Journal of Bioenergetics and Biomembranes, Vol. 30, No. 1, 1998

Cytochrome *c* Oxidase (Heme aa_3) from *Paracoccus denitrificans:* Analysis of Mutations in Putative Proton Channels of Subunit I

Ute Pfitzner,[1] Annette Odenwald,[1] Thomas Ostermann,[1] Lilli Weingard,[1] Bernd Ludwig,[1] and Oliver-Matthias H. Richter[1]

Received August 20, 1997; Accepted August 22, 1997

One of the challenging features of energy-transducing terminal oxidases, like the aa_3 cytochrome *c* oxidase of *Paracoccus denitrificans,* is the translocation of protons across the cytoplasmic membrane, which is coupled to the transfer of electrons to oxygen. As a prerequisite for a more advanced examination of the enzymatic properties, several amino acid residues, selected on the basis of recent three-dimensional structure determinations, were exchanged in subunit I of the *Paracoccus* enzyme by site-directed mutagenesis. The properties of the mutated oxidases were analyzed by different methods to elucidate whether they are involved in the coupled and coordinated transfer of protons via two different pathways either to the site of oxygen reduction or through the enzyme from the cytoplasm to the periplasmic side.

KEY WORDS: Terminal oxidase; redox coupling; electrochemical gradient; electron transport; energy transduction; proton translocation; crystal structure; site-directed mutagenesis.

INTRODUCTION

Several reasons make bacterial cytochrome *c* oxidases attractive objects to study their enzymatic and spectroscopic properties. Firstly, they show a much simpler subunit structure than their eukaryotic, mitochondrial counterparts. Secondly, they are easily amenable to genetic manipulation, in particular to site-directed mutagenesis methods which aim at identifying the role and contribution of individual amino acid residues to the catalytic process. Furthermore, a large body of data shows the evolutionary and structural relationship of the oxidases that form the so-called superfamily of terminal oxidases (Saraste, 1990). Apart from the *bd*-type quinol oxidases (Miller *et al.*, 1988) all members of this family of oxidases are characterized mainly by the structure of their subunit I and the cofactors that are bound to this subunit (Calhoun *et al.*, 1994 and below) and by their ability to establish an electrochemical gradient by vectorial translocation of protons across the membrane (Morgan *et al.*, 1994). The close similarity of their overall enzymatic properties strongly suggests that results obtained with bacterial oxidases apply to the mitochondrial oxidase as well. This even holds true when oxidases are compared that differ from each other with respect to their heme composition (Calhoun *et al.*, 1994) and/or the donor of electrons (cytochrome *c* or quinol).

If a final proof of the similarity of prokaryotic and eukaryotic terminal oxidases had been required, this need was certainly fulfilled by the recent crystallization of the prokaryotic aa_3 cytochrome *c* oxidase of *Paracoccus denitrificans* (Iwata *et al.*, 1995) and the mitochondrial cytochrome *c* oxidase (Tsukihara *et al.*, 1995). The comparison of their structures shows that the three subunits that form the core of the mitochondrial enzyme may be superimposed on their prokaryotic counterparts without any noticeable spatial differences. The crystal structures give an ample illustration of the bewildering complexity of the eukaryotic

[1] Molecular Genetics, Institute of Biochemistry, Biozentrum, Marie-Curie-Str. 9, D-60439 Frankfurt, Germany.

0145-479X/98/0200-0089$15.00/0 © 1998 Plenum Publishing Corporation

oxidase that consists of 13 different subunits compared to only 4 subunits that constitute the *Paracoccus* oxidase.

THE AA_3 CYTOCHROME *C* OXIDASE OF *PARACOCCUS DENITRIFICANS*

Paracoccus denitrificans is able to transduce energy under aerobic conditions by transferring electrons from NADH to oxygen via an electron transport chain that closely resembles the mitochondrial one as far as the involvement of enzyme complexes and the sequence of individual steps is concerned (John and Whatley, 1977; van Spanning *et al.*, 1995). This similarity was even interpreted in the context of the endosymbiotic theory with *Paracoccus* as a successor of the organism once engulfed by the primordial eukaryote (John and Whatley, 1977).

The cytochrome *c* oxidase of the "mitochondrial" aa_3-type is only one of three terminal oxidases of *P. denitrificans* (de Gier *et al.*, 1994). Besides a quinol oxidase (Richter *et al.*, 1994; de Gier *et al.*, 1994) that uses ubiquinol as the donor of electrons to reduce oxygen directly there is a second cytochrome *c* oxidase (Raitio and Wikström, 1994; de Gier *et al.*, 1996) with a different heme (cbb_3) and cofactor composition, lacking the Cu_A center. Although the expression of this oxidase is believed to be enhanced under conditions of low oxygen tension (de Gier *et al.*, 1994), it is able to replace the aa_3 oxidase in supporting aerobic growth of *Paracoccus*. Four different loci are involved in providing the genetic information for the aa_3 cytochrome *c* oxidase of *Paracoccus*. There are two genetic loci for isogenes coding for subunit I; in most instances only the *cta*DII gene is transcribed (Raitio *et al.*, 1990) while there is only one condition reported where a plasmid-coded *cta*DI gene was expressed (de Gier *et al.*, 1994). The *cta*C and *cta*E genes of the so-called *cta* operon code for subunits II and III of this oxidase (Raitio *et al.*, 1987; Steinrücke *et al.*, 1987) while yet another locus, *cta*H, codes for subunit IV (Witt and Ludwig, 1997).

