396AR	5'-CC GAT GGT CCA GTC **CGC** GTA GTG CGA GAG CG-3'

(pMOZI) and HB101 (pMOZII) cells (Sambrook *et al*., 1989). Five conserved amino acids were substituted with alanine using pMOZI DNA as a template DNA by site-directed mutagenesis using 31-35 bp primers (Table 2). The PCR product of each mutation was transformed into XL1-Blue cells and the region of interest (0.6 kb) sequenced by MWG Biotech AG and Iontek using the primer 578F 5' GGTGGCCTATCTGATCG 3'. The strains containing the mutated plasmids were named XL1-Blue/pMOZIS333A, XL1-Blue/pMOZIS340A, XL1-Blue/pMOZIT350A, XL1-Blue/pMOZIN390A and XL1-Blue/pMOZIT396A.

After verifying the mutations by sequencing and ensuring no unexpected errors had been introduced into the sequence by PCR a 0.6 kb *Bsa*AI fragment containing the region of interest was isolated. This *Bsa*AI fragment was used to replace the equivalent region of pMOZII and the resultant plasmid used to transform the HB101 strain of *E. coli* cell. Plasmids containing the mutated *Bsa*AI fragment cloned in the correct orientation were determined by digestion of the plasmid DNA with *Sal*I. These strains were named HB101/pMOZIIS333A, HB101/pMOZIIS340A, HB101/pMOZIIT350A, HB101/pMOZIIN390A and HB101/pMOZIIT396A respectively.

A 2.8 kb *Xho*I fragment containing the mutation was used to replace the equivalent region of the ccoN gene in pOX15 and the resultant plasmid used to transform the HB101 strain of *E. coli*. The plasmids that contained the insert in the correct orientation were determined by *Hind*III digestion, and the strains named HB101/pOX15S333A, HB101/pMOZIIS340A, HB101/pMOZIIT350A, HB101/pMOZIIN390A and HB101/pOX15T396A. Finally each of these five new plasmids were transferred into GK32 strain of *R. capsulatus* (Koch *et al*., 1998), carrying a deletion covering the *NO'* part of the *CcoNOQP* operon, by triparental mating in the presence of HB101/pRK2013 (Ditta *et al*., 1985) helper cell.

Site-directed mutagenesis. Ser333Ala, Ser340Ala, Thr350Ala, Asn390Ala and Thr3936Ala mutations were created by site-directed mutagenesis using pMOZI as template DNA. Synthetic oligonucleotides 31-35 bases long containing an alanine codon (GCC or GCG) in place of the original codon in the middle of their sequences were used. To create these mutations, PCR reactions were performed with the forward and reverse primers (Table 2) for each mutation using 50 ng of pMOZI DNA, 125 ng of each primer and 2.5 U/μl *Pfu* DNA polymerase (MBI Fermantase) with initial denaturation of 1 minute at 95°C followed by 12 cycles of 30 s at 95°C, 30 s at 65°C and 1 min at 72°C. The PCR products were used to transform XL1- Blue cells.

NADI staining of whole cells. This test is based on the ability of cytochrome *c* dependent terminal oxidases to catalyze the aerobic synthesis of indophenol blue from α-naphthol and DMPD (N, N-dimetyl-p-phenylenediamine). The appearance of a deep-blue coloration by indophenol blue is taken as a positive result. The presence of only one-type of cytochrome *c* dependent oxidase in *R. capsulatus* allows the use of the NADI (α-naphthol + DMPD + O_2 → Indophenol Blue + H_2O) reaction (Keilin, 1966) for identification of mutations that affect cytochrome cbb_3 oxidase activity. The GK32 mutant, Δ(*ccoNO'*::kan), was complemented with plasmids containing each of the desired mutations. After complementation, the effect of the mutatation on cytochrome *c* dependent oxidase activity was determined by overlaying the plates with a 1 : 1 (vol/vol) mixture of 35 mM α-naphthol in ethanol and 30 mM *N*,*N*-dimethyl-*p*-phenylene diamine in H_2O (Keilin, 1966). Under these conditions, colonies on MedA medium that contain an active cytochrome cbb_3 oxidase turn blue within 30 seconds.

