Biochimica et Biophysica Acta 1787 (2009) 626–634

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Review

Electron transfer and energy transduction in the terminal part of the respiratory chain — Lessons from bacterial model systems

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ARTICLE INFO

Article history:
Received 27 December 2008
Received in revised form 13 February 2009
Accepted 16 February 2009
Available online 4 March 2009

Keywords:
Cytochrome *c* oxidase
Complex-III
Proton pumping
Cofactor insertion
Thermus thermophilus
Paracoccus denitrificans

ABSTRACT

This review focuses on the terminal part of the respiratory chain where, macroscopically speaking, electron transfer (ET) switches from the two-electron donor, ubiquinol, to the single-electron carrier, cytochrome *c*, to finally reduce the four-electron acceptor dioxygen. With 3-D structures of prominent representatives of such multi-subunit membrane complexes known for some time, this section of the ET chain still leaves a number of key questions unanswered. The two relevant enzymes, ubiquinol:cytochrome *c* oxidoreductase and cytochrome *c* oxidase, appear as rather diverse modules, differing largely in their design for substrate interaction, internal ET, and moreover, in their mechanisms of energy transduction. While the canonical mitochondrial complexes have been investigated for almost five decades, the corresponding bacterial enzymes have been established only recently as attractive model systems to address basic reactions in ET and energy transduction. Lacking the intricate coding background and mitochondrial assembly pathways, bacterial respiratory enzymes typically offer a much simpler subunit composition, while maintaining all fundamental functions established for their complex "relatives". Moreover, related issues ranging from primary steps in cofactor insertion to supramolecular architecture of ET complexes, can also be favourably addressed in prokaryotic systems to hone our views on prototypic structures and mechanisms common to all family members.

1. Cytochrome *c* oxidase

Cytochrome oxidase is the terminal member of the electron transport chain of mitochondria and many bacteria. It catalyzes the reduction of molecular oxygen in a concerted four-electron transfer step, and uses the free energy of this reaction to establish a proton gradient across the membrane [1–4]. It may be considered a perfect molecular machine, coupling electron transfer to proton translocation with a stoichiometry of the pump of unity, i.e. 1 H^+ translocated per e^-. Thus, during a complete O_2 reduction cycle, out of 8 protons taken up from the inside (mitochondrial matrix or bacterial cytoplasm), four are translocated across the bilayer (vectorial or "pumped" protons) while another four, designated as "chemical" protons, are directed to the active site for water formation:

$$4\,\mathrm{cyt}\,c^{2+} + 8\,H^+_{in} + O_2 \rightarrow 2\,H_2O + 4\,\mathrm{cyt}\,c^{3+} + 4\,H^+_{out}.$$

Despite early 3-D structural information [5–8], the molecular mechanism(s) of the actual coupling process in this integral membrane protein is still not resolved. With no substantial redox-related conformational changes between different redox states observed, structure alone does not immediately provide functional clues for this crucial step in energy transduction (see also below).

Focusing here on the aa_3-type cytochrome *c* oxidase isolated from the soil bacterium *Paracoccus denitrificans* (see [9]), two different preparations have been studied in the past: originally isolated as a two-subunit enzyme (see Fig. 1) in the presence of Triton X-100 [10] and later as a four-subunit complex using dodecyl maltoside for solubilization [11,12], it became obvious that subunits I–III (however, not subunit IV, see [13]) exhibit high sequence identities with their corresponding mitochondrially coded subunits of the eukaryotic enzyme. Not unexpectedly, both these bacterial preparations turned out to be indistinguishable from the 13-subunit enzyme from mammalian mitochondria in terms of their energy transduction properties [12], and for their basic 3-D structure as deduced from X-ray crystal analyses (see above). Furthermore, subunits III and IV do not contribute to the redox-related signals as observed by FTIR, nor influence the ET reaction to any extent [14–15].

