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ABSTRACT

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Diss.

Cytochrome *c* oxidase is the last enzyme in mitochondrial and many bacterial respiratory chains. It catalyzes electron transfer from cytochrome *c* to molecular oxygen and transfers free energy of this reaction into a transmembrane proton electrochemical gradient. In bacteria, there are two main classes of terminal oxidases, cytochrome *c* - and quinol oxidases. The main difference between these oxidases lies in subunit II that is also believed to be the electron entry site. Cytochrome *c* oxidases contain a redox-active copper centre (Cu_A) in subunit II, whereas this copper centre has been lost from quinol oxidases during evolution.

In the present work, the C-terminal domain of subunit II from various bacterial terminal oxidases was expressed in *Escherichia coli* as a soluble protein. Using site-directed mutagenesis, it was possible to engineer copper centres similar to Cu_A and blue copper into the originally copperless quinol oxidase domain. This shows that subunit II in quinol- and cytochrome *c* oxidases must have a similar three-dimensional structure. Spectroscopic studies on the isolated *Paracoccus denitrificans* Cu_A-binding domain showed that it is similar to the centre A of nitrous oxide reductase. The spectrum of the isolated Cu_A centre is sensitive to pH, which is not the case for the Cu_A in the intact oxidase. The biochemical copper quantification and electrospray mass spectrometry showed that the engineered Cu_A-like centre as well as the isolated *Paracoccus* Cu_A centre contain two coppers. The EPR spectra, together with the quantification of EPR-detectable coppers, indicated that the two coppers form a dinuclear, mixed valence [Cu(1.5)...Cu(1.5)] centre. Site-directed mutagenesis showed that these coppers are ligated by two cysteine and two histidine residues.

The interaction of the oxidized Cu_A-binding domain with reduced cytochrome *c* was studied by stopped-flow spectroscopy. The reaction follows monophasic kinetics, indicating the presence of only one catalytically active cytochrome *c* binding site in this domain. The k_{on} rates and dissociation constants were similar to the ones that have been obtained earlier with the intact cytochrome *c* oxidase. This indicates that the Cu_A-binding domain contains a complete cytochrome *c* binding site. By site-directed mutagenesis, five conserved residues (two aspartates, two glutamates and a glutamine) in the Cu_A-binding domain were identified as cytochrome *c* binding residues.

Keywords: Electron transfer; cytochrome *c* oxidase; quinol oxidase; dinuclear Cu_A; *Escherichia coli*; *Paracoccus denitrificans*; site-directed mutagenesis.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals:

I Van der Oost, J., Lappalainen, P., Musacchio, A., Warne, A., Lemieux, L., Rumbley, J., Gennis, R. B., Aasa, R., Pascher, T., Malmström, B. G. & Saraste, M. 1992: Restoration of a lost metal-binding site: Construction of two different copper sites into a subunit of the *E.coli* cytochrome *o* quinol oxidase complex. - EMBO J. 11: 3209-3217.

II Kelly, M., Lappalainen, P., Talbo, G., Haltia, T., van der Oost, J & Saraste, M. 1993: Two cysteines, two histidines, and one methionine are ligands of a binuclear purple copper center. - J. Biol. Chem. 268: 16781-16787.

III Lappalainen, P., Aasa, R., Malmström, B. G. & Saraste, M. 1993: Soluble Cu_A-binding domain from the *Paracoccus* cytochrome *c* oxidase. - J. Biol. Chem. 268: 26416-26421.

IV Lappalainen, P., Watmough, N. J., Greenwood, C. & Saraste, M. 1995: Electron transfer between cytochrome *c* and the isolated Cu_A domain: Identification of substrate-binding residues in cytochrome *c* oxidase. - Biochemistry (in press).

V Farrar, J. A., Lappalainen, P., Zumft, W.G., Saraste, M. & Thomson, A. J. 1995: Spectroscopic and mutagenesis studies on the Cu_A centre from cytochrome *c* oxidase complex of *Paracoccus denitrificans*. (Submitted for publication in Eur. J. Biochem.)

Abbreviations:

CD	circular dichroism
ENDOR	electron nuclear double resonance
EPR	electron paramagnetic resonance
EXAFS	extended X-ray absorption fine structure
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
FPLC	fast protein liquid chromatography
Im^-	imidazolate
$ImHH^+$	imidazolium
K_D	dissociation constant
N_2OR	nitrous oxide reductase
ORF	open reading frame
PCR	polymerase chain reaction
NAD^+	nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonance
MCD	magnetic circular dichroism
Q	ubiquinone
QH_2	ubiquinol

CONTENTS

1 INTRODUCTION

Copper has a significant role in biological systems. In humans, it is the third most abundant trace element, after iron and zinc. Like most metals in living cells, copper carries out its various physiological functions in association with specific proteins. The function of copper proteins ranges from electron transfer and oxygen transport to active chemistry, such as incorporation of oxygen into substrates (reviewed by Adman 1991).

Copper belongs to the transition metals, which are characterized by having partially filled *d*-orbitals in many of their compounds. An important feature for the biological function of the transition metals is that they generally have more than one relatively stable valence state. In biological systems Cu^{2+} - Cu^{+} is a common oxidation-reduction system. Cu^{+} has all its *d*-orbitals filled and hence a spherically symmetrical electron cloud. Because Cu^{+} contains no unpaired electrons, its complexes are always diamagnetic and generally colourless. In contrast, the complexes of Cu^{2+} are generally coloured. Since Cu^{2+} has one electron less than can be accomodated by the five *d*-orbitals, it has an unpaired electron in all mononuclear complexes. The complexes are therefore paramagnetic and characterized by a spin of 1/2 (reviewed by Malkin & Malmström 1970). Because of the paramagnetism and coloured properties of Cu^{2+} compounds, many spectroscopic methods, in addition to classical biochemical methods, have turned out to be important for the current understanding of copper proteins. Electron paramagnetic resonance (EPR), electronic absorption, magnetic circular dichroism (MCD) and Raman spectroscopy have been important for probing the enviroment of the copper ions in proteins (see Darnall & Wilkins 1980).

According to the spectroscopic properties, copper-proteins have been classified into three categories (Malkin & Malmström 1970, Fee 1975).

Type 1 or blue copper proteins are characterized by an intense absorption near 600 nm and a relatively small hyperfine constant (A_{II}) in the EPR spectra. The single-domain blue copper proteins are invariably involved in electron transfer. X-ray diffraction and nuclear magnetic resonance (NMR) studies on several small blue copper proteins have shown that they are structurally homologous. The greek key b-barrel structure typical to the type 1 blue copper proteins is called the cupredoxin fold (reviewed by Adman 1991). Type 2 copper proteins are typically involved in chemical reactivity. Compared to the blue copper proteins, the EPR spectra of the type 2 copper sites have larger hyperfine constants that are comparable to those found in most Cu^{2+} complexes. The optical properties of the type 2 copper proteins are not clearly defined. In general, the extinctions are much lower than in blue copper proteins, but significantly higher than those found in most Cu^{2+} complexes (Malkin & Malmström 1970). Proteins containing the type 3 copper site form a structurally and functionally heteogeneous class. This copper site is characterized by antiferromagnetic coupling of a pair of copper atoms and a strong absorbance at 330 nm (Adman 1991). Recently, a fourth class of copper sites has been discovered. It consists of the electron transfer centres of nitrous oxide reductase (centre A) and cytochrome *c* oxidase (Cu_A centre) (reviewed by Kroneck et al. 1990, Malmström & Aasa 1993). The spectroscopic and biochemical features of this class of copper sites will be discussed in the following chapters.

2 REVIEW OF LITERATURE

2.1 Respiratory chains

2.1.1 Mitochondrial respiratory chain

The mitochondrial respiratory chain transfers electrons through a redox potential span of 1.1 V from the $NAD^+/NADH$ couple to the O_2/H_2O couple. The respiratory enzymes convert free energy released by this electron flow into a transmembrane electrochemical proton gradient. This gradient, referred to as the proton motive force, is used for energy-requiring reactions such as the synthesis of ATP, transport of ions and metabolites, and in the case of micro-organisms, for cell motility. In mitochondria, the respiratory enzymes are located in the inner membrane where the energy-yielding reactions result in outward proton translocation (reviewed by Hatefi 1985, Nicholls & Ferguson 1992, Trumpower & Gennis 1994).

Using detergents such as cholate and deoxycholate, the mitochondrial respiratory chain can be fractionated into four protein complexes (see Fig.1). NADH dehydrogenase (Complex I) catalyses the reaction:

$$NADH + ubiquinone + 5\ H^+(N) = NAD^+ + ubiquinol + 4\ H^+(P)$$

where *(N)* and *(P)* refer to the negative inner and positive outer side of the mitochondrial inner membrane. The enzyme has been isolated and characterized from bovine heart and filamentous fungus *Neurospora*

crassa. It is comprised of approximately 40 different subunits (reviewed by Weiss et al. 1991, Walker 1992). Seven subunits are encoded by mitochondrial DNA, and these subunits are probably located in the hydrophobic transmembrane fragment of the enzyme (Gibb & Ragan 1990). NADH dehydrogenases contain noncovalently bound flavin mononucleotide (FMN) and four iron-sulphur clusters as prosthetic groups. FMN appears to be the primary electron acceptor in Complex I. The electrons are transferred via the iron-sulfur clusters to ubiquinone. Little is known about the coupling of electron transfer to proton translocation in NADH dehydrogenase (reviewed by Weiss et al. 1991, Walker 1992). The structure of Complex I from *N.crassa* has been studied by electron microscopy of two-dimensional membrane crystals. The enzyme was found to be L-shaped. One arm is probably mainly buried in the membrane bilayer, whereas the other arm extensively protruds to one surface, probably into the matrix side in mitochondria (Leonard et al. 1987, Hofhaus et al. 1991).

In addition to Complex I, there are other pathways feeding electrons to ubiquinone. Succinate dehydrogenase (Complex II), and α-glycerophosphate dehydrogenase transfer electrons from redox couples with midpoint potentials close to 0 mV to the UQH_2/UQ couple with the midpoint potential of +60 mV. Consequently the limiting redox span (free energy) does not enable proton translocation (Hatefi 1985, Nicholls & Ferguson 1992). Succinate dehydrogenase that oxidizes succinate to fumarate, consists of four subunits. Two subunits form the soluble part of the complex and carry the succinate dehydrogenase activity, while the other two subunits are hydrophobic and anchor the catalytic subunits to the mitochondrial inner membrane. The catalytically active soluble part of the enzyme contains three iron-sulfur clusters and covalently bound FAD as prosthetic groups (Hatefi 1985). One of the anchoring subunits of bovine heart complex has been found to contain cytochrome b_{560}, but the role of this cytochrome in electron transfer has not yet been established (Yu et al. 1992).

Cytochrome bc_1 complex (Complex III) catalyses transfer of two electrons from ubiquinol to two molecules of cytochrome *c*. The reaction is coupled to proton translocation across the inner mitochondrial membrane:

$$QH_2 + 2\,H^+(N) + 2\,\text{cyt}\,c^{3+} = Q + 4\,H^+(P) + 2\,\text{cyt}\,c^{2+}$$

The mechanism of proton translocation in the bc_1 complex is relatively well understood (reviewed by Trumpower 1990). The so called Q cycle is a branched pathway of electron transfer from ubiquinol to cytochrome *c*. The protons are translocated by the cycling of quinol (QH_2) molecules from the negative to the positive side of the membrane and quinone (Q) from the positive to the negative side (Mitchell 1976). As in the case of Complex I, the best structural information on the cytochrome bc_1 complex is based on electron microscopic studies of membrane crystals (Karlsson et al. 1983). Biochemical experiments have shown that the mitochondrial cytochrome

bc$_1$ complex contains 9-11 subunits. Three of these contain all catalytically active prosthetic groups: two cytochromes *b*, cytochrome c_1 and a 2Fe:2S cluster in so called Rieske protein. The haems B and the iron-sulfur cluster are involved in the actual Q cycle, whereas the cytochrome c_1 is involved in the delivery of electrons to the soluble cytochrome *c*. The function of the additional subunits is still largely unknown. All subunits except for the cytochrome *b* are encoded by the nuclear DNA (reviewed by de Vries & Marres 1987, Trumpower & Gennis 1994).

Cytochrome *c* oxidase (Complex IV) is the last enzyme in the mitochondrial respiratory chain. It catalyses electron transfer from cytochrome *c* to molecular oxygen (reviewed by Babcock & Wikström 1992). The electron transfer is coupled to proton translocation in the following way:

$$4 \text{ cyt } c^{2+} + 8\ H^+(N) + O_2 = 4 \text{ cyt } c^{3+} + 4H^+(P) + 2\ H_2O$$

Cytochrome *c* oxidase consists of three mitochondrially-encoded subunits and up to ten nuclear encoded subunits. All the redox centres, two cytochromes and two copper centres, are located in two mitochondrially-encoded subunits (reviewed by Saraste 1990).

2.1.2 Bacterial respiratory chains

Oxidative phosphorylation is an important source of energy also in many genera of bacteria. The respiratory enzymes are located in the cytoplasmic membrane, and they translocate protons from the cytoplasm either to the periplasmic space (in Gram-negative bacteria) or to the extracellular mileu (in Gram-positive bacteria). Bacterial respiratory enzymes are structurally simpler and contain fewer subunits than their mitochondrial counterparts. This, together with the possibility of using random and site-directed mutagenesis, has made the bacterial proteins important for our current understanding of respiratory enzymes (reviewed by Anraku 1988, Nicholls & Ferguson 1992).

Bacterial respiratory systems have a greater diversity of electron transfer pathways than their mitochondrial counterparts. A bacterium may possess several different types of dehydrogenases and terminal oxidases, depending on available substrates and natural habitats. Bacterial respiratory components also interact with other electron transfer systems such as those involved in denitrification and photosynthesis (reviewed by Anraku 1988, Nicholls & Ferguson 1992). It is also typical for many bacteria that they are able to express alternative enzymes with similar activities. Yagi et al. (1988) have shown that *Thermus thermophilus* contains two different types of NADH dehydrogenases, one of which is homologous to the mitochondrial enzyme. Many bacterial species, like *Paracoccus denitrificans*, also express a number of different *c* -type cytochromes, which are able to transfer electrons to the terminal oxidases (Bolgiano et al. 1989)(see Fig.1).

Probably the most striking difference between the mitochondrial and bacterial respiratory systems lies in the terminal oxidases. All aerobic bacterial species that have been examined so far, are able to express multiple respiratory oxidases. Although these oxidases have different substrates (cytochrome *c* versus quinol), oxygen affinities and metal composition, most of them are members of the haem-copper oxidase superfamily (reviewed by Garcia-Horsman et al. 1994, Calhoun et al. 1994). The only well characterized exception is cytochrome *bd* quinol oxidase, which is not homologous to the haem-copper oxidases. (reviewed by Anraku 1988, Poole & Ingledew 1987).