Crystallization of the *Paracoccus* aa_3 cytochrome *c* oxidase finally proved that the enzyme complex consists of four different subunits. A small peptide originally proposed by Haltia (1990) as a fourth subunit was shown to be part of the complex (Iwata *et al.*, 1995). The function of this very small subunit with only one transmembrane helix remains uncertain as the deletion of its gene had no deleterious effects for the assembly or function of the oxidase (Witt and Ludwig, 1997). Subunit III is equally dispensable since an enzyme complex consisting only of the subunits I and II has been shown to be catalytically competent and completely functional (Ludwig and Schatz, 1980; Hendler *et al.*, 1991). On the other hand there have been speculations for the *Paracoccus* aa_3 oxidase that the spatial arrangement of the seven transmembrane helices of subunit III may reflect its involvement in binding cytochrome c_{552} (Iwata *et al.*, 1995), that this subunit may influence the conformation of subunits I and II (Echabe *et al.*, 1995), and that the absence or mutation of this subunit influences the efficency of energy conservation (Wu *et al.*, 1995).

The core of the aa_3 cytochrome *c* oxidase is composed of subunit II which carries the binuclear Cu_A site that serves as the entry point for electrons donated by cytochrome *c* (Hill, 1991) and subunit I which contains the other redox-active centers. These are a low-spin heme *a* accepting electrons from the Cu_A center and transferring them to the binuclear site of oxygen reduction which is formed by one copper atom called Cu_B and the high-spin heme a_3.

In addition to the redox-active atoms mentioned above, manganese is bound in substoichiometric amounts by specific amino acid residues in the two larger subunits (Hosler *et al.*, 1995; Witt *et al.*, 1997). The importance of this metal atom is not completely understood although some stabilizing role has been implied.

PROTON-PUMPING PATHWAYS IN CYTOCHROME *C* AND QUINOL OXIDASES

Due to the structural similarity between cytochrome *c* and quinol oxidases which was recently emphasized by the analysis of two-dimensional crystals of the *E. coli* bo_3 quinol oxidase (Gohlke *et al.*, 1997), it is generally assumed that protons are transferred within and translocated across subunit I via routes that are identical in both classes of enzymes. In fact, two distinct pathways for protons have been inferred from mutagenesis studies of the *E. coli* bo_3 quinol oxidase since the mutation of Asp135 to Asn exerts no major effect on the reduction of oxygen to water while the proton-pumping capacity of this oxidase is effectively abolished (Thomas *et al.*, 1993a).

Inspection of the three-dimensional structure of the *Paracoccus* cytochrome *c* oxidase also indicated

two possible proton transfer pathways (Iwata *et al.*, 1995). One such route for the delivery of protons to the binuclear center as the site of water formation (Fig. 1) was suggested to be composed mainly of residues Ser291, Lys354, Thr351, the hydroxyl group of heme a_3, and of Tyr280 (numbering refers to the residues in subunit I of the *Paracoccus* enzyme). The side chain of Lys354 may adopt different conformations to form hydrogen bonds either to Ser291 or to Thr351, respectively. This pathway has been dubbed "K channel" due to the involvement of the lysine residue.

Considerably more residues contribute to the pathway (often termed the "D channel") for protons to be translocated across the membrane. Asp124, Thr203, and Asn199 are located in the vicinity of the entrance of this channel (Fig. 1). The further route of protons is probably lined by Asn113, Asn131, and Tyr35 before a larger cavity is reached in the protein with Ser134, Ser189, and Ser193 as possible hydrogen-bonding partners for water molecules, which may also be important for the proton transfer to Glu278 (Iwata *et al.*, 1995). No definite suggestion could be deduced from the structure of this oxidase as to the further route of the protons once they gain access to Glu278. One alternative could be a pathway via the Cu_B ligand His325 and Asp399 to the periplasmic bulk phase while an equally likely route may involve one or both of the heme a_3 propionate groups.

This concept of two different proton conducting pathways has recently been challenged extensively. Although mutation of Lys362 of the *E. coli* bo_3 quinol oxidase to methionine leads to an inactive enzyme, it has been shown that this mutant oxidase is nevertheless able to catalyze the later steps of oxygen chemistry, provided suitable experimental conditions are established (Konstantinov *et al.*, 1997). This implies that only the first two of the four protons required for the formation of two water molecules are transferred via the K channel. The other two would reach the binuclear center via the D channel which is also used by the "pumped" protons. A total of six protons would thus have to be funneled through the D channel, although it is not immediately clear how chemical protons can be separated from pumped protons (see Wikström *et al.*, this volume). Perhaps there is no such mechanistic separation, and their fate is decided on momentary needs. A different challenge to the two-channel concept is the description of additional proton pathways invoked from the crystal structure of the bovine cytochrome *c* oxidase (Tsukihara *et al.*, 1996; see also below).