Receiving electrons from its single-electron donor cyt *c*, the charge is transferred to the Cu_A center in subunit II of oxidase, a homo-binuclear copper center of mixed valence, from where electrons reach redox centers in subunit I: first a heme *a* at a metal-to-metal distance of 19.5 Å, and then the binuclear center made up of heme a_3 and a copper ion termed Cu_B, both closely spaced (at around 4.5 Å) and

Abbreviations: ET, electron transfer; FeS, iron/sulfur center

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doi:10.1016/j.bbabio.2009.02.020

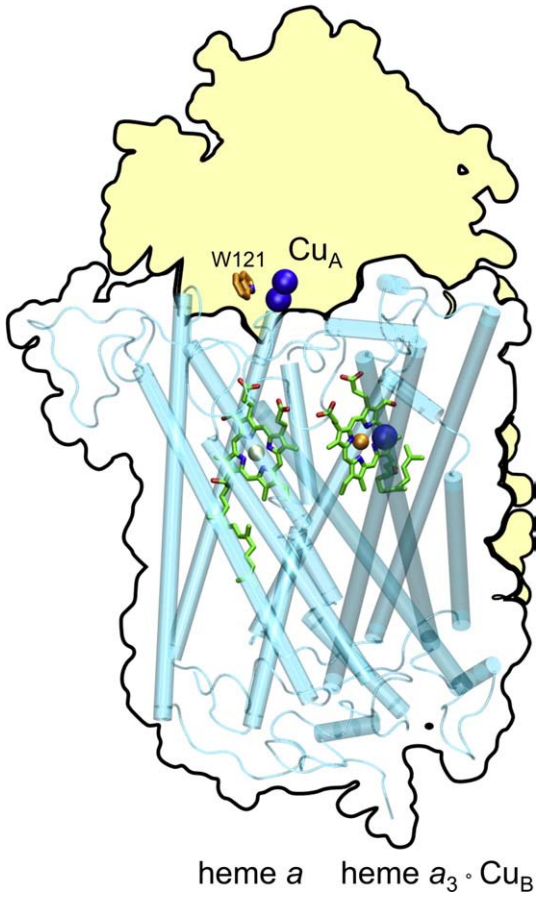

Fig. 1. Simplified 3-D structure of the two-subunit form of the *Paracoccus denitrificans* aa_3-type cytochrome *c* oxidase. White space-filling outline, subunit I with its 12 transmembrane helices (presented in light blue) and its redox centers heme *a* and the binuclear heme a_3 • Cu_B. Light green outline denotes subunit II with its large periplasmically oriented hydrophilic domain housing the Cu_A center; W121 specifies the surface-exposed tryptophan as the most crucial residue for ET from cytochrome *c*.

electronically coupled ([5], see also Fig. 2). This latter arrangement is considered the active site of the enzyme, where oxygen reduction occurs and most likely also the coupling to proton translocation. Importantly, this binuclear center is located by about one-third within the depth of the hydrophobic core of the membrane, with its shielded location making any charge movements energetically highly unfavourable without invoking a compensating countercharge movement to match the electroneutrality principle [16].

1.1. Cytochrome c interaction

Electron entry into the enzyme is mediated via cytochrome *c*; out of all *c*-type cytochromes studied in *P. denitrificans* [9,17] the membrane-anchored c_{552} was identified to best serve this purpose. A soluble fragment of this cytochrome was expressed and identified in both its redox states by NMR and X-ray approaches [18–20] which confirmed a high overall structural homology with the strongly basic mitochondrial cytochrome *c*. Despite pronounced differences in their p*I* values, their surface charge distribution around the potential docking site for oxidase is largely comparable: this hemisphere of the c_{552} molecule centered around the accessible heme cleft exposes a remarkable local concentration of up to 9 lysine residues [18]. This fact provides a reasonable explanation for the observation that for several prokaryotic oxidases, horse heart cytochrome *c* may well replace the endogenous bacterial cytochromes *c* in activity assays, and in the *Paracoccus* case, kinetic parameters for the oxidase reaction [21,22] revealed a clearly comparable catalytic efficiency for the mitochondrial *c*-cytochrome, however at different ionic strength optimum.