Isolation of chromatophore membranes and SDS-PAGE/TMBZ staining analysis. Before breaking the previously frozen *R. capsulatus* cells, pellets were resuspended in 10 ml of 50 mM MOPS/1 mM KCl solution containing, RNAse, DNAse (10 mg/ml), 1 mM $MgCl_2$ and 1 mM PMSF (Phenylmethanosulphonylfluoride) which was added just before breaking the cells and the sample mixed thoroughly. The cells were broken by a double passage through a French pressure cell (11000 psi) and the homogenate centrifuged at 10.000 × g for 45 min at 4°C to remove any broken cells. The resulting supernatant was centrifuged at 100,000 × g for 2 h at 4°C to sediment the membrane fragments. To remove any remaining cytoplasmic proteins the membrane fragments were washed and resuspended gently in 10 ml of 50 mM MOPS/1 mM KCl buffer with help of the paint brush and then centrifuged again at 100,000 × g at 4°C for two hours. Finally, the pellet was dissolved in 400 μl of 50 mM MOPS/1 mM KCl buffer contaning 1 mM PMSF and stored at −80°C. Total protein was measured using the method of Lowry (Lowry *et al*., 1951) using bovine serum albumin

as a standard. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described in (Schägger *et al*., 1987) with 16.5% polyacrylamide gels. Before loading samples on the polyacrylamide gel, they were dissolved in 5% β-mercaptoethanol and incubated at 37°C for five minutes. Gels were stained with 3,3',5,5'-tetramethylbenzidine (TMBZ) and H_2O_2 to reveal the heme peroxidase activity (Thomas *et al*., 1976).

TMPD-dependent oxidase activity of chromatophore membranes. N,N,N',N' tetramethy-p-phenylendiamine (TMPD) oxidase activity was measured using a Clark-type oxygen electrode (Oxygraph plus V1.00, Hansetech Instruments). The assay mixture containined 0.7 mM TMPD, 2 mM ascorbate, 10 μM myxothiazol and 0.1 mM cyanide in 50 mM Tris-HCl, 100 mM NaCl pH 8.0. All measurements were made at 25°C. The reaction was initiated by the addition of the membrane suspension (final concentration 100 μg/ml) to the cuvette. The net TMPD dependent oxidase activity was determined by subtraction of the endogenous respiratory rate (potassium cyanide insensitive rate of oxygen consumption).

Results

Mutagenesis of five conserved residues in CcoN. In this study, Ser333Ala, Ser340Ala, Thr350Ala, Asn390Ala and Thr396Ala mutants on subunit I of cbb_3-type oxidase in *R. capsulatus* were created by site-directed mutagenesis. Plasmid DNA was isolated from each of XL1-Blue/pMOZ1S333, XL1-Blue/pMOZ1S340, XL1-Blue/pMOZ1T350, XL1-Blue/pMOZ1N390 and XL1-Blue/pMOZ1T396 and a 0.6 kb region of DNA surrounding the mutation sequenced. When the results were compared with the sequence of the *R. capsulatus* cbb_3 oxidase from Gene Data Bank, (http://ergo.integratedgenomics.com) it was found that all plasmid DNAs had desired mutations, GCC or GCG respectively instead of CAC and there was no mutation other than that which was intended.

Effect of mutations on the catalytic activity of mutant enzymes. We first analyzed the effect of the five amino acid replacements on cytochrome *c* oxidase activity using the NADI test which allows rapid screening for cytochrome *c* dependent oxidase activity by observation of colony pigmentation. We performed the NADI staining test with whole cells of wild type (MT1131) and targeted mutants that had been grown under aerobic conditions, because this terminal oxidase is expressed under such conditions (Preisig *et al*., 1993; Koch *et al*. 1998). In this assay, TMPD is used as the artificial electron donor. As shown in Fig. 4, the negative control strain GK32 showed virtually no cbb_3 oxidase activity. On the other hand, the positive control strain, MT1131 and MT1131/pOX15, as well as mutant strains, GK32/pOX15S340A, GK32/pOX15T350A, GK32/pOX15T396A, displayed similar activity of the cbb_3 oxidase. While the cbb_3 oxidase activity detected in the strain GK2/pOX15S333A was significantly reduced as compared with that in the positive control strain, MT1131 and MT1131/pOX15, the activity of the MT1131/pOX15N390A was completely abolished.