Considering these uncertainties about the contribution of the two proposed channels to the overall movement of protons within or across the enzyme complex, it is beyond the scope of this review to address the even more speculative aspect of coupling of electron transfer to oxygen with the translocation of protons across the membrane, and the nature of the gating mechanism. This topic is covered by other reviews of this volume.

PUMP STOICHIOMETRY OF TERMINAL OXIDASES

It is generally accepted today that the ratio between electrons transferred to oxygen and concomitant translocation of protons across the membrane-incorporated oxidase complex is integer, as long as experimental conditions favor the strict coupling of both processes. In addition four protons are consumed in the reduction of one oxygen molecule.

The ability of oxidases to act as a proton pump can already be tested on the level of whole cells. In the absence of an alternative proton pumping cytochrome *c* oxidase (see below) *Paracoccus* cells should acidify the external medium with a stoichiometry of 3 protons for every electron transferred from succinate to oxygen via the the bc_1 complex and the aa_3 oxidase if any contribution by complex I (oxidizing NADH) is inhibited. This stoichiometry is decreased to 2 if the bc_1 complex is bypassed, and oxygen reduced only by the quinol oxidase.

One bias of the whole-cell approach, however, deserves mentioning. While a ratio of 3 H^+/e^- clearly indicates the presence of a redox-driven cytochrome *c* oxidase proton pump, values close to 2, on the other hand, may reflect two alternative situations: (i) with a high electron transport rate, it would indicate loss of proton pumping activity; (ii) with a low electron transfer rate, electrons are forced to predominantly use the quinol oxidase branch; any small contribution of a proton pumping cytochrome *c* oxidase is therefore difficult to assess experimentally.

Experimental conditions for proton pumping experiments presented here were essentially as described by Puustinen *et al.* (1989).

EXPRESSION AND PURIFICATION OF *PARACOCCUS AA*$_3$ CYTOCHROME *C* OXIDASE

Based on evidence from the 3-D structure of the *Paracoccus* and beef heart oxidases (Iwata *et al.*, 1995;