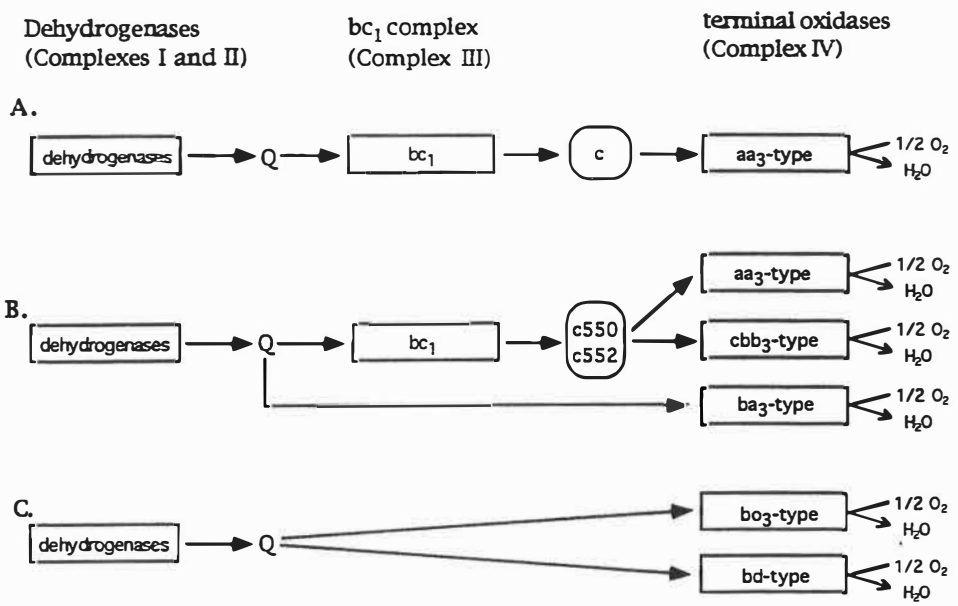

FIGURE 1. Model of respiratory chains in mitochondria and bacteria. The diagram summarizes the relationships between the electron transfer complexes in mitochondria (A), *Paracoccus denitrificans* (B) and *Escherichia coli* (C). In mitochondria, ubiquinone (Q) mediates electron transfer from the dehydrogenases to the cytochrome *bc1* complex, whereas cytochrome *c* transfers electrons from the cytochrome *bc1* complex to the terminal oxidase. *Paracoccus denitrificans* has three different types of terminal oxidases, two of which utilize cytochrome *c* as a substrate and one that is a quinol oxidase (de Gier et al. 1994, Richter et al. 1994). All the terminal oxidases of *Paracoccus* are members of the haem-copper oxidase superfamily. *Escherichia coli* is able to express two types of terminal quinol oxidases. Cytochrome *bd*, which does not belong to the haem-copper oxidase superfamily, has higher affinity for O_2 than cytochrome *bo3* (reviewed by Garcia-Horsman et al. 1994).

The expression and function of the terminal oxidases is regulated by enviromental and nutritional conditions. The most thoroughly characterized system at the moment is the regulation of *bo*- and *bd*-type terminal oxidases in *E.coli*. The expression of these oxidases is controlled at least by oxygen concentration and pH (Cotter et al. 1990). Models of the aerobic respiratory chains in mitochondria, *Paracoccus denitrificans* and *Escherichia coli* are shown in Fig.1.

2.2 Cytochrome oxidase

2.2.1 General features of haem-copper oxidases

The mammalian cytochrome *c* oxidase contains 13 subunits, three of which are encoded by mitochondrial DNA. Bacterial haem-copper oxidases typically consist of three subunits that have high sequence homology with the eukaryotic mitochondrially-encoded subunits (Saraste 1990). Although the bacterial oxidases are structurally simpler than their mitochondrial counterparts, they have been shown to pump protons and catalyse reduction of dioxygen as efficiently as the mitochondrial oxidases (Hendler at al. 1991, Hosler et al. 1992).

Bacterial terminal oxidases fall into two categories according to their electron donating substrate (see Fig.2). Bacterial cytochrome *c* oxidases, like their mitochondrial counterparts, receive electrons from cytochrome *c*. Quinol oxidases, that have so far been cloned and characterized from *Escherichia coli* (Chepuri et al. 1990), *Bacillus subtilis* (Santana et al. 1992), *Sulfolobus acidocaldarius* (Lübben et al. 1992) and *Paracoccus denitrificans* (Richter et al. 1994), use ubiquinol or menaquinol as a substrate.

The common feature for all haem-copper oxidases is that subunit I contains a hexa-coordinated low-spin haem and a binuclear center consisting of a copper atom (Cu_B) and a high-spin haem. A hexa-coordinated low-spin haem transfers electrons to the binuclear high-spin haem - Cu_B centre, where dioxygen is reduced to water. All members of the haem-copper oxidase superfamily have been shown to function as proton pumps (Calhoun et al. 1994, Raitio & Wikström 1994). The main structural difference between cytochrome *c*- and quinol oxidases concerns subunit II. Quinol oxidases do not contain any redox active metal centre in subunit II, whereas a typical cytochrome *c* oxidase has a redox active copper centre (Cu_A) in subunit II. However, bacterial cytochrome *c* oxidases also show many variations on the metal centres in subunit II. The *Bacillus* caa_3-type oxidases have a cytochrome *c* fused to subunit II (Saraste et al. 1991), and cbb_3-type oxidases have two membrane anchored cytochromes *c* instead of the classical Cu_A-binding subunit II as shown in Fig.2 (see Garcia-Horsman et al. 1994).

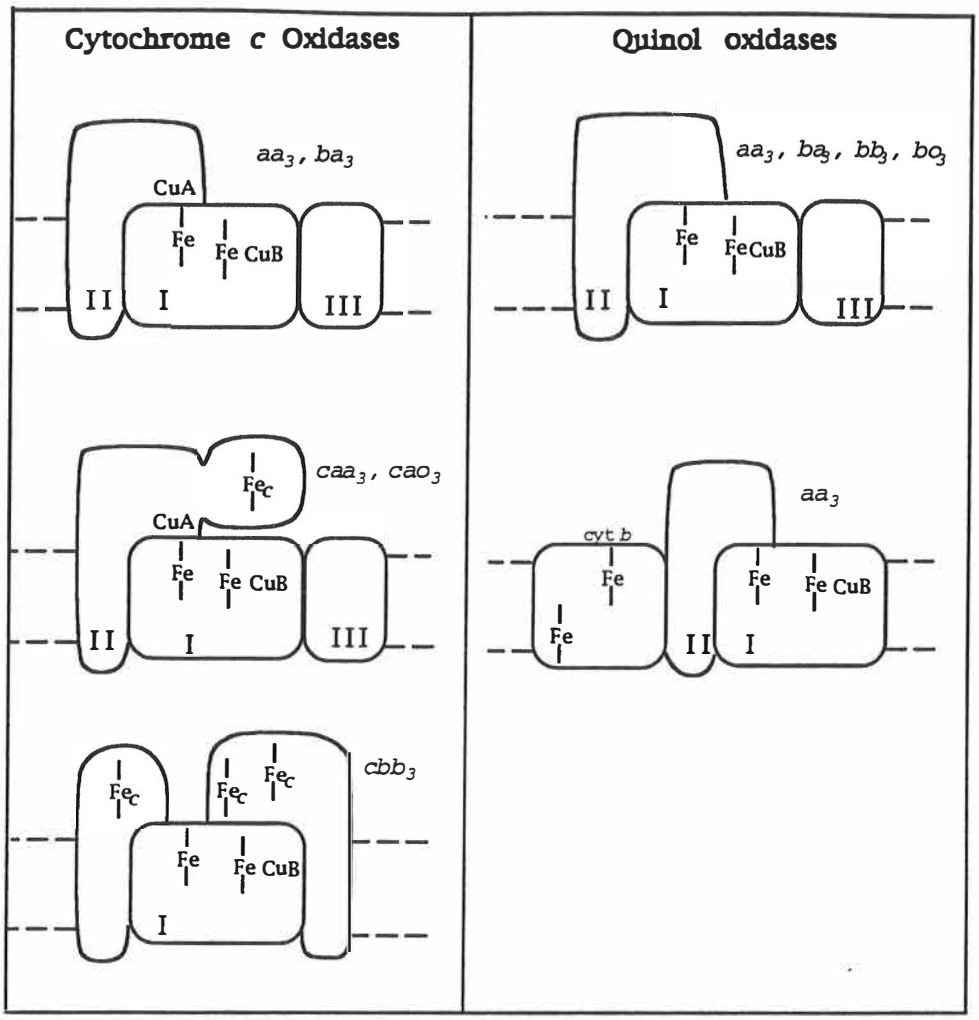

FIGURE 2. The different members of the haem-copper respiratory oxidase superfamily. The panel on the left illustrates the three classes of cytochrome *c* oxidases, and the right hand panel illustrates the two classes of quinol oxidases. All haem-copper oxidases share a homologous subunit I with identical sets of redox centres (a low-spin haem, a high-spin haem and Cu_B). The variety of haem combinations found in each subclass is shown in figure. Subunits that are homologous to the 'core' subunits of the mitochondrial cytochrome *c* oxidase are indicated by Roman numerals. Note that the *Sulfolobus acidocaldarius* aa_3-type quinol oxidase (below right) has probably a cytochrome *b* homologue complexed with the oxidase subunits I and II (Lübben et al. 1992). The figure is modified from Garcia-Horsman et al. (1994).

2.2.2 Subunit I

Subunit I of the haem-copper oxidases is a large membrane protein. According to hydropathy plots, this subunit contains 12 putative transmembrane helices in most oxidases. However, in the *E.coli* bo_3-type quinol oxidase, as well as in some related oxidases, it contains 3 additional transmembrane spans (Trumpower & Gennis 1994). The topology of this *E.coli* protein has been studied by using random gene fusions. These

experiments agree with the model that proposes 15 transmembrane segments (Chepuri & Gennis 1990). The location of the N- and C-termini on the periplasmic and cytoplasmic side of the membrane has been further confirmed by genetic fusions of subunits I, II and III of *E.coli* bo_3-type quinol oxidase (Ma et al. 1993).

Three different types of haems are found in the subunit I of haem-copper oxidases in a variety of combinations (see Fig.2). These are haems B (protohaem IX), O and A. It has been recently discovered that some Archaea also possess modified forms of haems O and A (Lübben & Morand 1994, Lübben et al. 1994a). Electron paramagnetic resonance (EPR) studies on oriented multilayer preparations of cytoplasmic membrane fragments have shown that both haems in the oxidases are oriented perpendicularly to the membrane plane (see Salerno & Ingledew 1991). Haem A or haem O is involved in the formation of the binuclear centre in the oxidases characterized so far. These haems have a hydroxyethyl-farnesyl side chain in position 2 of the porphyrin ring. It has been postulated, that this farnesyl side chain could be functionally important for the high-spin haem. However, the recent discovery of cbb_3-type cytochrome *c* oxidases argues against its essential role for the high-spin haem (reviewed by Mogi et al. 1994). The hexa-coordinated low-spin haem is responsible for the main part of optical absorption in red region. Haem B and haem O have very similar absorption spectrum, giving a red colour to the oxidase. Haem A has a formyl group that replaces a methyl group in position 8 of the porphyrin ring. The formyl group causes a shift in the spectrum which leads to a green colour for oxidases containing haem A in the low-spin position (reviewed by Garcia-Horsman et al. 1994).

Electron nuclear double resonance (ENDOR) and extended X-ray absorption fine structure (EXAFS) experiments have shown that Cu_B is coordinated to at least three nitrogens and probably also to one sulphur or chloride (Cline et al. 1983, Li et al. 1987). ENDOR has also provided evidence for bis-imidazole coordination of the low-spin haem (Martin et al. 1985). There are six histidines among the totally conserved residues in subunit I. The roles of these histidines as ligands for the haems and Cu_B have been confirmed by site-directed mutagenesis of the *E.coli* cytochrome bo_3 (Lemieux et al. 1992, Minagava et al. 1992) and the *R.sphaeroides* cytochrome aa_3 (Shapleigh et al. 1992). In addition to the histidines, a conserved tyrosine 288 in the cytochrome bo_3 of *E.coli* may be Cu_B ligand (Thomas et al. 1994). A detailed spectroscopic characterization of site-directed mutants has led to a consensus in the assignment of the ligands to the metal centres (Calhoun et al. 1993 a-c, Uno et al. 1994). The model for the coordination of the redox centres in the subunit I of the *E.coli* cytochrome bo_3 is shown in Fig.3.

Subunit I also contains several other well conserved residues in addition to the metal ligands. Site-directed mutagenesis have shown that the loop between the putative transmembrane helices IX and X forms a cap over the haem *a* - haem a_3 - Cu_B centre in the *R.sphaeroides* cytochrome aa_3 and is important for the structure of the active site (Hosler et al. 1994). Substitutions of conserved polar residues of the

transmembrane helix VIII and of the helix II>III loop of the cytochrome bo_3 subunit I lead to a loss of proton pumping activity without significantly affecting the spectroscopical properties of the redox centres. Therefore these residues might form a proton and/or water channels in the transmembrane part of subunit I (Thomas et al. 1993a,b).