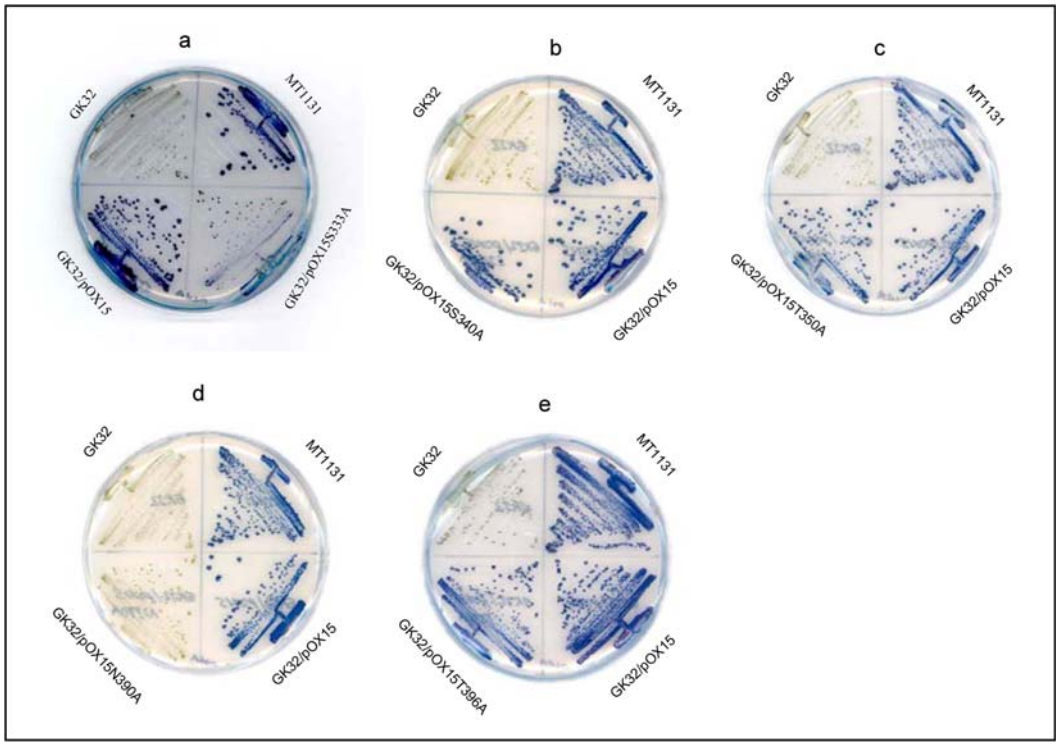

Fig. 4. NADI-stained plates each containing one of the desired mutations together with wild type (MT1131), negative control (GK32) and positive control (GK32/pOX15).

Table 3. Phenotypic and biochemical characterization of mutants

Strains	NADI Assay	Assembly	Biochemical properties[b]	
			c_o	c_p
GK32[a]	-	Not assembled	-	-
GK32/pOX15	+	Assembled	+	+
GK32/pOXI5S333A	(+)[c]	Assembled	+	+
GK32/pOXI5S340A	+	Assembled	+	+
GK32/pOXI5T350A	+	Assembled	+	+
GK32/pOXI5N390A	-	Not assembled	(+)[d]	-
GK32/pOXI5T396A	+	Assembled	+	+

[a]For detailed description of the strain, see the text.
[b]Based on TMBZ-stained SDS-PAGE.
[c]Very weak staining.
[d]Small amount of CcoO (c_o) were detectable.