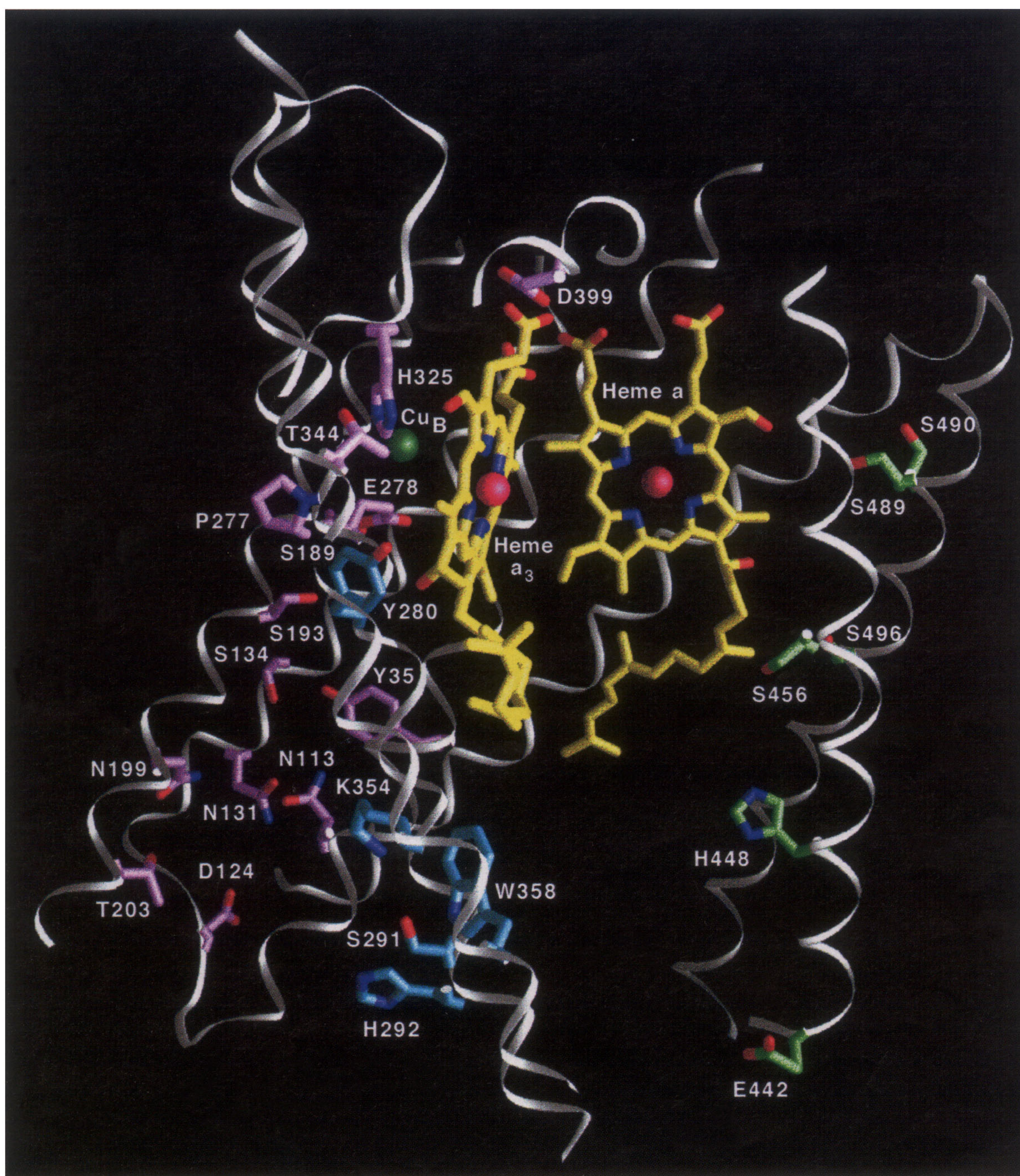

Fig. 1. Partial structure of subunit I of the *P. denitrificans* cytochrome *c* oxidase (Iwata *et al.*, 1995), showing residues in 8 out of 12 transmembrane helices, where mutations were introduced (see Table I). Carbon atoms are color coded according to their proposed contribution to one of three separate channels (see text for details). Magenta: channel D, cyan: channel K, and green: channel E.

Tsukihara *et al.*, 1996) and on earlier results of studies with the bo_3 quinol oxidase of *E. coli* and the aa_3 cytochrome *c* oxidase of *Rhodobacter sphaeroides*, several amino acid residues of the *Paracoccus* aa_3 cytochrome *c* oxidase were exchanged by site-directed mutagenesis (for experimental details see Witt *et al.*,

1995). Mutations were confirmed by DNA sequencing, and genes cloned into a derivative of the broad-host range plasmid pBBR1MCS (Kovach *et al.*, 1994). Besides an additional selection marker (Parales and Harwood, 1993) conferring resistance to streptomycin, the promoter region of the *cta* operon (Raitio *et al.*, 1987) was inserted to direct the expression of the *cta*DII gene.

Broad-host range plasmids carrying mutated *cta*DII genes for subunit I were conjugated (Gerhus *et al.*, 1990) into the *Paracoccus* host strain AO1 carrying the following features. This strain is a derivative of strain MR31 deleted in the chromosomal copy of the *cta*DII gene and its isogene *cta*DI (Raitio and Wikström, 1994). To obtain AO1, the alternative cbb_3 cytochrome *c* oxidase was inactivated additionally by insertion of a gentamycin resistance gene (Elzer *et al.*, 1995) into *cco*N coding for subunit I of this oxidase. Consequently, strain AO1 is not able to express any cytochrome *c* oxidase activity, but regains the ability to assemble an intact aa_3 cytochrome *c* oxidase once a functional copy of the *cta*DII gene is introduced *in trans*. When the host strain is complemented with a mutated *cta*DII gene, some characteristics of the mutant oxidase can easily be determined already on the level of membranes. Expression in strain AO1 of the aa_3 cytochrome *c* oxidase carrying the wild-type subunit I or mutated versions usually reached a level comparable to that observed with wild-type strains of *Paracoccus*.

The aa_3 cytochrome *c* oxidase was purified after solubilization of membranes by dodecyl maltoside (Hendler *et al.*, 1991), but essentially in a one-step procedure involving specific F_v antibody fragments and affinity purification of the resulting complexes on streptavidine sepharose (Kleymann *et al.*, 1995), usually followed by size exclusion chromatography to separate the oxidase complex from excess antibody fragments.

SITE-DIRECTED MUTAGENESIS OF RESIDUES LINING PUTATIVE PROTON TRANSFER PATHWAYS IN THE *PARACOCCUS* CYTOCHROME *C* OXIDASE

Determination of the structure of the *Paracoccus* aa_3 cytochrome *c* oxidase allowed us to investigate the possible involvement of specific amino acid residues in the transfer of protons either to the binuclear center of subunit I or across this subunit.

The mutations that were introduced at different positions of subunit I fall into three categories. Those thought to line the channel that transfers protons from the cytoplasmic to the periplasmic side (Iwata *et al.*, 1995) constitute one group (the D channel). Mutations of this group include Tyr35, Asn113, Asp124, Asn131, Ser134, Ser189, Ser193, Asn199, Thr203, Pro277, Glu278, Asp399, the double mutations Asn113/Asn131, Ser189/Ser193, and Asn199/Thr203, as well as His325 and Thr344 (see Table I). A second set of mutations is located in the other channel (the K channel): Ser291, His292, Lys354, Trp358, and Tyr280. The final category includes residues that were proposed to establish a separate proton transfer pathway, although no specific function was attributed to it (Tsukihara *et al.*, 1996). These mutations comprise Glu442, His448, Ser456, the double mutant Ser489/Ser490, and Ser496. For convenience, this putative channel will be called the E channel.

General Remarks

Several routine analyses were performed with each mutant cytochrome *c* oxidase after its purification. These include electrophoretic analysis of the subunit pattern, determination of the heme-to-protein ratio, and basic spectral characterizations. If not noted otherwise, all of these criteria were found in close agreement with those of the wild-type. In some instances, the above assignment of residues, especially to channels D and K (e.g., Thr344 and His325), may appear arbitrary, since according to current models both these channels should come in close contact at some point (Morgan *et al.*, 1994; Iwata *et al.*, 1995). Some of the residues might just serve a structural purpose.