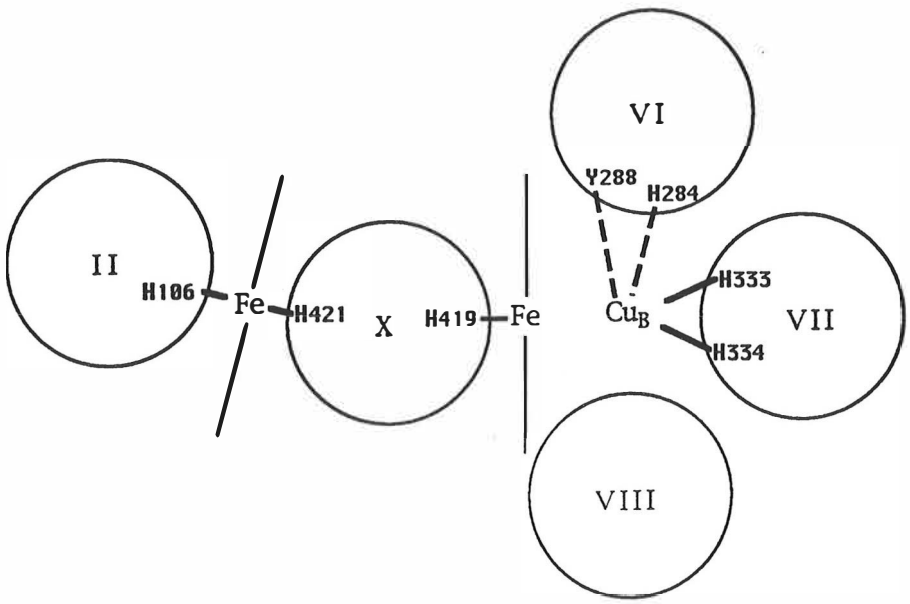

FIGURE 3. Model for the coordination of haem *b*, haem *o* and Cu_B in subunit I of the *Escherichia coli* bo_3-type oxidase. The six totally conserved histidines are located in helices II, VI, VII and X. Spectroscopic studies of site-directed mutants indicate that His106 and His421 are the axial ligands of the low-spin haem, whereas His419 is the proximal ligand of the high-spin haem (Calhoun et al. 1993b,c, Uno et al. 1994). Residues His333 and His334 have been confirmed to coordinate the Cu_B center (Calhoun et al. 1993a). The remaining invariant histidine (His284), which is probably also involved in coordination of Cu_B, has been proposed to function in proton translocation (Wikström et al. 1994). Recent studies indicate that the well conserved tyrosine (Tyr288) may also be a Cu_B ligand (Thomas et al. 1994).

The binuclear high-spin haem - Cu_B centre forms the catalytically active site of the oxidase, where dioxygen is reduced to water. Cu_B seems to function as a substrate entry point of the binuclear centre, controlling the access of exogenous ligands to the high-spin haem (Woodruff et al. 1991, Woodruff 1993). The two activities of the enzyme, oxygen reduction and proton translocation are structurally, kinetically and energetically intertwined. Catalysis is extremely rapid, since the maximal turnover of the isolated enzyme in steady state is more than 500 e^- per second. During catalysis, the binuclear centre cycles through intermediate states, which differ from each other depending on the valence, spin and ligation states

of the metals (reviewed by Babcock & Wikström 1992). Time resolved absorption (Han et al. 1990a) and Raman spectroscopy (Han et al. 1990b, Varotsis et al. 1993) have been valuable techniques in detecting these reaction intermediates. It is not yet fully understood how oxygen reduction is coupled to proton translocation. Wikström (1989) has shown that only two of the four electron transfers are coupled to proton translocation. Recently a chemical model for proton translocation has been proposed (Wikström et al. 1994). In this model, one of the histidine ligands of Cu_B cycles between its imidazolate (Im^-) and imidazolium ($ImHH^+$) forms. This could be a principal link between redox events and proton translocation.

2.2.3 Subunit II and the Cu_A centre

Subunit II is the most hydrophilic among the three core subunits of cytochrome oxidases. According to hydropathy plots, this subunit consists of a small hydrophilic N-terminal domain, two putative transmembrane helices connected by a short loop, and a larger hydrophilic C-terminal domain (Saraste 1990). However, subunits II in the oxidases of *S.acidocaldarius* and *Thermus thermophilus* are significantly smaller, containing only one predicted transmembrane helix and a hydrophilic C-terminal domain (Lübben et al. 1992, Mather et al. 1993). The topology of this subunit has been studied by gene fusions. These experiments have shown that the C-terminus of the *E.coli* cytochrome bo_3 is located in the periplasmic space (Chepuri & Gennis 1990, Ma et al. 1993).

It has been long predicted that subunit II of the cytochrome *c* oxidases harbours the Cu_A centre, since it shares sequence similarity with the small blue copper proteins (Steffens & Buse 1979). The fluorescence probe studies by Hall et al. (1988) showed that the Cu_A centre is indeed located in subunit II. In cytochrome *c* oxidases, the C-terminal domain of subunit II contains several totally conserved residues including two cysteines, two histidines, two aspartates and a glutamate. These residues are not found in quinol oxidases. It has been proposed that the cysteines and the histidines could coordinate the copper atom, whereas the carboxylates could be important for the interaction with cytochrome *c* (see Holm et al. 1987, Capaldi 1990). The cysteine-histidine ligation of Cu_A has been experimentally confirmed by ENDOR and EXAFS studies (Scott et al. 1986, Li et al. 1987, Martin et al. 1988). On the other hand, the sequence alignments between cytochrome *c* oxidase subunits II and nitrous oxide reductases suggests that these related copper centres would have six ligands: two cysteines, two histidines, one methionine and one aspartate (Steffens et al. 1993).

The optical spectrum of Cu_A is mostly covered by the strong absorption of the low-spin haem. However, MCD spectra have shown that Cu_A has transitions throughout the spectral region from 450 to 950 nm (Greenwood et al. 1983). EPR, MCD and EXAFS studies have shown that the Cu_A centre of cytochrome *c* oxidase is structurally similar to the

electron transfer copper centre A in a bacterial denitrification enzyme, nitrous oxide reductase (N_2OR)(Scott et al. 1989, Kroneck et al. 1990, Farrar et al. 1991). The EPR spectra of both centres have several unusual features. They both have anomalously low g-values as well as a narrow hyperfine coupling and an unusual broadening of spectra at temperatures above 150 K (Malmström & Aasa 1993). The latter has been proposed to arise from the interactions between Cu_A and other metal centres. However, recent resonance Raman studies indicate that Cu_A is independent of the other redox centres, and therefore its peculiar spectroscopic features must be due to the structure of the copper centre itself (Takahashi et al. 1993).

Two different models have been proposed in order to explain these features. According to the 'classical' model the Cu_A centre is mononuclear. The unusual EPR spectrum is explained as arising from delocalization of the unpaired electron to one or two cysteine sulphurs, indicating that a covalent interaction may exist between copper and cysteine ligands (Stevens et al. 1982, Martin et al. 1988). In the second model, the spectroscopic properties are explained by the dinuclear nature of Cu_A. The latter model was originally based on spectroscopic studies on the centre A of N_2OR. The EPR spectrum of N_2OR contains a well-resolved seven-line hyperfine structure at 9 GHz (X-band) with an intensity ratio 1:2:3:4:3:2:1 (Kroneck et al. 1988). In the intact cytochrome *c* oxidase, this hyperfine structure cannot be resolved at 9 GHz, but a weak seven-line signal has been detected at a lower frequency (S-band) (Antholine et al. 1992). Kroneck et al. (1988) have suggested that this feature arises from mixed valence [Cu(1.5)....Cu(1.5)] S=1/2 species with the unpaired electron delocalized between the two equivalent copper nuclei. The dinuclear nature of Cu_A has been also supported by several quantitative copper measurements, showing that cytochrome *c* oxidases contain approximately 2.5-3 copper atoms/2 hemes (Steffens et al. 1987, Öblad et al. 1989, Steffens et al. 1993). However, in contrast to the dinuclear model of Cu_A, Pan et al. (1991) have suggested that the extraneous copper of cytochrome *c* oxidase could be associated with subunit III and would play a role in the enzyme dimerization.

2.2.4 Subunit III

The hydropathy plot of subunit III suggests that this protein contains seven putative transmembrane helices in a typical cytochrome *c* oxidase. The *E.coli* cytochrome bo_3 as well as some other bacterial oxidases have a short version of subunit III with only five transmembrane segments. However, in these cases the oxidase complexes have a longer version of subunit I, indicating that the two first N-terminal helices of subunit III can be "moved" to the C-terminus of subunit I (Saraste 1990, Trumpower & Gennis 1994). In the thermophilic organisms *Sulfolobus acidocaldarius* and *Thermus thermophilus*, a genetic fusion between subunits I and III has also been reported (Mather et al. 1993, Lübben et al. 1994b).

The function of subunit III is not known. A gene deletion study has shown that it is important for the assembly of functional oxidase (Haltia et al. 1989). This subunit harbours two conserved carboxylates in the middle of hydrophobic sequences, which have been proposed to be involved in proton translocation. However, the substitution of these residues in the *Paracoccus* cytochrome aa_3 results in a structurally and functionally normal enzyme (Haltia et al. 1991). Calorimetric studies using bacterial and mitochondrial oxidases have shown that subunits I and II form a highly cooperative complex, while subunit III is less stable and and forms a structurally independent domain (Rigell et al. 1985, Rigell & Freire 1987, Haltia et al. 1994). Subunit III is also often partially lost during enzyme purification, indicating that it interacts loosely with subunits I and II. The remaining two-subunit version of the *P.denitrificans* oxidase has been shown to be a proton pump (Hendler et al. 1991). However, recent studies by Haltia and co-workers have shown that the absence of subunit III in the *P.denitrificans* cytochrome aa_3 leads to a turnover-induced loss of electron transfer activity (Haltia et al. 1994).

2.3 The reaction of cytochrome *c* oxidase with cytochrome *c*

Cytochrome *c* oxidases react with reduced cytochrome *c* with rates around 10^7 $M^{-1}s^{-1}$, and the formation of the complex between cytochrome *c* and oxidase is believed to be the rate-limiting step in this reaction (Andreasson 1975, Antalis & Palmer 1982). This fast reaction phase is followed by a much slower phase with a rate of 1-3 s^{-1}. The origin of the biphasic kinetics is not well understood. Purified mammalian cytochrome *c* oxidase has been shown to bind two molecules of cytochrome *c* in two distinct binding sites with $K_D=10^{-8}$ M and $K_D=10^{-6}$ M (Ferguson-Miller et al. 1976, Rieder & Bosshard 1978). It has been suggested that the two kinetic phases could be due to two distinct high- and low- affinity binding sites for cytochrome *c* (Ferguson-Miller et al. 1976, Garber & Margoliash 1990). In contrast, transient-state kinetic studies by Antalis & Palmer (1982) suggested that cytochrome oxidase contains only one kinetically active cytochrome *c* binding site, with $K_D=10^{-6}$ M. Brzezinski & Malmström (1986) have further suggested that the biphasic kinetics could arise from two different conformational stages of the proton-pumping enzyme in which the primary electron acceptor has different reduction potentials.

The interaction between cytochrome *c* and cytochrome *c* oxidase is strongly dependent on ionic strength, indicating that electrostatic interactions are important for the formation of the complex (Antalis & Palmer 1982). The interaction of cytochrome *c* with the high-affinity site has been shown to involve a ring of seven lysine residues around the haem edge (Rieder & Bosshard 1978). Subunit II in cytochrome *c* oxidase contains well conserved aspartates and glutamates, which are believed to interact with the conserved lysines in cytochrome *c* (Capaldi 1990, Saraste 1990). The location of the cytochrome *c* binding sites has been mapped

using several different methods including chemical modification (Seiter at al. 1979, Bisson & Montecucco 1982, Millett et al. 1983), cross-linking (Erecinska 1977, Briggs & Capaldi 1978) and monoclonal antibodies (Taha & Ferguson-Miller 1992). The results indicate that in the mitochondrial oxidase, the high-affinity binding site is located in subunit II, with some nuclear-encoded subunits constituting part of the binding site. The location of the kinetically active cytochrome *c* binding site in subunit II is also supported by kinetic electron transfer studies, which have shown that the Cu_A centre is the primary electron acceptor in cytochrome *c* oxidase (Hill 1991, Nilsson 1992, Pan et al. 1993).

2.4 Evolution of cytochrome oxidase

Aerobic organisms are found in all three major domains of living systems: eubacteria, archaebacteria and eukaryotes. In eukaryotes, aerobic respiration occurs in mitochondria and photosynthesis takes place in chloroplasts. Mitochondria are derived from purple bacteria, whereas chloroplasts are derived from the endosymbiosis of cyanobacteria. Since cytochrome oxidases and aerobic respiration are found in all three major kingdoms of life, it was believed that respiration evolved separately in these evolutionary lines after the atmospheric oxygen concentration increase due to the emergence of photosynthesis (Broda & Peschek 1979). However, during the past years cytochrome oxidase genes from a number of different sources, including archaebacteria, have been cloned and sequenced. A comprehensive evolutionary analysis of these sequences has demonstrated that all haem-copper oxidases are related, and therefore derived from a common ancestor. The ancient oxidase has been present in the common ancestor of archaebacteria and eubacteria, whereas oxygenic photosynthesis has evolved later in eubacteria. Furthermore, phylogenetic trees show that quinol oxidases have evolved from cytochrome *c* oxidases (Castresana et al. 1994)(see Fig.4). The data proposing that respiration evolved prior to photosynthesis is further supported by the discovery of a new class of cytochrome *c* oxidases from *Bradyrhizobium japonicum* (Preisig et al. 1993). This novel class of oxidases functions in an enviroment with a very low oxygen concentration, and according to phylogenetic trees, it seems to be the most ancient type of cytochrome oxidase (see Fig.4).

The recent sequencing of the operon encoding nitric oxide reductase (Zumft et al. 1994) has revealed interesting relationship between respiration and denitrification. Nitric oxide reductase contains two subunits, NorB and NorC. Saraste & Castresana (1994) and Van der Oost et al. (1994) have noticed that NorB shares significant sequence homology with subunit I of the haem-copper oxidases. It contains the conserved histidines that are involved in the coordination of the redox centres in subunit I. A phylogenetic tree that is based on an alignment of bacterial and archaean oxidases with NorB, shows that NorB is the most distant member of the haem-copper oxidase superfamily (Fig.4). This suggests that

respiration might have evolved from denitrification (Saraste & Castresana 1994). A second link between cytochrome *c* oxidases and denitrification enzymes is Cu_A. The Cu_A-binding domain of subunit II is homologous to the C-terminal Cu-binding sequence of N_2OR (Buse & Steffens 1991, Zumft et al. 1992). It is possible that the Cu_A site has evolved from the related site in N_2OR (Saraste & Castresana 1994).

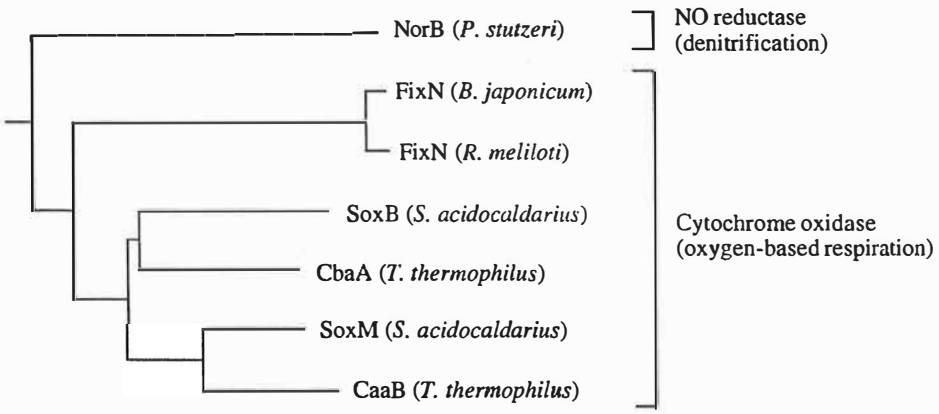

FIGURE 4. A phylogenetic tree of subunit I of cytochrome oxidases and the integral membrane subunit (NorB) of nitric oxide reductase. The most divergent members of the oxidase family have been chosen. The phylogenetic tree shows that NorB is the most divergent member of the oxidase superfamily. FixN is the closest branch to NorB, suggesting that the latter is related to the primitive cytochrome oxidase. Figure is taken from Saraste & Castresana (1994).