Table 4. TMPD oxidase activities in chromatophores from various *R. capsulatus* strains grown chemoheterotrophically in MPYE medium

Strains	O_2 consumption (μmol/ml)[a]		% of wt activity
	Ascorbate-TMPD	Ascorbate-TMPD-KCN	
MT1131	78,7	3,0	73
GK2/POX15	106,3	3,4	100
Mutants			
GK32	2,5	2,6	0
GK32/pOX15S333A	16,2	3,6	15,5
GK32/pOX15S340A	45,5	2,5	41,8
GK32/pOX15T350A	59,3	2,5	57,2
GK32/pOX15N390A	3,3	3,4	0
GK32/pOX15T396A	53,8	3,1	44,7

[a]Values shown are means of at least three independent measurements. The net TMPD oxidase activity was determined by subtraction of the endogenous respiratory rate from that induced by KCN, potassium cyanide; wild type, wt.

Catalytic activity of the cytochrome *cbb*$_3$ oxidase. Since the activity of the cbb_3 oxidase can be determined roughly by NADI staining test, oxygen consumption rate of chromatophore membranes of the wild type and mutants are measured polarographically in the presence of ascorbate and TMPD. The cytochrome *c* oxidase catalyzes the reduction of O_2 as monitored by oxygen uptake using TMPD/ascorbate as electron donors. Cytochrome *c* O_2 uptake activity is inhibited by low concentrations of cyanide (10 μM KCN). The cbb_3 oxidase O_2 consumption rates detected in the strains, GK32/pOX15S340A, GK32/pOX15T350A and GK32/pOX15T396A, were reduced by approximately 41.8%, 57.2% and 44.7%, respectively, as compared with that in the positive control strain, MT1131 and MT1131/pOX15. Although, the S333A mutant was normally assembled in the membrane and retained its stability, its O_2 consumption rate was significantly reduced, 15.5% of the wild type activity. The N390A mutant led to the almost complete loss of the activity of the cbb_3 oxidase. Taken together, the results presented in Table 4 indicated that S333 and N390 amino acids are important residues for activity and assembly or stability of the cbb_3 oxidase in *R. capsulatus*, respectively.

Presence of subunits containing c-type heme in *cbb*$_3$-type oxidase. Four distinct membrane-bound *c*-type cytochromes, with M_r of 32, 31, 29, and 28 kDa, are readly detected in chromotophore membranes of a wild type *R. capsulatus* strain, MT1131, grown aerobically on MPYE enriched medium. Of these, the 31-kDa protein is the cytochrome c_1 subunit of the cytochrome bc_1 complex (Jenney *et al*., 1994), the 29 kDa protein is the membrane-assosiated electron carrier cytochrome c_y (Jenney *et al*., 1993). The c_o, 28 kDa, and c_p, 32 kDa, subunits are corresponding to CcoO and CcoP of the cbb_3 oxidase.

To determine whether the assembly of the cbb_3 oxidase in the membrane was affected by replacement of five conserved residues in CcoN, the chromatophore membranes from both the controls and the mutants were electrophoresed on 16.5% Schägger type SDS-polyacrylamide gels that were subsequently stained with 3,3',5,5'-tetrametylbenzidine. The 3,3',5,5'-

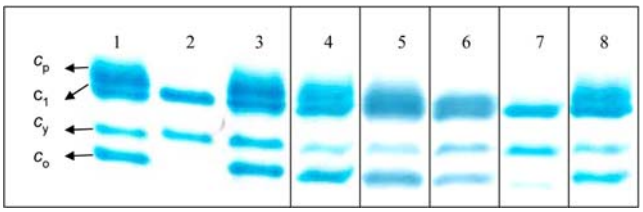

Fig. 5. Cytochrome *c* profile of MT1131/pOX15, GK32, GK32/pOX15, GK32/pOX15S333A, GK32/pOX15S340A, GK32/pOX15T350A, GK32/pOX15N390A and GK32/pOX15T396A strains. Cytochrome *c* profiles of chromatophore membranes of *R. capsulatus* strains grown in enriched MPYE medium under respiratory conditions. Approximately 100 µg of protein was loaded in each lane of a 16.5% SDS-PAGE. After electrophoresis, *c*-type cyts were detected by TMBZ staining. Lane 1; MT1131/pOX15, lane 2: GK32, lane 3: GK32/pOX15, line 4: GK32/pOX15S333A, lane 5: GK32/pOX15S340A, lane 6: GK32/pOX15T350A, lane 7: GK32/pOX15N390A and lane 8: GK32/pOX15T396A. The cyt c_p and c_o are the subunits II and III of cyt cbb_3 oxidase, and cyts c_1 and c_y correspond to the cyt c_1 subunit of the bc_1 complex and the membrane-attached electron carrier c_y.