THE D CHANNEL

By definition the channel through which protons are pumped across the membrane has to traverse the protein. Residues from several transmembrane helices are thought to contribute to this channel. In the *Paracoccus* enzyme, residues from helices II, III, IV, and VI seem to be involved. The carboxyl group of Asp124 which lies at the entry of this channel in the loop between helix II and III is of prime importance for the

Table I. Activities of Purified Mutant Oxidases from *Paracoccus denitrificans* and Proton-to-Electron Stoichiometries Measured in Intact Cells

Mutation[a]	Helix[b]	Activity (%)[c]	H^+/e^- ratio[d]
	channel D		
Y35F	I	59	3.0
N113D	II	67	2.9
N113V	II	16	3.2
N113V/N131D	III/II	96[e]	2.5
S134A	III	94	2.9
D124N	loop II/III	5	2.1
D124S	loop II/III	7	1.9
N131D	III	62	3.0
N131V	III	8	2.2
S189A	IV	85	3.1
S193A	IV	73	3.0
S189A/S193A	IV/IV	104	3.3
N199V	IV	174	3.1
N199D/T203V	IV/IV	72	2.6
T203V	IV	72	3.0
P277G	VI	2.5	nd[f]
E278Q	VI	2	2.0
E278D	VI	60	2.9
H325N	loop VII/VIII	3	2.3
T344D	VIII	1.2	nd[f]
T344V	VIII	1.2	2.2
D399L	loop IX/X	12	2.0
D399N	loop IX/X	110	3.0
	channel K		
Y280H	VI	0.9	nd[f]
S291A	VI	105	3.2
H292L	VI	115	3.2
K354M	VIII	0	1.8
W358F	VIII	105	3.0
	channel E		
E442Q	XI	42[e]	3.1
H448L	XI	5[e]	1.8
S456A	XI	81[e]	2.9
S489A/S490A	XII/XII	106[e]	2.9
S496A	XII	67[e]	2.9

[a] Position of introduced mutation in subunit I.
[b] According to the crystal structure of Iwata *et al.* (1995).
[c] Activities are given relative to a wild-type oxidase expressed and purified under the same conditions. All oxidases are isolated as a complex with F_v antibody fragments. 100% is equivalent to a turnover number of about 200 sec^{-1} at 20 μM cytochrome *c* (for details of the assay conditions see Witt *et al.*, 1995).
[d] Proton-to-electron ratio of whole cells respiring on succinate determined according to Puustinen *et al.* (1989).
[e] Measured only on the level of isolated membranes.
[f] nd, not determined.

maintenance of the proton pumping capacity as has been shown for the quinol oxidase of *E. coli* (Thomas *et al.*, 1993a) and the aa_3 cytochrome *c* oxidase of *R. sphaeroides* (Fetter *et al.*, 1995). Experiments with whole cells give no indication for proton pumping if Asp124 of the *Paracoccus* enzyme is replaced by asparagine, in line with results obtained with the aforementioned oxidases, but this may simply be the consequence of the low residual enzymatic activity (see above and Table I). This is about 5% for the *Paracoccus* mutant and comparable to Asp132Asn in *Rhodobacter,* while Asp135Asn in *E. coli* still exhibits 45% of the wild-type electron transfer activity. The same residue has also been mutated to serine in *Paracoccus* (Table I), to alanine in *Rhodobacter* (same consequences as the Asp132Asn mutation; Fetter *et al.*, 1995), and to lysine in *E. coli* (Garcia-Horsman *et al.*, 1995). This illustrates to some extent the problem of comparing the effect of mutations if different amino acid residues are used for the replacement. Comparing identical substitutions in this position, results may be, and have been in the past, interpreted as a decoupling of electron transfer from proton translocation. Yet, electron transfer is not unaffected, and the different levels of residual activity may support the notion that the stringency of interaction between both processes may be higher in cytochrome *c* oxidases than in the quinol oxidase. While a number of alternative explanations may be plausible, too, this mutual influence is in accordance with the view that most of the protons which are required for both processes share this same D channel pathway. A mutation in an essential residue of this channel would therefore lead to loss of proton pumping (decoupling), but, to a varying degree in the two different groups of oxidases, also decrease the efficiency of water formation, as reflected by a diminished electron transfer rate.

The situation is very much different for the mutations Asn113Asp and Asn113Val which in *Paracoccus* retain significant enzymatic and proton pumping activity. The low electron transfer activity of the Asn113Val oxidase probably reflects a reduced stability of the purified enzyme as the activity of the mutant oxidase in membranes reaches about 50% of the wild-type value. In contrast, mutations of the equivalent residue (Asn124) in *E. coli* to aspartate and histidine both abolish proton transfer, though retaining considerable electron transfer activity (56% and 16% respectively). The *E. coli* mutant Asn124Gln, on the other hand, shows wild-type characteristics (Garcia-Horsman *et al.*, 1995).