3 AIM OF THE STUDY

As discussed in chapter 2.2.3, the Cu_A centre in cytochrome *c* oxidase is probably located in the hydrophilic C-terminal domain of subunit II. This domain harbours also several conserved carboxylates, that are believed to form the cytochrome *c* binding site (Capaldi 1990, Saraste 1990). The structure of the Cu_A centre has been the focus of several studies during recent years. EPR data and quantitative copper/protein measurements have indicated that this centre might contain two copper atoms (Malmström & Aasa 1993). However, since cytochrome *c* oxidase is a large multi-subunit enzyme and contains other metal centres in addition to Cu_A, detailed examination of this copper centre has been difficult. The present work has focused to isolating Cu_A-binding domains from cytochrome *c* oxidases. These domains would be free from any other metal centres, and hence suitable for a number of biochemical studies. More specifically, the aims of this study have been:

1. To express Cu_A-binding domains from different bacterial sources for biochemical, spectroscopic and structural studies.

2. To quantify the number of coppers in the Cu_A centre - is the Cu_A a mononuclear or a dinuclear copper centre?

3. To identify the Cu_A-binding residues by site-directed mutagenesis.

4. To identify the cytochrome *c* binding residues in cytochrome *c* oxidase.

5. To crystallize the isolated Cu_A-binding domain, and to solve its three-dimensional structure.

4 MATERIALS AND METHODS

The materials and methods are described in detail in the original publications I-V.

Bacterial strains and plasmids

The expression of cytochrome oxidase domains was carried out in *E.coli* BL21(DE3)(F^-, *ompT*, r_B- m_B-, l lysogen containing the T7 RNA polymerase gene under the *lacUV5* promoter). The modified pET vector used as an expression plasmid was a derivative of pET3d (Studier et al. 1990, I). The *E.coli* cells were grown in Luria Broth medium at 37 °C under good aeration, and expression was induced with isopropyl-thio-β-D-galactoside as described (I, III). The *Paracoccus denitrificans* cytochrome *c-550* was purified from a strain carrying pEG400-*cyc*A -plasmid (obtained from Jan-Willem de Gier & John van der Oost, Free University of Amsterdam), grown at 30 °C on succinate-based minimal-medium (Ludvig 1986) under anaerobic conditions as described (IV).

Site-directed mutagenesis

The oligonucleotide primers for subcloning and mutagenesis are described in the original publications I-V. The mutations were introduced with polymerase chain reaction (PCR) following the overlap extension method (Higuchi et al. 1988). All PCR constructs were sequenced, and those containing undesired mutations were discarded. The standard methods of molecular biology are described by Ausubel et al. (1991).

Protein purification

The bacterial cells were broken by a French press. The membranes and insoluble fragments were pelleted by ultracentrifugation as described (I-V). *Paracoccus* cytochrome *c-550*, as well as the Cu_A domain from *Synechocystis PCC-6803* and *Rhodobacterium sphaeroides* were expressed as soluble proteins in the cytoplasm and purified from the supernatant by a combination of anion exchange and gel filtration chromatography. The *E.coli* CyoA fragments were purified from the soluble fraction by a combination of anion exchange, metal affinity and gel filtration chromatography (I). The wild type and mutant *P.denitrificans* Cu_A-domains formed inclusion bodies in the *E.coli* BL21(DE3) cells. These were solubilized in 7 M urea and the proteins were refolded by gradually decreasing the urea concentration as described (III). The refolded proteins were purified by anion exchange and gel filtration chromatography. All the columns were run with a Pharmacia FPLC instrument at room temperature.

Spectroscopic methods

Optical absorption spectra were recorded with a Perkin Elmer Lambda 2 spectrophotometer at room temperature. Circular dichroic (CD) spectra were recorded on a Jasco-710 instrument. The protein concentrations were 20 μM for near-UV CD and 40 μM for the temperature denaturation scanning. Electrospray mass spectra were recorded with a Sciex API III instrument. The EPR spectra in publication II were recorded with Bruker ER 200D-SRC X-band spectrofotometer. The EPR and MCD -spectra in publication V were recorded with a Bruker ER-200D and JASCO J-500D instruments respectively.

Rapid kinetic measurements

The kinetics of the electron transfer reaction between reduced cytochrome *c* and the oxidized Cu_A-binding domain was monitored by stopped-flow spectroscopy. The oxidation of cytochrome *c* was followed by the decrease of absorbance at 550 nm and the reduction of Cu_A by the decrease of absorbance at 800 nm. Before the experiments, the samples of *Paracoccus* (see above) and horse heart (type IV, Sigma) cytochromes *c* were reduced with an excess of sodium dithionite or ascorbate. The reducing agent was subsequently removed by gel filtration in Sephadex G-25 columns (PD-10, Pharmacia). The stopped-flow experiments were carried out with a Bio-Logic (SMF-3) apparatus at EMBL and with Bio-Sequential apparatus at the University of East Anglia as described in (IV).

Other methods

The millimolar extinction coefficients at 278 nm for the purified proteins were based on protein concentrations determined by quantitative amino

acid analysis. The copper content of the protein samples was determined by the biquinone method (Broman et al. 1962). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was carried out using the buffer system of Laemmli (1970). Protein crystallization was performed using the vapour diffusion method. The search for the initial crystallization conditions was carried out by factorial experimental design (Carter 1992).

5 REVIEW OF THE RESULTS

5.1 Spectroscopic properties of isolated Cu_A-domains

In the intact cytochrome *c* oxidase, the optical spectrum of Cu_A is covered by the strong absorption of the low-spin haem. For this reason the optical spectrum of Cu_A in the 400 - 700 nm region cannot be resolved, and only a weak absorption peak around 800 nm has been assigned to Cu_A (Malmström and Aasa 1993). However, the EPR spectrum of Cu_A is similar to the spectrum of centre A in N_2OR, suggesting similarities also in the optical properties between these two copper centres (Kroneck et al. 1990).

We have expressed Cu_A-binding domains from aa_3-type cytochrome *c* oxidases of *Paracoccus denitrificans* (III), *Rhodobacterium sphaeroides* and *Synechocystis PCC-6803*. In addition, we have been able to reconstitute a Cu_A-like and blue copper centre into the homologous CyoA subunit of the *E.coli* cytochrome bo_3 quinol oxidase. These mutants were designed by using local sequence alignments of small blue copper proteins and subunit II in cytochrome *c* and quinol oxidases. The possible blue copper and Cu_A ligands were introduced into the soluble CyoA fragment (I). The comparison of optical spectra of the Cu_A-binding fragments is shown in Fig.5. These spectra have a strong absorbance maximum at 460-540 nm and a smaller peak around 800 nm. They are very similar to the optical spectra reported earlier for N_2OR (Riester et al. 1989). The presence of a peak at 800 nm region confirms its earlier assignment to Cu_A in the intact oxidase. These results are also in a good agreement with the MCD studies on intact

oxidase, which have demonstrated that Cu_A has transitions throughout the spectral region from 450 to 950 nm (Greenwood et al. 1983).

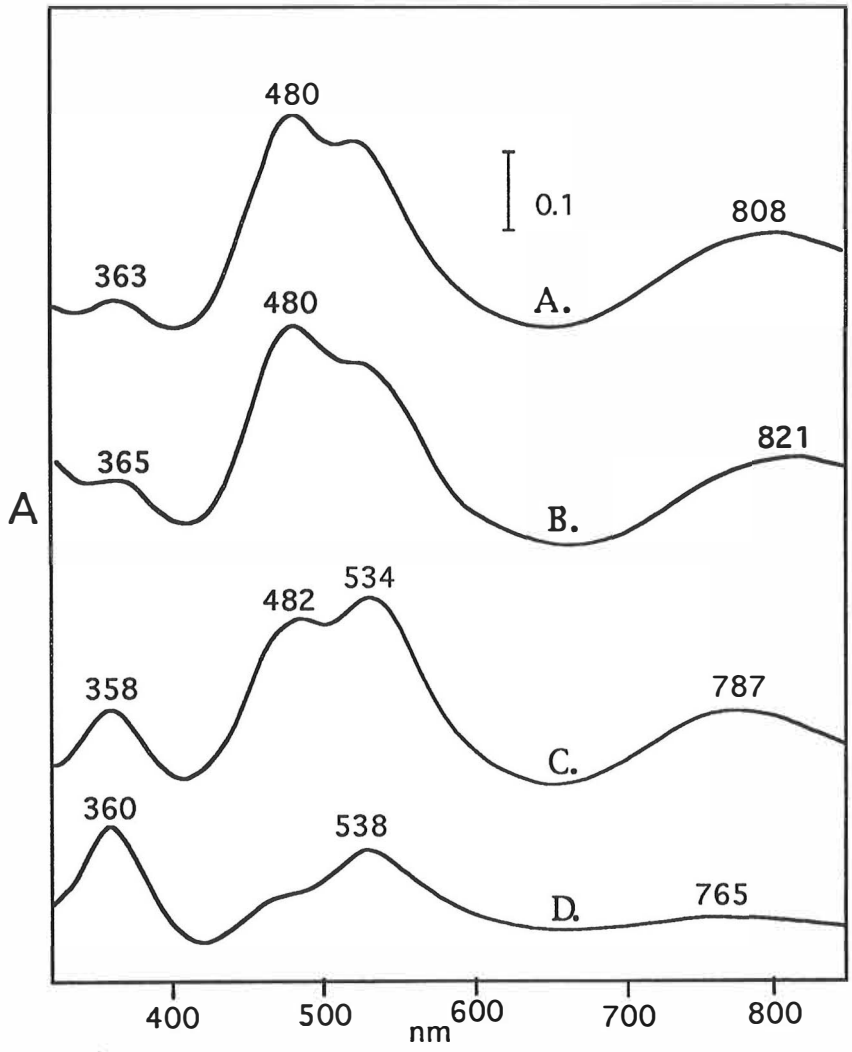

FIGURE 5. Optical spectra of isolated Cu_A-binding domains. The figure shows the spectra of the domains from *Paracoccus denitrificans* aa_3-type oxidase (A), *Rhodobacterium spheroides* aa_3-type oxidase (B), *Synechocystis PCC-6803* aa_3-type oxidase (C) and Cu_A-like site engineered into *Escherichia coli* bo_3-type oxidase (D). The spectra were recorded with 100 μM protein samples in 20 mM Bis-Tris (pH 7.0).

A special feature of these isolated cytochrome *c* oxidase Cu_A-binding domains is the pH-induced change of the spectra. At alkaline pH, there is a dramatic decrease in absorbance throughout the 400 - 850 nm region. In the case of the *Paracoccus* and *Rhodobacterium* domains, this feature is combined with a strong increase in absorbance at 370 nm. The pK for this

reversible change is about 8.2 (III). A similar pH dependence is not seen in the engineered Cu_A-like centre (I) or in the intact cytochrome *c* oxidase.

At neutral pH the X-band EPR spectra of the *Paracoccus* (III and V) and *Synechocystis* (unpublished observations) Cu_A-binding domains as well as the spectrum of engineered Cu_A-like centre (I), are nearly identical to the spectra earlier observed with the intact oxidase complex. As in the case of the intact oxidase, the clear seven-line hyperfine signal typical to N_2OR could not be detected with these Cu_A-fragments. The EPR spectra showed also a similar temperature-dependent broadening as observed with the Cu_A of the intact cytochrome oxidase (I, III). This confirms that the unusual saturation properties of Cu_A (reviewed by Malmström & Aasa 1993) are due to the peculiar structure of this copper centre rather than its interactions with other redox centres.

5.2 Cu_A is a dinuclear centre

In order to examine the number of coppers in the centre, we studied the copper content of the isolated domains by the analytical biquinoline method and electrospray mass spectrometry. In publication I, we compared the copper content of the native CyoA domain and the domain with the engineered CuA-like centre (purple CyoA). The purple CyoA contained approximately 1.1 extra coppers in comparison to the colourless wild type protein. However, also the native quinol oxidase domain seemed to bind copper, which caused difficulties in the accurate quantification. After the revised purification protocol (developed by Dr. Mark Kelly) the purple CyoA preparations were shown to contain approximately 1.5 coppers more than the wild type protein (II). The presence of two coppers in the engineered Cu_A-like centre was confirmed by electrospray mass spectrometry. The results from these experiments demonstrated that the mass of purple CyoA is 128 daltons higher than the one of the corresponding copperless apoprotein. This is almost exactly twice the mass of a copper atom (II).

Electrospray mass spectrometry was also applied to the *Paracoccus* Cu_A-binding domain (III). The mass difference between Cu_A-containing and apoprotein was approximately 128 Da, supporting the dinuclear nature of native Cu_A. This was confirmed by chemical copper/protein measurements, which gave a value of 1.9 (mol/mol) both for the low- and high-pH forms. In both, approximately 50 % of the copper was EPR detectable. In the case of low-pH (native) form, this is in a good agreement with the mixed valence nature of this dinuclear copper centre (III). At alkaline pH, the EPR spectum of isolated Cu_A-binding domains aquire a type-2 character with a four-line hyperfine signal. Together with copper/protein measurements, this indicates that at high pH the unpaired electron is localized to only one of the coppers, and hence the centre loses its mixed-valence features (III, V).

5.3 Identification of the Cu_A ligands

It has been proposed that subunit II has a similar three-dimensional fold to the small blue copper proteins (Saraste 1990, I). In the blue-copper proteins, the copper is coordinated to two histidines, a cysteine and a methionine. One of the histidine ligands is located between β-strands C and D, whereas the other three ligands are located in the loop between β-strands F and G, using the nomenclature for amicyanin (Adman 1991).

The mutagenesis studies showed that at least two cysteines, two histidines and a methionine are important for the engineered centre (II). Two cysteines, one histidine and the methionine are located in the region corresponding to the amicyanin FG-loop, whereas the other histidine is located between the β-strands C and D, using the nomenclature of amicyanin. The replacement of any of these residues leads to the loss of copper and to significant spectral changes. The substitution of the conserved glutamate, that is located between the two conserved cysteines, does not lead to any significant changes in the optical spectrum or in the copper content (II).