tetrametylbenzidine-mediated heme peroxidase activity reveals two strong bands with apparent molecular masses of 32 and 28 kDa which correspond to the two *c*-type heme containing subunits CcoP and CcoO, respectively. Figure 5 shows that the Ser333Ala, Ser340Ala, Thr350Ala and Thr396Ala mutant strains have similar levels of CcoP and CcoO in the membrane as the positive control strains. However the Asn390Ala mutant showed significant differences in their cyt *c* profile. The 32-kDa subunit CcoP (cyt c_o) was missing, while the 28-kDa subunit CcoO (cyt c_o) was reduced in the Asn390Ala mutant. Overall, the data in Fig. 5 suggests that Asn390Ala mutation leads to either some assembly defect or instability in the cbb_3 oxidase complex in the membrane which results in an almost complete loss of oxidase activity. Thus, Asn390A mutant is considered to be good candidate for purification and the further spectrophotometric analysis.

Discussion

Sequence comparisons indicate that despite a conserved membrane topology and metal ligands the catalytic subunits (CcoN) of the cbb_3-type oxidases are quite distinct from subunit I both the type A (aa_3-type oxidases) oxidases and the aberrant type B (ba_3-type oxidases) oxidases. Specifically none of the elements of the D-channel are present and the tyrosine on helix VI (Tyr 280 in *R. sphaeroides* cytochrome aa_3) which is covalently linked to a histidine ligand of Cu_B and terminates the K-channel is absent. Without an authentic three-dimensional structure of a cbb_3-type oxidase and limited experiments that inform on the roles of individual residues in CcoN, it is not yet possible to conclude whether cytochrome cbb_3 oxidases have independently evolved a distinctive method for coupling oxygen reduction to proton translocation. An alternative possibility is that the mechanism of energy transduction might be functionally conserved in the cbb_3-type oxidases. Homology modeling (Sharma *et al.* 2006) and mass spectrometric analysis (Rauhamaki *et al.*, 2006) of the CcoN subunit of the *R. sphaeroides* cbb_3 type oxidase lend some support to that view. It appears that in the cbb_3 oxidases and that it is possible to identify a tyrosine helix VII (Tyr311 in the *R. sphaeroides* cbb_3 type oxidase) that forms a covalent link to the histidine residue in helix VI that ligates Cu_B. Moreover this tyrosine could cap an alternative K-channel which would include Ser340 which was one of the targets for mutagenesis in the present study, which also included Ser333, and Thr350 in helix VIII and Asn390 and Thr396 which are located in the loop between helix IX and helix X.

Helix VIII. In the *R capsulatus* cbb_3 oxidase, two polar residues in helix VIII, serine (Ser333) and threonine (Thr350), are absolutely conserved whilst a third residue serine (Ser340) is represented by threonine/serine represents in almost all other oxidases (Fig. 2). Of these three conserved residues only Ser340 has been definitely implicated in the alternative K-channel, although others may have a role. Our data suggests that none of these residues are critical for maintaining either the structure or the function of the oxidase in the membrane. However, substitution of Ser333 with alanine was found to significantly reduce oxidase activity (15% of wild type), whilst substitution of Ser340 with alanine reduced the activity to about 50% of wild type enzyme.

It has been established for a number of years that the helix VIII of bo_3-type quinol oxidase of *E. coli* has a highly conserved face containing several polar residues which could play a role in facilitating proton and/or water movement during turnover of the oxidase. These include the highly conserved polar residues, Thr352 and Thr359 (homologous to Ser333 and Ser340 of cbb_3 oxidase in *R. capsulatus*), which form part of the typical K-channel of Type A oxidases. Mutation of either of these two residues caused a virtual elimination of oxidase activity (Thomas *et al.*, 1993). Substitution of the equivalent residues, Thr352 and Thr359, by alanine in the *R. sphaeroides* cytochrome aa_3, also indicates the importance of these residues for the (re-)reduction of the active site of the oxidase during turnover (Hosler *et al.*, 1996).