Yet another mutant that impairs proton transfer in *E. coli* is Asn142Val, while the effect of the mutation Asn142Asp is less clear (some pumping may be attrib-

utable to this mutant). Asn142Gln again shows wild-type behavior (Garcia-Horsman *et al.*, 1995). The mutation Asn131Val of the oxidase in *Paracoccus* has a lower electron transfer activity than in *E. coli* (8% instead of 22%) while currently no unequivocal conclusion on the proton pumping capacity can be drawn from whole-cell measurements. In contrast, Asn131Asp is clearly comparable to the wild-type enzyme although the electron transfer activity is somewhat reduced (62%).

Another mechanistically important amino acid residue in the pathway for the pumped protons is Glu286 in the *E. coli* enzyme. The alanine mutant is nearly devoid of electron and proton transfer activity (Thomas *et al.*, 1993a), as is the glutamine mutant (5% residual activity; Wikström, personal communication), which had earlier been reported to perform both functions (Thomas *et al.*, 1993a). The equivalent mutation leaves the *Rhodobacter* enzyme completely inactive (Mitchell *et. al.*, 1995). The same holds true for the *Paracoccus* Glu278Gln mutant with a residual activity of 2% close to the limit of reliability of the assay employed. The conservative replacement Glu278Asp leads to a functional, proton-transferring enzyme (Table I). The mutation Pro277Gly shows even more severe effects in *Paracoccus* than does Pro285Gly in *E. coli* (29% of wild-type activity, no proton pumping; Svensson-Ek *et al.*, 1996) since the activity is not only reduced to about 3% in the *Paracoccus* enzyme but the mutant enzyme displays distorted spectroscopic properties; not the least, substoichiometric ratios of heme to protein and probably a lack of Cu_B are observed (not shown).

One residue that would at best show an indirect contribution to proton transfer is Thr344 of the *Paracoccus* enzyme. Replacement by aspartate and valine results in an enzyme with only marginal enzymatic activity and a decreased copper content in the latter (not shown). Mutation of the equivalent Thr352 to alanine causes a considerable loss of enzymatic activity in *E. coli,* while replacement by asparagine and serine retains much of the enzymatic activity (Thomas *et al.*, 1993b). The mutation Thr352Ala results in a severely reduced enzymatic activity in *Rhodobacter* (Hosler *et al.*, 1996). His325Asn, the *Paracoccus* equivalent of one of the three Cu_B ligands, is virtually devoid of enzymatic activity, although copper determination by TXRF shows partial occupancy of Cu_B in the binuclear site (Wittershagen, unpublished). Distortion of the binuclear site may obscure a possible role of this residue in proton transfer according to the histidine shuttle model (Iwata *et al.*, 1995).

The replacements Ser134Ala, Ser189Ala (no equivalent mutations published for the oxidases of *E. coli* or *Rhodobacter*), and the mutations Ser193Ala, Asn199Val, and Thr203Val for which similar mutations were constructed in *Rhodobacter,* as well as the unprecedented double mutants Asn113Val/Asn131Asp, Ser189Ala/Ser193Ala, and Asn199Asp/Thr203Val all result in functional enzymes with electron transfer activity ranging from 44% to 105%. The latter two double mutants may become more interesting, however, as measurements with whole cells indicate that proton pumping efficiency is reduced in these mutant oxidases (Table I). Asp399 is included in this group of residues as it has been speculated that it may be located in the exit part of the D channel. The mutation to asparagine does not alter the properties of the oxidase while the mutation Asp399Leu reduces the activity to about 10%. The influence of this mutation is accompanied by some changes in the redox FTIR[2] spectrum (Hellwig, unpublished). Mutations of the equivalent Asp407 of *Rhodobacter* to alanine, asparagine, and cysteine result in enzymes with properties nearly identical to wild-type (Qian *et al.*, 1997). Therefore, these three replacements do not indicate an essential role of this aspartate in proton translocation in the *Rhodobacter* oxidase, although structure determination shows that residue Asp399 is in close contact to propionate groups of heme a_3 (Iwata *et al.*, 1995).

Results of site-directed mutagenesis for residues of the D channel support the notion that there are qualitative differences between the quinol and cytochrome *c* oxidases as can be inferred from the different effects exerted by the mutations Asn113Asp in *Paracoccus* and Asn124Asp in *E. coli* which retain or abolish proton pumping (see above). On the other hand, more results of equivalent mutations in other oxidases are necessary to evaluate whether the contribution of this residue reflects a genuine difference between these two types of oxidases.

THE K CHANNEL

Spatially confined to residues of helices VI and VIII, side chains thought to constitute the K channel

[2] Abbreviations: FTIR: Fourier transform infrared spectroscopy; TXRF total reflection X-ray fluorescence.

could direct protons to the binuclear center (Iwata *et al.*, 1995). Ser291 and His292 are close to the cytoplasmic side of this channel in the *Paracoccus* enzyme. Their mutation to alanine and leucine, respectively, is without significant influence on either the transfer of electrons or protons. As can be seen from the structure the mouth of the K channel is relatively wide, which may explain the above results. The same holds true for the Trp358Phe mutant of *Paracoccus* (Table 1). The most important side chain for the functionality of this channel seems to be the one of a highly conserved lysine. Mutation of Lys354 to methionine in *Paracoccus* leaves the enzyme virtually inactive as are the equivalent mutant enzymes of *E. coli* and *Rhodobacter* (Thomas *et al.*, 1993b; Hosler *et al.*, 1996). Interestingly, the mutant enzymes Lys362Met und Lys362Leu of *E. coli* still show an isotopic effect (Svennson *et al.*, 1995) indicative of the involvement of proton uptake reactions.