A more careful mutagenesis study that was carried out with the native *Paracoccus* Cu_A-binding domain is presented in (V). All the conserved, putative copper ligands of this domain were mutagenized and the optical spectra and copper/protein ratios were determined for all of the mutants. EPR- and MCD spectra were recorded from each mutant showing changes in the absorption properties. As in the case of the engineered centre, only two cysteines, two histidines and a methionine seemed to be crucial for the Cu_A site. However, replacement of the methionine either with threonine, cysteine, leucine or isoleucine seemed to cause problems in protein folding, as monitored by an analytical anion exchage chromatography. Whereas the wild type and the other mutant proteins eluted as a sharp peak at 0.45 M NaCl, the methionine mutants eluted as several small peaks between 0.4 and 0.6 M NaCl (V). These results indicate that this methionine is probably important for the folding of the Cu_A-binding domain. The structural role of this methionine is further supported by the fact that it is also conserved in the copperless quinol oxidase subunit II.

Mutagenesis of the putative Cu_A-ligands was not able to identify any residues that would be responsible for the pH sensitivity of the isolated Cu_A centre. However, the replacement of histidine 252 by asparagine led to a protein with an optical spectrum similar to the high-pH form. The spectrum of this mutant was not pH sensitive, and it retained these specific spectral features also at neutral and acidic pH. Also the EPR-spectrum of this mutant is typical for the type 2 copper site, but the g_{II} as well as hyperfine coupling are somewhat different from the wild type high-pH form (V).

5.4 Reaction of the Cu_A-binding domain with cytochrome *c*

Cu_A has been shown to be the electron entry site in cytochrome *c* oxidases (see e.g. Hill 1994). The location of the cytochrome *c* binding site has been studied using several different methods (see chapter 2.3). The results have indicated that the binding site is mainly located in subunit II. Since the binding is strongly dependent on ionic strength, it has been suggested that the conserved carboxylates in subunit II would interact with the conserved lysines around the heme edge of cytochrome *c* (Holm et al., 1987, Capaldi 1990).

The reaction between the isolated *Paracoccus* Cu_A-binding domain and cytochromes *c* was studied by optical spectroscopy (III) and kinetically with stopped-flow spectroscopy (IV). At low ionic strength, the Cu_A-binding domain oxidizes *Paracoccus* cytochrome *c-550* and horse mitochondrial cytochrome *c* at rates of $1.5x10^6$ and $3x10^5$ $M^{-1}s^{-1}$, respectively. The reaction follows monophasic kinetics, indicating the presence of only one kinetically competent cytochrome *c* binding site within the domain. The reaction rates are strongly dependent on ionic strength. The slower rates and the stronger ionic-strength dependence of the reaction with horse cytochrome *c* shows, that this reaction is less specific.

We identified five conserved residues (two aspartates, two glutamates and a glutamine) in the Cu_A-binding domain that are involved in cytochrome *c* binding. Substitution of these residues resulted in a 30-80% decrease in the reaction rates with *Paracoccus* cytochrome *c-550*. Simultaneous substitution of the three absolutely conserved carboxylates led to a 95 % decrease in the reaction rates, showing that the effects of these substitutions are cumulative (IV).

5.5 Crystallization of the Cu_A-binding domain

There is no high resolution structure available for cytochrome *c* oxidase or N_2OR. The C-terminal CyoA fragment of the *E.coli* cytochrome bo_3 has been crystallized (Van der Oost et al. 1993) and the structure has been recently solved (Wilmanns et al. unpublished). This quinol oxidase domain does not harbour Cu_A and the structure will not give direct information about the structure of this copper centre.

Purified *Paracoccus, Rhodobacterium* and *Synechocystis* Cu_A-binding domains have been used for crystallization trials. The *Paracoccus* domain crystallizes at pH 3.5-4.5 without any precipitants. The crystals are thin needles that grow in bundles (III). The experiments to produce better quality crystals, by using different additives as well as screening for other crystallization conditions, have been unsuccesful. The crystallization experiments were also expanded to include the various *Paracoccus* domain mutants. Fig.6 shows crystals obtained with the K219Q mutant. These crystals grow at pH 3.5-4.5 as the wild type crystals. However, the

crystals are significantly thicker and less branched than the ones obtained with the wild type protein. At the time of the writing, the experiments to improve the quality of these crystals are being undertaken.

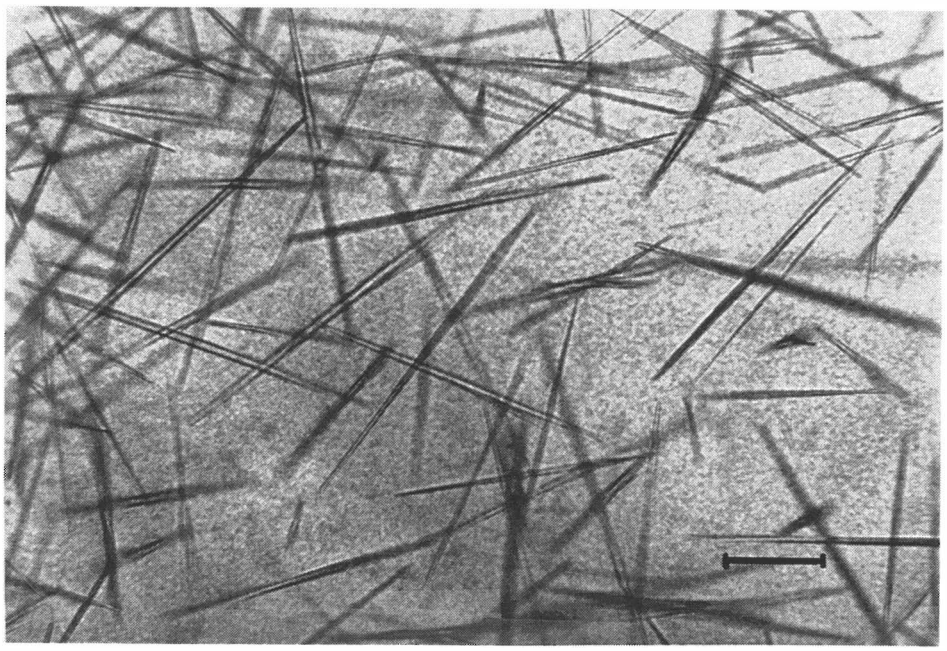

FIGURE 6. The crystals of the K219Q mutant Cu_A-domain. These crystals were grown by vapour diffusion in hanging drops against a reservoir buffer containing 0.1 M sodium acetate (pH 4.0). The protein concentration was 4 mg/ml. The scale bar is 0.1 mm.

6 DISCUSSION

6.1 Structure of the Cu_A centre

The synthesis of spectroscopical data including ENDOR, EXAFS and MCD spectroscopy has led to the model of a mononuclear Cu_A centre (see chapter 2.2.3). This has been challenged in particular by the EPR spectroscopy which has argued for a dinuclear mixed-valence [Cu(1.5)...Cu(1.5)] centre (reviewed by Kroneck et al. 1990, Malmström & Aasa 1993).

The results in publications II and III show that both the engineered Cu_A-like centre and native Cu_A centre contain two coppers. The electrospray mass spectrometry showed that the mass difference between apo protein and copper-binding purple protein is almost exactly the mass of two copper atoms. Chemical copper/protein measurements gave values of 1.5 and 1.9 for the engineered and for the *Paracoccus* Cu_A centre respectively, indicating that the engineered centre is probably not fully occupied with copper. Von Wachenfeldt et al. (1994) have recently shown that also the Cu_A centre isolated from the *Bacillus subtilis* cytochrome *caa*$_3$ contains two coppers. In theory, it is possible that the fragments would contain a mononuclear Cu_A and a second copper bound to an other part of the protein. However, the substitution of a single Cu_A ligand, the distant histidine in the C-D loop, leads to the loss of both coppers. This shows that the coppers must be located in close proximity to each other (II, V).

Our results are in good agreement with the mixed-valence [Cu(1.5)...Cu(1.5)] model first presented by Kroneck et al (1988). Approximately half of the copper in the *Paracoccus* Cu_A-domain is EPR detectable (III) and a weak 7-line splitting can be resolved in the X-band

spectrum of this domain (V). The redox titrations with cytochrome *c* demonstrated that this domain acts as an one- electron acceptor (IV). A possible explanation for the lack of a clear 7-line signal in the case of cytochrome oxidase Cu_A centre might be that the chemical enviroments of two coppers are not as identical as those in the centre A of N_2OR. At high pH, the EPR spectrum of the isolated *Paracoccus* Cu_A-binding domain aquire the character of a type 2 copper centre, with a 4-line hyperfine structure in the g_{II} region. Since only half of the copper is EPR detectable, it seems that the unpaired electron is localized to one nucleus (III, V). This indicates that at alkaline pH the copper nuclei are no longer equivalent, which might reflect the loss or addition of a ligand to one copper. A similar pH dependence has not been observed with the intact cytochrome *c* oxidase. This indicates, that Cu_A becomes accessible to solvent in the isolated form. However, Baker & Palmer (1987) have shown that a long (several hours) incubation of the beef heart enzyme at pH 11 leads to the appearance of a new EPR signal. This EPR spectrum is similar to the high-pH form of the isolated *Paracoccus* Cu_A centre.

Only two cysteines, two histidines and a methionine are crucial for the Cu_A site. The substitution of any other conserved residues, including the totally conserved carboxylates, did not lead to spectral changes or alter the copper content (V). The cysteine - histidine ligation of Cu_A is also in agreement with the earlier EXAFS and ENDOR data (Scott et al. 1986, Li et al. 1987, Martin et al. 1988). Furthermore, it seems that the conserved methionine is important for structural reasons, and is probably not a direct ligand for the Cu_A (V). This is supported by the fact that this methionine is conserved also in the majority of the quinol oxidases, that do not have Cu_A (see Saraste 1991, Richter et al. 1994). It has also been noticed that in the small blue copper protein, azurin, the corresponding methionine is not an essential copper ligand (Karlsson et al. 1991).

Two possible models for the coordination of Cu_A, which are based on site-directed mutagenesis and biochemical as well as spectroscopic data (II, III, V, Wachenfeldt et al. 1994, Antholine et al. 1992), are presented in Fig.7. In both models, the copper nuclei are chemically equivalent. The main difference is that in model A, the cysteine thiolates serve as bridging ligands for coppers, whereas a direct copper-copper bond is present in model B. The latter model is supported by the recent EXAFS work carried out with the *Bacillus* domain. The simulations of EXAFS spectra and the comparison with an inorganic compound having a direct Cu(1.5)-Cu(1.5) bond suggested Cu-Cu distance of 2.5 Å (Blackburn et al. 1994). However, so far no evidence for a Cu-Cu vibration has been obtained in the Raman resonance study with Cu_A-binding domains (Andrew et al. 1994).

The reason for the dinuclear nature of Cu_A centre is not yet understood. Several studies have shown that Cu_A functions as an electron transfer centre in cytochrome *c* oxidase (Kobayashi et al. 1989, Hill 1991, Nilsson 1992, Pan et al. 1993, Hill 1994, IV). Li et al. (1991) have proposed that the Cu_A-centre would be involved in proton pumping. However, the absence of Cu_A in quinol oxidases that are capable of pumping protons makes this hypothesis unlikely. One possibility is that the dinuclear,

mixed-valence Cu_A functions as a short electron conductor between cytochrome *c* and heme *a*. The dinuclear nature of Cu_A would in this model decrease the effective distance between these two redox centres (Lappalainen & Saraste 1994).

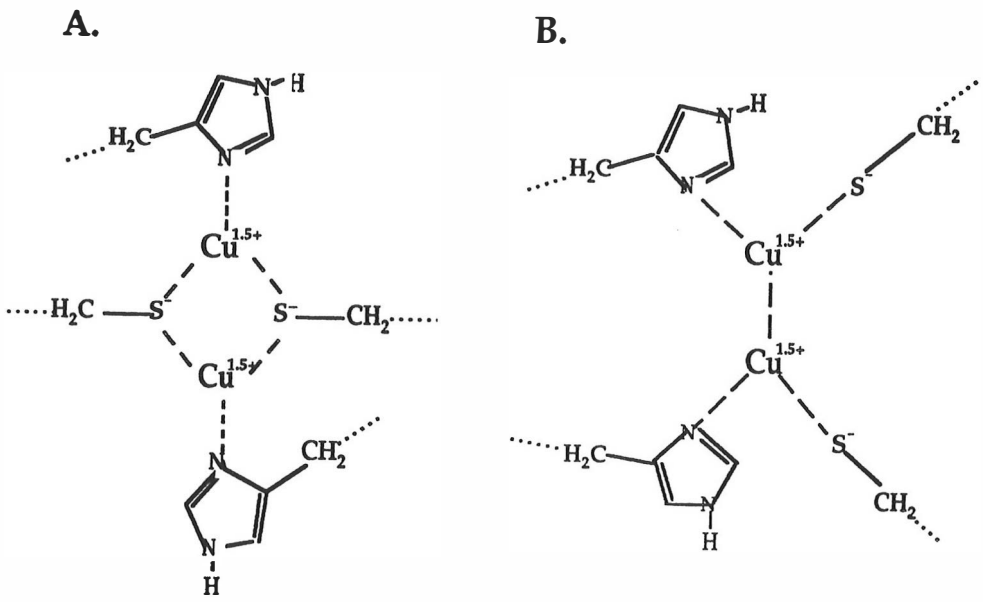

FIGURE 7. Two alternative models for coordination of Cu_A. The set of copper ligands, two cysteines and two histidines, were determined by site-directed mutagenesis in article V. In the first model (A), the two coppers are bridged by the cysteine thiolates. This model allows a relatively long Cu - Cu distance. In the second model (B), the ligands for each copper are one cysteine, one histidine and the other copper.

6.2 The Cu_A centre is the electron entry site of cytochrome *c* oxidase

Article IV shows that the reaction between the isolated Cu_A-binding domain and cytochrome *c* follows monophasic kinetics indicating that the isolated Cu_A-binding domain consequently contains only one catalytically active cytochrome *c* binding site. The K_D for the Cu_A-binding domain/cytochrome *c-550* complex is 1.5 uM, as calculated from the kinetic data. This is similar to what has been earlier reported for the intact oxidase (Antalis & Palmer 1982), indicating that the domain contains a complete cytochrome *c* binding site (IV). The reaction between the isolated domain and cytochrome *c-550* has also a similar ionic-strength dependence as the one reported earlier for the intact oxidase (IV, Antalis & Palmer 1982). This again supports the location of a complete binding-site within the Cu_A-binding domain. However, the k_{on} rates for the reaction between the isolated *Paracoccus* Cu_A-binding domain and cytochrome *c-550* are approximately 10-fold lower than those reported for the intact mitochondrial oxidase (e.g Andreasson 1975, Antalis & Palmer 1982). It is still unclear, whether cytochrome *c550* or *c552* in *Paracoccus* is better substrate for cytochrome aa_3 (see e.g. Berry & Trumpower 1985). If *c552* is the natural substrate, this could explain the relative slowness of the reaction between Cu_A-binding domain and cytochrome *c550*.