The underlying concept, developed originally long ago (see e.g. [23]), of a strong positive surface charge governing oxidase (and as well, cytochrome bc_1, see below) interaction, was put to proof in the bacterial case by exchanging each of the lysine residue individually by neutral side chains [22]: Phenotypes of every c_{552} variant were found to be affected in both standard kinetic parameters, K_M and v_{max}, to a similar extent, while no particular lysine position stood out significantly, arguing against any specific localized side chain charge (or charge pair from either interacting docking surface). Along with other examples (e.g. [24]), this is taken as evidence for a delocalized surface charge effect crucial for orienting interacting partner proteins into their docking position, see also below.

Early evidence based on crosslinking experiments clearly pointed at subunit II of the mitochondrial oxidase as the (main) docking site for cytochrome *c* [25], later well appreciated for the fact that this subunit, more precisely its hydrophilic C-terminal domain (see Fig. 1), ligands the Cu_A center, conclusively shown to be the first ET acceptor in oxidase [26,27]. Extensive site-directed mutagenesis experiments on surface-exposed acidic residues, largely conserved in corresponding subunit II sequences of other oxidases, were performed [28–31]. Analysis by turnover and pre-steady-state kinetics of such oxidase variants established that an extended lobe of negative charges is responsible for docking the substrate cytochrome *c*, drastically influencing the kinetic efficiency (both K_M and v_{max}).

Still another group of subunit II mutants provided insight into the docking process: surface-exposed, yet hydrophobic amino acids cluster in a patch within the negatively charged lobes above the Cu_A site. In these variants, kinetic parameters were severely affected, showing mostly a sharp drop in v_{max}. The most drastic phenotypic change was obtained when replacing W121 (Fig. 1; unless stated otherwise, all numbering follows the *P. denitrificans* nomenclature):

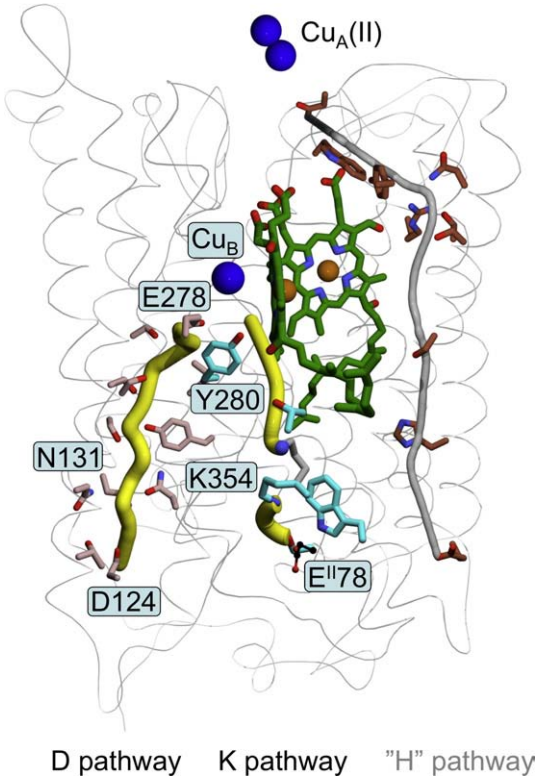

Fig. 2. Redox centers of the *P. denitrificans* aa_3 oxidase (see also Fig. 5) with the α-trace of subunit I in the background, modelling the two canonical protonic pathways (D, K; in yellow) and the hypothetical "H pathway" (grey), each lined by selected side chains referred to in the text.

unlike for other neighbouring aromatic residues, any exchange for this tryptophan tested [30,31], left the enzyme almost inactive in ET, while hardly affecting its K_M value.

Taken together, data for site-specific alterations in the docking site of subunit II for its substrate cytochrome *c* allow to draw the conclusion that the interaction of both redox proteins proceeds in several steps: (i) Oppositely charged surface potentials govern the long-range orientation and initial encounter of both partner domains, followed by (ii) a fine-tuning of the interaction geometry mediated by hydrophobic patches into an optimal position to finally (iii) allow the ET step to reduce the Cu_A site, located within 5 Å of the crucial W121 side chain (see Fig. 1). Slight differences in the presumed docking sites on subunit II were revealed [31] for the endogenous cytochrome c_{552} and for horse heart cytochrome *c*, for practical purposes often used as a substitute. For the bacterial cytochrome, no redox-related differences in its interaction with the Cu_A domain were observed in an NMR approach employing soluble fragments [32], at the same time showing by multi-frequency pulse EPR spectroscopy [33] that any interaction between the two (soluble) modules is only transient in nature and is not restricted to any single, unique complex, but rather suggests a broad distribution of multiple interaction complexes.