It would appear that our data is consistent with the phenotypes of K-channel mutants in the canonical oxidases. Further studies using purified enzyme will be required to confirm the proposed role of these two residues in this pathway. Further investigation of the Thr350Ala mutant will also be on interest as there is little information on the role of this conserved in any class of oxidase yet our data clearly shows that mutation leads to impairment of activity.

IX-X Loop. Residues corresponding to Asn390 have been intensively studied in aa_3-type oxidase of *P. denitrificans*

(Pfitzner *et al*., 2000) and *R. sphaeroides* (Qian *et al*., 1997). Replacements of the aspartate (Asp399), located at this position of aa_3-type oxidase in *P. denitrificans* showed that the aspartate residue (Asp399) on the A-type oxidase (aa_3) of *P. denitrificans* plays an essential role at the subunit I/II interface on the periplasmic side. Three different replacements at this position (Asp399Leu, Asp399Asn and Asp399Glu) have been studied. Although Asp399Leu mutant led to almost complete inhibition of catalytic activity, both the Asp399Asn and Asp399Glu mutants result in a moderate reduction of the activity (60% and 40%, respectively) (Pfitzner *et al*., 2000).

Previously, aspartate (Asp399) had been assumed to play a role at the potential proton exit site (Iwata *et al*., 1995; Thomas *et al*., 1993; Pfitzner *et al*., 1998; Behr *et al*., 1998). Substitutions of aspartate of cytochrome aa_3 oxidase in *P. denitrificans* for asparagine and glutamate, showed that a hydrophobic residue in this position may cause a structural change, possibly altering the H-bonding pattern around the propionate of heme a_3 (Behr *et al*., 1998). Two residues in the periplasmic loop between helices IX and X, aspartate (Asp412) and threonine (Thr413), are also functionally conserved and, in the case of the type-A heme copper oxidase (*R. sphaeroides*), have been proposed to play role in the exit of pumped protons and capping the active site, respectively (Hosler *et al*., 1994).

Substitution of the asparagine residue at position 390 in subunit I of the *R. capsulatus* cbb_3-type oxidase leads to a lack stability and loss of activity. As demonstrated in Fig. 5, CcoO is either not made or easily degraded, moreover CcoP is barely detectable. This suggests a role for this residue in stabilizing assembly of the complex by interacting with one of the other subunits, probably CcoO, with which CcoN can form a catalytically competent sub-complex (de Gier *et al*., 1996; Zuffery *et al*., 1996). The lack of CcoO means that there is no route for the electrons to enter the enzyme complex from TMPD and accounts for the complete loss of activity.

There is less data on the effects of mutating residues equivalent Thr396 in subunit I of the *R. capsulatus* cbb_3-type oxidase in the canonical oxidases. In the aa_3 oxidase from *R. sphaeroides* substitution of Thr413 with asparagine does not perturb the spectroscopic properties of the enzyme and causes slightly lower electron transfer activity and normal proton pumping (Hosler *et al*., 1994; Fetter *et al*., 1995). Consequently it is of some interest that in *R. capsulatus* cbb_3-type oxidase substitution of Thr396 with alanine leads to a 55% loss of activity. Purification of this mutant form of the cbb_3 oxidase will reveal if this loss of activity is due to perturbation of the environments of the hemes as has been demonstrated for loopIX-X mutants in the class A oxidases (Hosler *et al.*, 1994) This is the first study using site-directed mutagenesis of the conserved residues on helix VIII and the loop between helix IX and X of the type C cytochrome oxidase (the cbb_3 oxidase). We have constructed five mutant forms of the enzyme in which changing conserved polar residues to alanine leads to as loss of activity and assembly of a stable oxidase complex. All five are excellent candidates for purification and further analysis of the electron and proton-transferring pathways which will doubtless inform on the exact function(s) of these amino acids.

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