Subunit II of cytochrome *c* oxidase has several well-conserved carboxylates which are not found in the homologous subunit of quinol oxidases. These residues may be involved in cytochrome *c* binding via electrostatic interactions with the ring of conserved lysines that surround the heme edge of cytochrome *c* (Saraste 1990). Also, the crystal structure of cytochrome *c*: cytochrome *c* peroxidase -complex has suggested that the electrostatic interactions between the conserved lysines of cytochrome *c* and carboxylates of peroxidase might be important for the formation of the complex (Pelletier & Kraut 1992).

All three totally conserved carboxylates (D206, D221 and E246) in the *Paracoccus* domain are involved in cytochrome *c* binding (IV). Labelling studies have earlier indicated that only the residue corresponding to E246 is protected upon binding of cytochrome *c*, and hence could be involved in this interaction (Millett et al. 1983). The strongest decrease in electron transfer rates was found with the mutant D206N (IV). This aspartate is also the only totally concerved carboxylate within the homologous sequence shared by subunit II and nitrous oxide reductase (Zumft et al. 1992). Also N_2OR probably receives electrons from cytochrome *c*, and this aspartate could be crucial for cytochrome *c* binding in both enzymes. The simultaneous substitution of all three invariant carboxylates (D206N, D221N, E246Q) showed that the effects of these substitutions are probably cumulative (IV). In addition to these totally concerved carboxylates, we tested the roles of a less conserved glutamate (E154) and an invariant glutamine (Q148) for cytochrome *c* binding. The substitution of these residues led to an approximately 60 % decrease in the reaction rates (IV).

The glutamine is probably involved in forming hydrogen bonds with cytochrome *c*, whereas the glutamate probably plays a similar role as the three invariant carboxylates. Labelling studies have earlier indicated that in the mitochondrial oxidase also the residue corresponding to this less conserved glutamate (E154) could be involved in cytochrome *c* binding (Millett et al. 1983).

6.3 Towards a three-dimensional structure of cytochrome oxidase

The crystallization of cytochrome oxidase has been successful in some cases (see e.g Yoshikawa, et al. 1988), but so far there are no reports on crystals with a sufficient quality for structure determination. The three-dimensional structural information of cytochrome oxidase is solely based on electron microscopic studies with two-dimensional membrane crystals (Frey, et al., 1978; Fuller, et al., 1979; Valpuesta, et al., 1990, Frey & Murray 1994, Crum et al. 1994). The structure of beef heart cytochrome oxidase, determined to a resolution of 15 Å, shows that a substantial part of the molecule is buried in the lipid bilayer. The molecule extends out of the membrane, and it seems to contain cavities (Valpuesta, et al., 1990). It is difficult to build models about the location of subunits and active sites based on this low-resolution structure partly because the mitochondrial enzyme contains 13 subunits.

Since crystallization of cytochrome oxidase has proven to be difficult, the present structural information on this enzyme relies on alternative methods such as site-directed mutagenesis and spectroscopy. The ligands for the haems and Cu_B in the subunit I have been successfully identified by a combination of site-directed mutagenesis and various spectroscopic methods (reviewed by Calhoun et al., 1994). The ligands are located in five putative transmembrane helices, and this mutagenesis work has made it possible to prepare rough models for the orientation of these helices relative to each other. The electron transfer studies have given further information about the distances between the redox centres (reviewed by Hill, 1993). In the future, it will probably be possible to design and synthesize model peptides that would mimic the structure of the core parts of cytochrome oxidase. These molecular 'maquettes' would be based on the available structural information for oxidase, but would be structurally simpler and more suitable for further structural work (Robertson et al., 1994).

In article I, we used a mutagenesis approach in order to get indirect information about the structure of cytochrome oxidase subunit II. The successful reconstitution of a copper centre similar to Cu_A into the originally copperless quinol oxidase domain showed that subunits II in cytochrome *c*- and quinol oxidases share similar three-dimensional folds. Furthermore, the successful engineering of a blue copper like centre into this quinol oxidase domain indicates that this domain in cytochrome

oxidase probably contains at least partial structural similaritiy to the cupredoxin fold (I). This has been confirmed by the structure determination of this domain (Wilmanns et al. unpublished). The identification of the Cu_A ligands and cytochrome *c* binding residues (articles IV and V) combined with the structure of the copperless quinol oxidase domain, will make it possible to build a reliable model for the structure of the cytochrome *c* oxidase Cu_A-binding domain.

7 CONCLUSIONS

The study presented here is part of a larger project, the aim of which is to study the structure - function relationship of cytochrome oxidase. The work has focused on various bacterial oxidases, since they are structurally simpler than their mitochondrial counterparts and allow genetic manipulation. My project has been biochemical and structural characterization of the cytochrome *c* oxidase subunit II. The main focus of this study has been to characterize the structure and coordination of the copper centre (Cu_A) that is located in this subunit. Another important focus has been the characterization of the interaction of this domain with cytochrome *c*.

My main conclusions are:

1. The C-terminal domain of subunit II in cytochrome *c* oxidases and quinol oxidases share a homologous three-dimensional structure which is similar to the cupredoxin fold of small blue-copper proteins.

2. The Cu_A centre is located in subunit II of cytochrome *c* oxidases, and is spectroscopically similar to the centre A of nitrous oxide reductase.

3. Cu_A is a dinuclear, mixed-valence copper centre. The major ligands for the two coppers are two histidines and two cysteines.

4. The Cu_A-domain in cytochrome *c* oxidases contains a complete cytochrome *c* binding site. Conserved carboxylates as well as a conserved glutamine are involved in cytochrome *c* binding.

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This work is dedicated to my parents, Aili and Unto Lappalainen.

Yhteenveto

Sytokromioksidaasin kaksiytiminen Cu_A keskus

Sytokromioksidaasi on viimeinen entsyymi sekä mitokondrion että monien bakteerien hengitysketjuissa. Se katalysoi elektronin siirtoa sytokromi *c*:ltä hapelle ja muuntaa tästä reaktiosta vapautuvan energian membraaninläpäiseväksi protonigradientiksi. Aerobisissa bakteereissa esiintyy kahta eri tyyppiä sytokromioksidaaseja, jotka vastaanottavat elektroneja joko sytokromi *c*:ltä tai kinoleilta. Merkittävin ero näiden oksidaasi-tyyppien välillä sijaitsee entsyymin alayksikössä II. Sytokromi *c* oksidaasien alayksikkö II sisältää kuparikeskuksen (Cu_A), kun taas kinoli-oksidaaseista tämä kuparikeskus on hävinnyt evoluution myötä.

Sytokromioksidaasin alayksikön II karboksipää muodostaa suurehkon, liukoisen proteiinidomeinin, jossa kuparikeskuksen uskottiin sijaitsevan. Tässä työssä useiden eri bakteerilajien sytokromi *c* oksidaasien alayksikön II C-terminaalista proteiinidomeinia tuotettiin liukoisessa muodossa *Escherichia colissa*. Kohdennetun mutageneesin avulla onnistuimme tekemään sekä kupari A:n että sinisen kuparikeskuksen kaltaiset keskukset kinolioksidaasin alunperin kuparittomaan proteiinidomeiniin. Tämä osoittaa, että alayksiköt II kinoli- ja sytokromi *c* oksidaaseissa omaavat keskenään samankaltaiset kolmiulotteiset rakenteet.

Toisin kuin kokonaisessa sytokromioksidaasissa, eristetyn Cu_A keskuksen optinen spektri on herkkä pH:n muutoksille. Tämä osoittaa, että Cu_A on enemmän hautautunut kompleksissa. Biokemialliset kuparimääritykset sekä massaspektrometria osoittivat, että Cu_A -keskus sisältää kaksi kuparia. Elektronin paramagneettinen resonanssi (EPR) spektrit yhdessa kupari kvantitoinnin kanssa tukevat mallia, jossa nämä kaksi kuparia muodostavat kaksiytimisen, seka-valenssisen [Cu(1.5)...Cu(1.5)] keskuksen. Kohdennetun mutageneesin avulla osoitimme, että kaksi histidiiniä ja kaksi kysteiiniä toimivat näiden kuparien ligandeina.

Cu_A -domeinin ja sytokromi *c*:n välistä interaktiota tutkittiin "stopped-flow" spektroskopian avulla. Reaktio noudatti yksifaasista kinetiikkaa, jonka perusteella domeini sisältää vain yhden katalyyttisesti aktiivisen sytokromi *c*:n sitoutumiskohdan. Reaktionopeudet ja dissosiaatiovakiot olivat samankaltaisia kuin aiemmat tulokset kokonaisella sytokromioksidaasilla. Näin ollen Cu_A -domeini näyttää sisältävän sytokromi *c*:tä sitovan alueen kokonaisuudessaan. Kohdennetun mutageneesin avulla osoitimme, että ainakin viisi aminohappoa Cu_A -domeinissa (kaksi asparagiinihappoa, kaksi glutamiinihappoa sekä yksi glutamiini) ovat tärkeitä sytokromi *c*:n sitoitumiselle.

REFERENCES

Adman, E. T. 1991: Copper protein structures. - Adv. Prot. Chem 42:145-197.

Andreasson, L-E. 1975: Characterization of the reaction between ferrocytochrome *c* and cytochrome *c* oxidase. - Eur. J. Biochem. 53: 591-597.

Andrew, C. R., Han, J., de Vries, S., van der Oost, J., Averill, B. A., Loehr, T. M., Sanders-Loehr, J. 1994: Cu_A of cytochrome *c* oxidase and the A site of N_2O reductase are tetrahedrally distorted type 1 Cu cysteinates. - J. Am. Chem. Soc. 116: 10805-10806.

Anraku, Y. 1988: Bacterial electron transfer chains. - Annu. Rev. Biochem. 57: 101-132.

Antalis, T. M. & Palmer, G. 1982: Kinetic characterization of the reaction between cytochrome oxidase and cytochrome *c*. - J. Biol. Chem. 257: 6194-6206.

Antholine, W. E., Kastrau, D. H. W., Steffens, G. C. M., Buse, G., Zumft, W. G. & Kroneck, P. M. H. 1992: A comparative EPR investigation of the multicopper proteins nitrous-oxide reductase and cytochrome *c* oxidase. - Eur. J. Biochem. 209: 875-881.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. 1991: Current protocols in molecular biology. John Wiley and Sons, New York.

Babcock, G. T. & Wikström, M. 1992: Oxygen activation and the conservation of energy in cell respiration. - Nature 356: 301-309.

Baker, G. M. & Palmer, G. 1987: Effect of high pH to the spectral and catalytic properties of beef heart cytochrome oxidase. - Biochemistry 26: 3038-3044.

Berry, E. A. & Trumpower, B. L. 1985: Isolation of ubiquinol oxidase from *Paracoccus denitrificans* and resolution into cytochrome bc_1 and cytochrome c-aa_3 complexes. - J. Biol. Chem. 260: 2458-2467.

Bisson, R. & Montecucco, C. 1982: Different polypeptides of bovine heart cytochrome *c* oxidase are in contact with cytochrome *c*. - FEBS. Lett. 150: 49-53.

Blackburn, N. J., Barr, M. E., Woodruff, W. H., van der Oost, J. & de Vries, S. 1994: Metal-metal bonding in biology: EXAFS evidence for a 2.5 Å copper-copper bond in the Cu_A center of cytochrome oxidase. - Biochemistry 33: 10401-10407.

Bolgiano, B., Smith, L. & Davies, H. C. 1989: Electron transport in a cytochrome *c*-deficient mutant of *Paracoccus denitrificans*. - Biochim. Biophys. Acta 973: 227-234.

Brzezinski, P. & Malmström, B. G. 1986: Electron-transport-driven proton pumps display nonhyperbolic kinetics: Simulation of the steady-state kinetics of cytochrome *c* oxidase. - Proc. Natl. Acad. Sci. 83: 4282-4286.

Briggs, M. M. & Capaldi, R. A. 1978: Cross-linking studies on a cytochrome *c* - cytochrome *c* oxidase complex. - Biochem. Biophys. Res. Comm. 80: 553-559.

Broda, E. & Peschek, G. A. 1979: Did respiration or photosynthesis come first? - J. Theor. Biol. 81: 201-212.

Broman, L., Malmström, B. G., Aasa, R. & Vänngård, T. 1962: Quantitative electron spin resonance studies on native and denatured ceruloplasmin and laccase. - J. Mol. Biol. 5: 301-310.

Buse, G. & Steffens, G. C. M. 1991: Cytochrome oxidase in *Paracoccus denitrificans*: Protein, chemical, structural and evolutionary aspects. - J. Bioenerg. Biomembr. 23: 269-289.

Calhoun, M. W., Hill, J. J., Lemieux, L. J., Ingledw, J., Alben, J. O. & Gennis, R. B. 1993a: Site-directed mutants of the cytochrome *bo* ubiquinol oxidase of *Escherichia coli*: Amino acid substitutions for two histidines that are putative Cu_B ligands. - Biochemistry 32: 11524-11529.

Calhoun, M. W., Lemieux, L. J., Thomas, J. W., Hill, J. J., Chepuri-Goswitz, V., Alben, J. O. & Gennis, R. B. 1993b: Spectroscopic characterization of mutants supports the assignment of histidine-419 as the axial ligand of heme *o* in the binuclear center of the cytochrome *bo* ubiquinol oxidase from *Escherichia coli*. - Biochemistry 32: 13254-13261.

Calhoun, M. W., Thomas, J. W., Hill, J. J., Hosler, J. P., Shapleigh, J. P., Tecklenburg, M. M. J., Ferguson-Miller, S., Babcock, G. T., Alben, J. O. & Gennis, R. B. 1993c: Identity of the axial ligand of the high-spin heme in cytochrome oxidase: Spectroscopic characterization of mutants in the *bo*-type oxidase of *Escherichia coli* and the aa_3-type oxidase of *Rhodobacter sphaeroides*. - Biochemistry 32: 10905-10911.

Calhoun, M. V., Thomas, J. W. & Gennis, R. B. 1994: The cytochrome oxidase superfamily of redox-driven proton pumps. - TIBS. 19: 325-330.