A quite different scenario of ET chain interactions seems to prevail e.g. in the *Thermus thermophilus* case: in its terminal branch (see also below), a soluble cytochrome c_{552} had been identified as the donor to the cytochrome ba_3-type oxidase (as reviewed in [34]). Later, with 3-D and further kinetic information available for either component [35–38], it became obvious that potential interaction sites (for both the c_{552} and the hydrophilic Cu_A domain in subunit II) lacked the typical clusters of charged surface-exposed residues, nor did it reveal a tight electrostatic complex between both reaction partners at low ionic strength as would be expected for an ionic interaction. According to a systematic Brønsted analysis, its interaction mode with the heterologously expressed Cu_A binding domain [39,40] turned out to be largely governed by hydrophobic forces between the two macromolecules [41], favoured over electrostatic interactions at such elevated growth temperatures. Unlike for the corresponding *Paracoccus* ET couple [42] where between two and three effective charges on either interacting surface appear responsible, under these thermophilic growth conditions less than one nominal charge on either molecule contributes to complex formation [41,42]. Based on a combined NMR and computational approach, a model for the transient docking complex and a suggestion for an ET pathway between the two redox centers has been put forward [43].

A further complication in ET is encountered when interacting *c*-type cytochromes mediating ET between complexes III and IV are covalently fused to either of the complexes' subunits, such as in the *Thermus* cytochrome caa_3 complex [44,45]. *A priori*, this may impede the otherwise free 3-D mobility, or at least rotational freedom of a membrane-anchored version of a cytochrome *c*. Such fused *c*-domains may either show flexible domain movements to interact both with its electron donor or acceptor subunits, or alternatively, unlike typical *c*-cytochromes, provide different entry and exit paths for the electron, if indeed solidly "wired" to its remaining subunit domain.

Higher-order associations between respiratory complexes, termed supercomplexes, have not only been described for the mitochondrial inner membrane, but have also been found in less specialized membrane systems such as the bacterial cytoplasmic membrane: there, due to their distinctly lower specific ET complex contents, they can hardly be mistaken for an aggregation artefact. Surprisingly, a well-defined supercomplex stoichiometry has been found for *Paracoccus* cytoplasmic membranes: a respiratory supercomplex consists of one copy of complex-I, four monomers of complex-III, and four copies of c_{552} [46]. Next to stabilization of the otherwise labile (*Paracoccus*) complex-I, supercomplexes in general provide efficient substrate channeling, thus avoiding diffusional encounters in the cytoplasmic membrane for this "NADH oxidase" complex. Remarkably, no other components of the highly branched ET chain of *Paracoccus* have been found associated with this rather "mitochondrion-like" supercomplex or "respirasome" composition, thus obviously actively excluding e.g. alternative terminal oxidases from a supercomplex arrangement in *Paracoccus* [46].

1.2. Proton pathways and coupling concepts

More than three decades ago [47] it was recognized that cytochrome *c* oxidase, like other members of the superfamily of heme–copper oxidases (see [48]), not only reduces molecular oxygen to water, but also acts as a proton pump, thus adding to the proton motive force already generated in the course of oxygen reduction for which electrons and protons come from opposite sides of the membrane (the intermembrane space and the mitochondrial matrix, in bacteria the periplasm and the cytoplasm, resp.).