As with the Tyr288Phe mutant from *E. coli* the corresponding Tyr280His mutant of *Paracoccus* has only very low enzymatic activity (proton pumping has not been assayed). Again this would be consistent with an important function of this residue for the catalytic mechanism, but functions other than the transfer of protons are possible. For example, loss of stabilizing interactions with other residues or a structural distortion due to different spatial requirements of the newly introduced side chain may explain the observed result, too.

The results of site-directed mutagenesis experiments obtained thus far for residues lining the K channel in the *Paracoccus* oxidase are consistent with results of earlier studies with equivalent mutations in other oxidases. Taken together, lysine 354 is obviously important for the transfer of protons toward the site of oxygen reduction while tyrosine 280 (288 in the *E. coli* enzyme) and threonine 344 (352 in *E. coli,* see above) may be involved in reactions other than proton transfer (Iwata *et al.*, 1995).

THE E CHANNEL

Analysis of the cytoplasmic surface of the *Paracoccus* enzyme reveals three different sinks, two of which represent the entries of the above discussed K and D channels. Glu442 is located in the vicinity of the third entry; therefore this channel is termed the E channel. The determination of the structure of the bovine cytochrome *c* oxidase led Tsukihara *et al.* (1996) earlier to claim a third channel with a rather constricted diameter at certain sites along helices XI and XII. Replacement of Glu442 by glutamine in *Paracoccus* causes a notable decrease of enzymatic activity to about 40% of the wild-type enzyme but is not accompanied by a loss of proton pumping activity (Table I). The electron transfer rates of the mutations Ser456Ala, Ser496Ala and the double mutant Ser489Ala/Ser490Ala are all in the range between 70% and 106%. No inhibition of proton pumping activity of whole cells is observed with any of these mutant oxidases with the exception of His448Leu. An oxidase that contains this mutation displays a severe disturbance of its heme *a* content and consequently is devoid of enzymatic activity. As a Western blot analysis proves the presence of at least subunits I and II of this oxidase, and since the distance of the histidine side chain of this residue to heme *a* or heme a_3 is >18 Å and ~7 Å respectively, there is no obvious explanation for the observed loss of the cofactor. Further studies will be required to elucidate the reason for this behavior.

No published data for mutations in those two transmembrane helices are available to safely draw general conclusions from the above results with the *Paracoccus* cytochrome *c* oxidase. Nevertheless, at least for this specific oxidase there are at present no data that indicate an involvement of amino acid residues of this region in the overall catalytic mechanism. Given the general structural and functional similarity of enzymes of the superfamily of terminal oxidases these results may also apply to other oxidases, disproving the existence of an additional proton transfer pathway in this section of the enzyme.

ACKNOWLEDGMENT

We thank Mårten Wikström (Helsinki), So Iwata (Uppsala), and Hartmut Michel (Frankfurt) for helpful discussions, Mirja Raitio (Helsinki) for providing strain MR31, Petra Hellwig (Erlangen) for FTIR data, Axel Wittershagen (Frankfurt) for metal determinations by TXRF, and Christian Lücke (Frankfurt) for the graphical realization of Fig. 1. Andrea Herrmann, Rüdiger Noll, and Werner Müller provided excellent technical assistance. This work was supported by DFG (SFB 472) and Fonds der Chemischen Industrie.

REFERENCES

Calhoun, M. W., Thomas, J. W., and Gennis, R. B. (1994). *Trends Biochem. Sci.* **19**, 325–330.

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., and van der Oost, J. (1994). *Mol. Microbiol.* **13**, 183–196.

de Gier, J.-W. L., Schepper, M., Reijnders, W. N. M., van Dyck, S. J., Slotboom, D. J., Warne, A., Saraste, M., Krab, K., Finel, M., Stouthamer, A. H., van Spanning, R. J. M., and van der Oost, J. (1996). *Mol. Microbiol.* **20**. 1247–1260.

Echabe, I., Haltia, T., Freire, E., Gõni, F. M., and Arrondo, J. L. R. (1995). *Biochemistry* **34**, 13565–13569.

Elzer, P. H., Kovach, M. E., Phillips, R. W., Robertson, G. T., Peterson, K. M., and Roop, R. M. (1995). *Plasmid* **33**, 51–71.

Fetter, J. R., Qian, J., Shapleigh, J., Thomas, J. W., Garcia-Horsman, A., Schmidt, E., Hosler, J., Babcock, G. T., and Gennis, R. B. (1995). *Proc. Natl. Acad. Sci. USA* **92**, 1604, 1608.

Garcia-Horsman, J. A., Puustinen, A., Gennis, R. B., and Wikström, M. (1995). *Biochemistry* **34**, 4428–4433.