Capaldi, R. A. 1990: Structure and function of cytochrome *c* oxidase. - Annu. Rev. Biochem. 59: 569-596.

Carter, C. W. 1992: Design of crystallization experiments and protocols. - In: Crystallization of nucleic acids and proteins. Practical approach (eds Ducruix, A. & Giege, R.) pp. 47-72. Oxford University Prss, Oxford.

Castresana, J., Lübben, M., Saraste, M. & Higgins, D. G. 1994: Evolution of cytochrome oxidase, an enzyme older than atmospheric oxygen. - EMBO J. 13: 2516-2525.

Chepuri, V., Lemieux, L., Au, D. C-T. & Gennis, R. B. 1990: The sequence of the *cyo* operon indicates substantial structural similarities between the cytochrome *o* ubiquinol oxidase of *Escherichia coli* and the aa_3-type family of cytochrome *c* oxidases. - J. Biol. Chem. 265: 11185-11192.

Chepuri, V. & Gennis, R.B. 1990: The use of gene fusions to determine the topology of all of the subunits of the cytochrome *o* terminal oxidase complex of *Escherichia coli*. - J. Biol. Chem. 265: 12978-12986.

Cline,J., Reinhammar, B., Jensen, P., Venters, R. & Hoffman, B. M. 1983: Coordination environment for the type 3 copper center of tree laccase and Cu_B of cytochrome *c* oxidase as determined by electron nuclear double resonance. - J. Biol. Chem. 258: 5124-5128.

Cotter, P. A., Chepuri, V., Gennis, R. B. & Gunsalus, R.P. 1990: Cytochrome *o* (*cyo* ABCDE) and *d*(*cyd*AB) oxidase gene expression in *Escherichia coli* is regulated by oxygen, pH, and the *fnr* gene product. - J. Bact. 172: 6333-6338.

Crum, J., Gruys, K. J. & Frey, T. G. 1994: Electron microscopy of cytochrome *c* oxidase crystals: Labelling of subunit III with a monomaleimide undecagold cluster compound. - Biochemistry 33: 13719-13726.

Darnall, D. W. & Wilkins, R. G. 1980: Methods for determining metal ion enviroments in proteins. Elsevier Nort Holland, Inc, New York.

de Gier, J-W. L., Lübben, M., Reijnders, W. N. M., Tipker, C. A., Slotboom, D-J., van Spanning, R. J. M., Stouthamer, A. H. & van der Oost J. 1994: The terminal oxidases of *Paracoccus denitrificans*. - Mol. Michrobiol. 13: 183-196.

de Vries, S. & Marres, C. A. M. 1987: The mitochondrial respiratory chain of yeast. Structure and biosynthesis and the role in cellular metabolism. - Biochim. Biophys. Acta 895: 205-239.

Erecinska, M. 1977: A new photoaffinity-labeled derivative of mitochondrial cytochrome *c*. - Biochem. Biophys. Res. Comm. 76: 495-501.

Farrar, J. A., Thomson, A. J., Cheesman, M. R., Dooley, D. M. & Zumft, W. G. 1991: A model of the copper centres of nitrous oxide reductase (*Pseudomonas stuzeri*): Evidence from optical, EPR and MCD spectroscopy. - FEBS Lett. 294: 11-15.

Fee, J. A. 1975: Copper proteins: Systems containing the "blue" copper center. - Struct. Bonding 23: 1-60.

Ferguson-Miller, S., Brautigan, D. L. & Margoliash, E. 1976: Correlation of the kinetics of electron transfer activity of various eucaryotic cytochromes *c* with binding to mitochondrial cytochrome *c* oxidase. - J. Biol. Chem. 251: 1104-1115.

Frey, T. G., Chan, S. H. P. & Schatz, G. 1978: Structure and orientation of cytochrome *c* oxidase in crystalline membranes. - J. Biol. Chem. 253: 4389-4395.

Frey, T. G & Murray, J. M. 1994: Electron microscopy of cytochrome *c* oxidase crystals: Monomer-dimer relationship and cytochrome *c* binding site. - J. Mol. Biol. 237: 275-297.

Fuller, S. D., Capaldi, R. A. & Henderson, R. 1979: Structure of cytochrome *c* oxidase in deoxycholate-derived two-dimensional crystals. - J. Mol. Biol. 134: 305-327.

Garber, E. A. E. & Margoliash, E. 1990: Interaction of cytochrome *c* with cytochrome *c* oxidase; An understanding of the high- to low-affinity transition. - Biochim. Biophys. Acta 1015: 279-287.

Garcia-Horsman, J. A., Barquera, B., Rumbley, J., Ma, J. & Gennis, R. B. 1994: The superfamily of heme-copper respiratory oxidases. - J. Bact. 176: 5587-5600.

Gibb, G. M. & Ragan, C. I. 1990: Identification of the subunits of bovine NADH dehydrogenase which are encoded by the mitochondrial genome. - Biochem. J. 265: 903-906.

Greenwood, C., Hill, B. C., Barber, D., Eglinton, D. G. & Thomson, A. J. 1983: The optical properties of Cu_A in bovine cytochrome *c* oxidase determined by low-temperature magnetic-circular-dichroism spectroscopy. - Biochem. J. 215: 303-316.

Hall, J., Moubarak, A., O'Brien, P., Pan, L. P., Cho, I. & Millett, F. 1988: Topological studies of monomeric and dimeric cytochrome *c* oxidase in identification of the copper A site using a fluorescence probe. - J. Biol. Chem. 263: 8142-8149.

Haltia, T., Finel, M., Harms, N., Raitio, M., Wikström, M. & Saraste, M. 1989: Deletion of the gene for subunit III leads to defective assembly of bacterial cytochrome oxidase. - EMBO J. 8: 3571-3579.

Haltia, T., Saraste, M. & Wikström, M. 1991: Subunit III of cytochrome *c* oxidase is not involved in proton translocation: a site-directed mutagenesis study. - EMBO J. 10: 2015-2021.

Haltia, T., Semo, N., Arrondo, J. L. R., Goni, F. M. & Freire, E. 1994: Thermodynamic and structural stability of cytochrome *c* oxidase from *Paracoccus denitrificans*. - Biochemistry 33: 9731-9740.

Han, S., Ching, Y. & Rousseau, D. L. 1990a: Primary intermediate in the reaction of mixed-valence cytochrome *c* oxidase with oxygen. - Biochemistry 29: 1380-1384.

Han, S., Ching, Y. & Rousseau, D. L. 1990b: Ferryl and hydroxy intermediates in the reaction of oxygen with reduced cytochrome *c* oxidase. - Nature 348: 89-90.

Hatefi, Y. 1985: The mitochondrial electron transport and oxidative phosphorylation system. - Annu. Rev. Biochem. 54: 1015-1069.

Hendler, R. W., Pardhasaradhi, K., Reynafarje, B. & Ludwig, B. 1991: Comparison of energy-transducing capabilities of the two- and three-subunit cytochromes aa_3 from *Paracoccus denitrificans* and the 13-subunit beef heart enzyme. - Biophys. J. 60: 415-423.

Higuchi, R., Krummel, B. & Saiki, R. K. 1988: A general method of in vitro preparation and specific mutagenesis of DNA fragments: Study of protein and DNA interactions. - Nucl. Acid Res. 16: 7351-7367.

Hill, B. C. 1991: The reaction of the electrostatic cytochrome *c* - cytochrome oxidase complex with oxygen. - J. Biol. Chem. 266: 2219-2226.

Hill, B. C. 1993: The sequence of electron carriers in the reaction of cytochrome *c* oxidase with oxygen. - J. Bioenerg. Biomembr. 25: 115-120.

Hill, B. C. 1994: Modelling the sequence of electron transfer reactions in the single turnover of reduced, mammalian cytochrome *c* oxidase with oxygen. - J. Biol. Chem. 269: 2419-2425.

Hofhaus, G., Weiss, H. & Leonard, K. 1991: Electron microscopic analysis of the peripheral and membrane parts of mitochondrial NADH dehydrogenase (Complex I). - J. Mol. Biol. 221: 1027-1043.

Holm, L., Saraste, M. & Wikström, M. 1987: Structural models of the redox centres in cytochrome oxidase. - EMBO J. 6: 2819-2823.

Hosler, J. P., Fetter, J., Tecklenburg, M. M. J., Espe, M., Lerma C. & Ferguson-Miller, S. 1992: Cytochrome aa_3 of *Rhodobacter sphaeroides* as a model for mitochondrial cytochrome *c* oxidase: Purification, kinetics, proton pumping, and spectral analysis. - J. Biol. Chem. 267: 24264-24272.

Hosler, J. P., Shapleigh, J. P., Tecklenburg, M. M. J., Thomas, J. W., Kim, Y., Espe, M., Fetter, J., Babcock, G. T., Alben, J. O., Gennis, R. B. & Ferguson-Miller, S. 1994: A loop between transmembrane helices IX and X of subunit I of cytochrome *c* oxidase caps the heme *a*-heme a_3-Cu_B center. - Biochemistry 33: 1194-1201.

Karlsson, B., Hovmöller, R., Weiss, H. & Leonard, K. 1983: Structural studies of cytochrome reductase: Subunit topography determined by electron microscopy of membrane crystals of a subcomplex. - J. Mol. Biol. 165: 287-302.

Karlsson, B. G., Nordling,M., Pascher, T., Tsai, L-C., Sjölin, L & Lundberg, L. G. 1991: Casette mutagenesis of Met121 in azurin from *Pseudomonas aeruginosa*. - Prot. Engineering 4: 343-349.

Kobayashi, K., Une, H. & Hayashi, K. 1989: Electron transfer process in cytochrome oxidase after pulse radiolysis. - J. Biol. Chem. 264: 7976-7980.

Kroneck, P. M. H., Antholine, W. A., Riester, J. & Zumft, W. G. 1988: The cupric site in nitrous oxide reductase contains a mixed-valence [Cu(II), Cu(I)] binuclear center: a multi-frequency electron paramagnetic resonance investigation. FEBS Lett. 242: 70-74.

Kroneck, P. M. H., Riester, J., Zumft, W. G. & Antholine, W. E. 1990: The copper site in nitrous oxide reductase. - Biol. Metals 3: 103-109.

Laemmli, U. K. 1970: Cleavage of structural proteins during the asembly of the head of bacteriophage T4. - Nature 227: 680-685.

Lappalainen, P. & Saraste, M. 1994: The binuclear Cu_A centre of cytochrome oxidase. - Biochim. Biophys. Acta. 1187: 222-225.

Lemieux, L. J., Calhoun, M. W., Thomas, J. W., Ingledew, J. & Gennis, R. B. 1992: Determination of the ligands of the low-spin heme of the cytochrome *o* ubiquinol oxidase complex using site-directed mutagenesis. - J. Biol. Chem. 267: 2105-2113.

Leonard, K., Haiker, H. & Weiss, H. 1987: Three dimensional structure of NADH:ubiquinone reductase (complex I) from *Neurospora* mitochondria determined by electron microscopy of membrane crystals. - J. Mol. Biol. 194: 277-286.

Li, M. P., Gelles, J., Chan, S. I., Sullivan, R. J. & Scott, R. A. 1987: Extended X-ray absorption fine structure of copper in Cu_A-depleted, p-(hydroxymercuri)benzoate-modified, and native cytochrome *c* oxidase. - Biochemistry 26: 2091-2095.

Li, Z., Larsen, R. W., Pan, L. & Chan, S. I. 1991: The effects of p-hydroxymercuribenzoic acid modification and heat treatment on the Cu_A reduction potential of cytochrome *c* oxidase. - J. Biol. Chem. 266: 22858-22865.

Lübben, M., Kolmerer, B. & Saraste, M. 1992: An archeabacterial terminal oxidase combines core structures of two mitochondrial respiratory complexes. - EMBO J. 11: 805-812.

Lübben, M. & Morand, K. 1994: Novel prenylated hemes as cofactors of cytochrome oxidases. - J. Biol. Chem. 269: 21473-21479.

Lübben, M., Warne, A., Albracht, S. P. J. & Saraste, M. 1994a: The purified SoxABCD quinol oxidase complex of *Sulfolobus acidocaldarius* contains a novel haem. - Mol. Michrobiol. 13: 327-335.

Lübben, M., Arnaud, S., Castresana, J., Warne, A., Albracht, S. P. J. & Saraste, M. 1994b: A second terminal oxidase in *Sulfolobus acidocaldarius*. - Eur. J. Biochem. 224: 151-159.

Ludvig, B. 1986: Cytochrome *c* oxidase from *Paracoccus denitrificans*. - Methods Enzymol. 126: 153-159.

Ma, J., Lemieux, L. & Gennis, R. B. 1993: Genetic fusion of subunits I, II, and III of the cytochrome *bo* ubiquinol oxidase from *Escherichia coli* results in a fully assembled and active enzyme. - Biochemistry 32: 7692-7697.

Malkin, R. & Malmström BG. 1970: The state and function of copper in biological systems. - Adv. Enzymol. 33: 177-244.

Malmström, B. G. & Aasa, R. 1993: The nature of the Cu_A center in cytochrome *c* oxidase. - FEBS. Lett. 325: 49-52.

Martin, C. T., Scholes, C. P. & Chan, S. I. 1985: The identification of histidine ligands to cytochrome *a* in cytochrome *c* oxidase. - J. Biol. Chem. 260: 2857-2861.

Martin, C. T., Scholess, C. P. & Chan, S. I. 1988: On the nature of cysteine coordination to Cu_A in cytochrome *c* oxidase. - J. Biol. Chem. 263: 8420-8429.

Mather, M. W., Springer, P., Hensel, S., Buse, G. & Fee, J. A. 1993: Cytochrome oxidase genes from *Thermus thermophilus*. Nucleotide sequence of the fused gene and analysis of the deduced primary structures for subunits I and III. - J. Biol. Chem. 266: 5025-5035.

Millett, F., de Jong, C., Paulson,L. & Capaldi, F. 1983: Identification of specific carboxylate groups on cytochrome *c* oxidase that are involved in binding cytochrome *c*. - Biochemistry 22: 546-552.

Minagawa, J., Mogi, T., Gennis, R. B. & Anraku, Y. 1992: Identification of heme and copper ligands in subunit I of the cytochrome *bo* complex in *Escherichia coli*. - J. Biol. Chem. 267: 2096-2104.

Mitchell, P. 1976: Possible molecular mechanisms of the protonmotive function of cytochrome systems. - J. Theor. Biol. 62: 327-367.

Mogi, T., Saiki, K. & Anraku, Y. 1994: Biosynthesis and functional role of haem O and haem A. - Mol. Microbiol. 14: 391-398.