Gerhus, E., Steinrücke, P., and Ludwig, B. (1990). *J. Bacteriol.* **172**, 2392–2400.

Gohlke, U., Warne, A., and Saraste, M. (1997). *EMBO J.* **16**, 1181–1188.

Haltia, T. (1990). *Biochemistry* **33**, 9731–9740.

Hendler, R. W., Pardhasaradhi, K., Reynafarje, B., and Ludwig, B. (1991). *Biophys. J.* **60**, 415–423.

Hill, B. C., (1991). *J. Biol. Chem.* **266**, 2219–2226.

Hosler, J. P., Espe, M. P., Zhen, Y., Babcock, G. T., and Ferguson-Miller, S. (1995). *Biochemistry* **34**, 7586–7592.

Hosler, J. P., Shapleigh, J. P., Mitchell, D. M., Kim, Y., Pressler, M. A., Georgiou, C., Babcock, G. T., Alben, J. O., Ferguson-Miller, S., and Gennis, R. B. (1996). *Biochemistry* **35**, 10776–10783.

Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995). *Nature* **376**, 660–669.

John, P., and Whatley, F. R. (1977). *Biochim. Biophys. Acta* **463**, 129–153.

Kleymann, G., Ostermeier, C., Ludwig, B., Skerra, A., and Michel, H. (1995). *Biotechnology* **13**, 155–160.

Konstantinov, A.A., Siletsky, S. A., Mitchell, D., Kaulen, A. D., and Gennis, R. B. (1997). *Proc. Natl. Acad. Sci USA*, **94**, 9085–9090.

Kovach, M. E., Phillips, R. W., Elzer, P. H., Roop, R. M., and Peterson, K. M. (1994). *Biotechniques* **16**, 800–802.

Ludwig, B., and Schatz, G. (1980). *Proc. Natl. Acad. Sci. USA* **77**, 196–200.

Miller, M. J., Hermodson, M., and Gennis, R. B. (1988). *J. Biol. Chem.* **263**, 5235–5240.

Mitchell, D. M., Aasa, R., Ädelroth, P., Brzezinski, P., and Malmström, B. G. (1995). *FEBS Lett.* **374**, 371–374.

Morgan, J. E., Verkhovsky, M. I., and Wikström, M. (1994). *J. Bioenerg. Biomembr.* **26**, 599–608.

Parales, R. E., and Harwood, C. S. (1993). *Gene* **133**, 23–30.

Puustinen, A., Finel, M., Virkki, M., and Wikström, M. (1989). *FEBS Lett.* **249**, 163–167.

Qian, J., Shi, W. J., Pressler, M., Hogansson, C., Mills, D., Babcock, G. T., and Ferguson-Miller, S. (1997). *Biochemistry* **36**, 2539–2543.

Raitio, M., Jalli, T., and Saraste, M. (1987). *EMBO J.* **6**, 2825–2833.

Raitio, M., Pispa, J. M., Metso, T., and Saraste, M. (1990). *FEBS Lett.* **261**, 431–435.

Raitio, M., and Wikström, M. (1994). *Biochim. Biophys. Acta* **1186**, 100–106.

Richter, O.-M. H., Tao, J.-s., Turba, A., and Ludwig, B. (1994). *J. Biol. Chem.* **37**, 23079–23086.

Saraste, M. (1990). *Quart. Rev. Biophys.* **23**, 331–366.

Steinrücke, P., Steffens, G. C. M., Panskus, G., Buse, G., and Ludwig, B. (1987). *J. Biol. Chem.* **266**, 7676–7681.

Svensson, M., Hallen, S., Thomas, J. W., Lemieux, L., Gennis, R. B., and Nilsson, T. (1995). *Biochemistry* **34**, 5252–5258.

Svensson-Ek, M., Thomas, J. W., Gennis, R. B., Nilsson, T., and Brzezinski, P. (1996). *Biochemistry* **35**, 13673–13680.

Thomas, J. W., Puustinen, A., Alben, J. O., Gennis, R. B., and Wikström, M. (1993a). *Biochemistry* **32**, 10923–10928.

Thomas, J. W., Lemieux, L., Alben, J. O., and Gennis, R. B. (1993b). *Biochemistry* **32**, 11173–11180.

Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1995). *Science* **269**, 1069–1074.

Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996). *Science* **272**, 1136–1144.

van Spanning, R. J. M., de Boer, A. P. N., Reijnders, W. N. M., de Gier, J.-W. L., Delorme, C. O., Stouthamer, A. H., Westerhoff, H. V., Harms, N., van der Oost, J. (1995). *J. Bioenerg. Biomembr.* **27**, 499–512.

Witt, H., and Ludwig, B. (1997). *J. Biol. Chem.* **272**, 5514–5517.

Witt, H., Zickermann, V., and Ludwig, B. (1995). *Biochim. Biophys. Acta* **1230**, 74–76.

Witt, H., Wittershagen, A., Bill, E., Kolbesen, B. O., and Ludwig, B. (1997). *FEBS Lett.* **409**, 128–130.

Wu, S., Moreno-Sanchez, R., and Rottenberg, H. (1995). *Biochemistry* **34**, 16298–16305.