Nicholls, D. G. & Ferguson, S. J. 1992: Respiratory chains. In: Bioenergetics 2, pp. 107-156. Academic Press Limited, 24-28 Oval Road, London.

Nilsson, T. 1992: Photoinduced electron transfer from tris(2,2'-bipuridyl)ruthenium to cytochrome *c* oxidase. - Proc. Natl. Acad. Sci. 89: 6497-6501.

Öblad, M., Selin, E., Malmström, B., Strid, L., Aasa, R. & Malmström, B. G. 1989: Analytical characterization of cytochrome oxidase preparations with regard to metal and phospholipid contents, peptide composition and catalytic activity. - Biochim. Biophys. Acta. 975: 267-270.

Pan, L. P., Li, Z., Larsen, R. & Chan, S. I. 1991: The nature of CuX in cytochrome *c* oxidase. - J. Biol. Chem. 266: 1367-1370.

Pan, L. P., Hibdon, S., Liu, R-Q, Durham, B. & Millett, F. 1993: Intracomplex electron transfer between ruthenium-cytochrome *c* derivates and cytochrome *c* oxidase. - Biochemistry 32: 8492-8498.

Pelletier, H. & Kraut, J. 1992: Crystal structure of a complex between electron transfer partners, cytochrome *c* peroxidase and cytochrome *c*. - Science 258: 1748-1755.

Poole, R. K. & Ingledew, W. J. 1987: Pathways of electrons to oxygen. In: *Escherichia coli* and *Salmonella typhimurium* molecular and cellular biology, vol. 1. (eds F. C. Neidhant et al.), pp. 170-200. Washington D.C, American Society of Michrobiology.

Preisig, O., Anthamatten, D. & Hennecke, H. 1993: Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen-fixing endosymbiosis. - Proc. Natl. Acad. Sci. 90: 3309-3313.

Raitio, M. & Wikström, M. 1994: An alternative cytochrome oxidase of *Paracoccus denitrificans* functions as a proton pump. - Biochim. Biophys. Acta. 1186: 100-106.

Richter, O-M. H., Tao, J., Turba, A. & Ludwig, B. 1994: A cytochrome ba_3 functions as a quinol oxidase in *Paracoccus denitrificans*. - J. Biol. Chem. 269: 23079-23086.

Rieder, R. & Bosshard, H. R. 1978: The cytochrome *c* oxidase binding site on cytochrome *c*: Differential chemical modification of lysine residues in free and oxidase-bound cytochrome *c*. - J. Biol. Chem. 253: 6045-6053.

Riester, J., Zumft, W.G. & Kroneck, P. M. H. 1989: Nitrous oxide reductase from *Pseudomonas stutzeri*: Redox properties and spectroscopic characterization of different forms of the multicopper enzyme. - Eur. J. Biochem. 178: 751-762.

Rigell, C. W., de Saussure, C. & Freire, E. 1985: Protein and lipid structural transitions in cytochrome *c* oxidase-dimyristylphosphatidylcholine reconstitutions. - Biochemistry 24: 5638-5646.

Rigell, C. W. & Freire, E. 1987: Differential detergent solubility investigation of thermally induced transitions in cytochrome *c* oxidase. - Biochemistry 26: 4366-4371.

Robertson, D. E., Farid, R. S., Moser, C. C., Urbauer, J. L., Mulholland, S. E., Pidikiti, R., Lear, J. D., Wand, J., DeGrado, W. F. & Dutton, P. L. 1994: Design and synthesis of multi-haem proteins. - Nature 368: 425-432.

Salerno, J. C. & Ingledew, W. J. 1991: Orientation of the haems of the ubiquinol oxidase:O_2 reductase, cytochrome *bo* of *Escherichia coli*. - Eur. J. Biochem. 198: 789-792.

Santana, M., Kunst, F., Hullo, M. F., Rapoport, G., Danchin, A. & Glaser, P. 1992: Molecular cloning, sequencing, and physiological characterization of the *qox* operon from *Bacillus subtilis* encoding the *aa_3-600* quinol oxidase. - J. Biol. Chem. 267: 10225-10231.

Saraste, M. 1990: Structural features of cytochrome oxidase. - Q. Rev. Biophys. 23: 331-366.

Saraste, M., Metso, T., Nakari, T., Jalli, T., Laureus, M. & van der Oost, J. 1991: The *Bacillus subtilis* cytochrome *c* oxidase; variations on a conserved protein theme. - Eur. J. Biochem. 195: 517-525.

Saraste, M. & Castresana, J. 1994: Cytochrome oxidase evolved by tinkering with denitrification enzymes. - FEBS Lett. 341: 1-4.

Scott, R. A., Schwartz, J. R. & Cramer, S. P. 1986: Structural aspects of the copper sites in cytochrome *c* oxidase. An X-ray absorption spectroscopic investigation of resting-state enzyme. - Biochemistry 25: 5546-5555.

Scott, R. A., Zumft, W. G., Coyle, C. L. & Dooley, D. M. 1989: *Pseudomonas stuzeri* N_2O reductase contains Cu_A-type sites. - Proc. Natl. Acad. Sci. 86: 4082-4086.

Seiter, C. H. A., Margalit & R. Perreault, R. A. 1979: The cytochrome *c* binding site on cytochrome *c* oxidase. - Biochem. Biophys. Res. Com. 86: 473-477.

Shapleigh, J. P., Hosler, J. P., Tecklenburg, M. M. J., Kim, Y., Babcock, G. T., Gennis, R. B. & Ferguson-Miller, S. 1992: Definition of the catalytic site of cytochrome *c* oxidase; specific ligands of heme *a* and the heme a_3-Cu_B center. - Proc. Natl. Acad. Sci. 89: 4786-4790.

Steffens, G. C. M. & Buse, G. 1979: Studies on cytochrome *c* oxidase, IV; primary structure and function of subunit II. - Hoppe-Seyler's Z. Physiol. Chem. 360: 613-619.

Steffens, G. C. M., Biewald, R. & Buse, G. 1987: Cytochrome *c* oxidase is a three-copper, two-heme-A protein. - Eur. J. Biochem. 164: 295-300.

Steffens, G. C. M., Soulimane, T. & Buse, G. 1993: Stoichiometry and redox behaviour of metals in cytochrome *c* oxidase. - Eur. J. Biochem. 213: 1149-1157.

Stevens, T. H., Martin, C. T., Wang, H., Brudvig, G. W., Scholes, C. P. & Chan, S. I. 1982: The nature of Cu_A in cytochrome *c* oxidase. - J. Biol. Chem. 257: 12106-12113.

Studier, F., Rosenberg, A. H., Dunn, J. J & Dubendorff, J. W. 1990: Use of T7 RNA polymerase to direct expression of cloned genes. - Methods Enzymol. 185: 60-89.

Taha, T. S. M. & Ferguson-Miller, S. 1992: Interaction of cytochrome *c* with cytochrome *c* oxidase studied by monoclonal antibodies and a protein modifying reagent. - Biochemistry 31: 9090-9097.

Takahashi, S., Ogura, T, Shinzawa-Itoh, K., Yoshikawa, S. & Kitagawa, T. 1993: Resonance Raman studies on the Cu_A site of cytochrome *c* oxidase using a multichannel scanning Raman spectrometer with a CCD detector. - Biochemistry 32: 3664-3670.

Thomas, J. W., Puustinen, A., Alben, J. O., Gennis, R. B. & Wikström, M. 1993a: Substitution of asparagine for aspartate-135 in subunit I of the cytochrome *bo* ubiquinol oxidase of *Escherichia coli* eliminates proton-pumping activity. - Biochemistry 32: 10923-10928.

Thomas, J. W., Lemieux, L. J., Alben, J. O. & Gennis, R. B. 1993b: Site directed mutagenesis of highly conserved residues in helix VIII of subunit I of the cytochrome *bo* ubiquinol oxidase from *Escherichia coli*; An amphipathic transmembrane helix that may be important in conveying protons to binuclear center. - Biochemistry 32: 11173-11180.

Thomas, J. W., Calhoun, M. W., Lemieux, L. J., Puustinen, A., Wikström, M., Alben, J. O. & Gemmis, R. B. 1994: Site-directed mutagenesis of residues within helix VI in subunit I of the cytochrome bo_3 ubiquinol oxidase from *Escherichia coli* suggests that tyrosine 288 may be a Cu_B ligand. - Biochemistry. 33: 13013-13021.

Trumpower, B. L. 1990: The protonmotove Q cycle. - J. Biol. Chem. 265: 11409-11412.

Trumpower, B. L. & Gennis, R. B. 1994: Energy transduction by cytochrome complexes in mitochondrial and bacterial respiration: The enzymology of coupling electron transfer reactions to transmembrane proton translocation. - Annu. Rev. Biochem. 63: 675-716.

Uno, T., Mogi, T., Tsubaki, M., Nishimura, Y. & Anraku, Y. 1994: Resonance Raman and Furier transform infrared studies on the subunit I histidine mutants of the cytochrome *bo* complex in *Escherichia coli*. - J. Biol. Chem. 269: 11912-11920.

Valpuesta, J. M., Henderson, R. & Frey, T. G. 1990: Electron cryo-microscopic analysis of crystalline cytochrome oxidase. - J. Mol. Biol. 214: 237-251.

van der Oost, J., Musacchio, A., Pauptit, R. A., Ceska, T. A., Wierenga, R. K. & Saraste, M. 1993: Crystallization and preliminary X-ray analysis of the periplasmic fragment of CyoA - a subunit of the Escherichia coli cytochrome *o* complex. - J. Mol. Biol. 229: 794-796.

van der Oost, J., de Boer, A. P. N., de Gier, J-W. L., Zumft, W. G., Stouthamer, A. H. & van Spanning, R. J. M. 1994: The heme-copper oxidase family consists of three distinct types of terminal oxidases and is related to nitric oxide reductase. - FEMS Michrobiol. Lett. 121: 1-10.

Varotsis, C., Zhang, Y., Appelman, E. H. & Babcock, G. T. 1993: Resolution of the reaction sequence during the reduction of O_2 by cytochrome oxidase. - Proc. Natl. Acad. Sci. 90: 237-241.

von Wachenfeldt, C., de Vries, S., & van der Oost, J. 1994: The Cu_A site of the *caa*$_3$-type oxidase of *Bacillus subtilis* is a mixed-valence binuclear copper centre.- FEBS Lett. 340: 109-113.

Walker, J. E. 1992: The NADH:ubiquinone oxidoreductase (complex I) of respiratory chains. - Q. Rev. Biophys. 25: 253-324.

Weiss, H., Friedrich, T., Hofhaus, G. & Preis, D. 1991: The respiratory-chain NADH dehydrogenase (complex I) of mitochondria. - Eur. J. Biochem. 197: 563-576.

Wikström, M. 1989: Identification of the electron transfers in cytochrome oxidase that are coupled to proton-pumping. - Nature 338: 776-778.

Wikström, M., Bogachev, A., Finel, M., Morgan, J. E., Puustinen, A., Raitio, M., Verkhovskaya, M. & Verkhovsky, M. I. 1994: Mechanism of proton translocation by the respiratory oxidases: The histidine cycle. - Biochim. biophys. acta 1187: 106-111.

Woodruff, W. H., Einarsdottir, O., Dyer, R. B., Bagley, K. A., Palmer, G., Atherton, S. J., Goldbeck, R. A., Dawes, T. D. & Kliger, D. S. 1991: Nature and functional implications of the cytochrome a_3 transients after photodissociation of CO-cytochrome oxidase. - Proc. Natl. Acad. Sci. 88: 2588-2592.

Woodruff, W. H. 1993: Coordination dynamics of heme-copper oxidases. The ligand shuttle and the control and coupling of electron transfer and proton translocation. - J. Bioenerg. Biomembr. 25: 177-188.

Yagi, T., Hon-nami, K. & Ohnishi, T. 1988: Purification and characterization of two types of NADH-quinone reductase from *Thermus thermophilus* HB-8. - Biochemistry 27: 2008-2013.

Yoshikawa, S., Tera, T., Takahashi, Y., Tsukihara, T. & Caughey, W. S. 1988: Crystalline cytochrome *c* oxidase of bovine heart mitochondrial membrane: composition and X-ray diffraction studies. - Proc. Natl. Acad. Sci. 85: 1354-1358.

Yu, L., Wei, Y. Y., Usui, H. & Yu, C. A. 1992: Cytochrome b_{560} (QPs1) of mitochondrial succinate-ubiquinone reductase; Immunochemistry, cloning and nucleotide sequencing. - J. Biol. Chem. 267: 24508-24515.

Zumft, W. G., Dreusch, A., Löchelt, S., Cuypers, H., Friedrich, B. & Schneider, B. 1992: Derived amino acid sequences of *nos*Z gene (respiratory N_2O reductase) from *Alcaligenes eutrophus*, *Pseudomonas aeruginosa* and *Pseudomonas stutzeri* reveal potential copper-binding residues. - Eur. J. Biochem. 208: 31-40.

Zumft, W. G., Braun, C. & Cuypers, H. 1994: Nitric oxide reductase from *Pseudomonas stutzeri*: Primary structure and gene organization of novel bacterial cytochrome *bc* complex. - Eur. J. Biochem. 219: 481-490.

ORIGINAL PAPERS

I

Restoration of a lost metal-binding site: Construction of two different copper sites into a subunit of the *E.coli* cytochrome *o* quinol oxidase complex

by

John van der Oost, Pekka Lappalainen, Andrea Musacchio, Antony Warne, Laura Lemieux, Jon Rumbley, Robert B. Gennis, Roland Aasa, Torbjörn Pascher, Bo G. Malmström & Matti Saraste

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II

Two cysteines, two histidines and one methionine are ligands of a binuclear purple copper site

by

Mark Kelly, Pekka Lappalainen, Gert Talbo, Tuomas Haltia, John van der Oost & Matti Saraste

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III

Soluble Cu_A-binding domain from the *Paracoccus* cytochrome *c* oxidase

by

Pekka Lappalainen, Roland Aasa, Bo G. Malmström & Matti Saraste

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IV

Electron transfer between cytochrome *c* and the isolated Cu_A-domain: Identification of substrate-binding residues in cytochrome *c* oxidase

by

Pekka Lappalainen, Nicholas J. Watmough, Colin Greenwood & Matti Saraste

Biochemistry (in press)

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V

Spectroscopic and mutagenesis studies on the Cu_A centre from cytochrome *c* oxidase complex of *Paracoccus denitrificans*

by

Jaqueline A. Farrar, Pekka Lappalainen, Walter G. Zumft, Matti Saraste & Andrew J. Thomson

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