ET proceeds from reduced cytochrome *c* via Cu_A and heme *a* to the binuclear site (see Fig. 2) although the redox potentials of the latter two are initially not arranged in the expected sequence [49]. Protonation of a nearby site is required to raise the midpoint potential of the heme a_3/Cu_B pair sufficiently [50,51] which now can accept this electron, together with a proton to maintain electroneutrality [16]. Uptake of this "chemical" proton leads to release of the proton from the pump site to the outside via a largely unknown pathway. Neither the exact nature of the pump site [52] nor the exit pathway are known (but see [53]). An aspartate residue, D399, located in the vicinity of the heme a_3 propionate, on the other hand, may share a proton with this propionate and, moreover, may interact with Cu_B liganding histidines, thereby regulating proton translocation [54]. Occupation of the low-spin heme site by heme *a* is obviously favoured by formation of a hydrogen bond between R54 and the formyl group of the heme moiety as the replacement R54M can no longer provide this stabilizing interaction, explaining the increased occupancy of this low-spin site by heme *o*. This leads to a drastic drop in heme *a* midpoint potential which was thought to result in electron flow from Cu_A directly to heme a_3, without transient reduction of the low-spin heme [55], disfavoured in wild type due to its larger distance (by roughly 2.5 Å) than to heme *a*. However, careful examination of this mutant enzyme based on optical and electrometric measurements clearly contradicted any bypass of electrons from Cu_A to the binuclear site [56].

Initially, oxygen reduction and proton pumping was assumed to involve two different proton pathways for chemical and pumped protons, respectively. Early mutagenesis experiments with the corresponding quinol oxidase of *E. coli* indeed revealed the existence of two separate proton input channels (see e.g. [57,58]). Inspection of the three-dimensional structure of the *Paracoccus* cytochrome *c* oxidase (as well as other oxidases) also indicated two possible proton transfer pathways [5].

A channel leading to the active site is possibly formed by S291, K354, T351, the hydroxyl groups of heme a_3 and Y280 (see Fig. 2, K pathway). The side chain of the essential K354 may adopt different conformations to form hydrogen bonds either to S291 or to T351, respectively (indicated in Fig. 2 by different colours). As K354 is located well within the membrane dielectric, experiments were conducted to identify catalytically important residues located at the entrance of this pathway by mutagenesis studies; all mutant enzymes, however, maintained wild type behaviour [59]. In other oxidases [60,61] a glutamate residue in subunit II was identified as the possible entry point for protons into the K pathway, for the fact that mutations in this residue influence the rate of heme a_3 reduction under anaerobic conditions. For the *Paracoccus* oxidase the possible involvement of the corresponding residue ($E^{II}78$, displayed in Fig. 2 in ball and stick representation) could not be substantiated as neither proton pumping, overall catalytic activity, nor heme a_3 reduction was affected to an extent expected for a canonical K pathway residue [62].

The other proton pathway [5] initially thought to be exclusively used for pumped protons, is specified in Fig. 2 by only a few key amino acid residues (for an overview of mutations introduced in either channel of the *Paracoccus* enzyme, see [59,63]). This pathway proceeds from the essential D124 at the entrance (therefore the designation "D channel"), via N131 (see below), to E278 which is considered a likely "gating" candidate (discussed below).

For the bovine cytochrome *c* oxidase a third proton-conducting channel (the H pathway) has been proposed [64], however, a mutagenesis approach did not reveal the existence of such a third pathway for the *Paracoccus* oxidase [59]. More recently additional mutations were introduced in the *Paracoccus* oxidase addressing further residues potentially involved in proton transfer through the H channel as suggested on the basis of uncoupled mutants in the bovine enzyme [65]; again, all mutants displayed wild type electron transfer and proton-pumping activity [66]. These studies, along with corresponding results obtained for a related bacterial oxidase [67], may point at potential differences between the eukaryotic and bacterial enzymes.

It turned out rather early that the situation concerning proton input channels is more complicated than anticipated. K362M of the *E. coli* bo_3 quinol oxidase results in an inactive enzyme, nevertheless able to catalyze the later steps of oxygen chemistry [68]. Obviously, not all four protons required for the formation of both water molecules are transferred via the K channel. Results obtained with single electron reduction of wild type and mutant *Paracoccus* oxidases indicate that the transfer of the first electron to the oxidized enzyme leads to the charge-compensating uptake of one proton through the K pathway [69] while transfer of the second electron is linked to proton uptake via the D [70] or K pathway [71]. Anaerobic reduction of the *Paracoccus* oxidase even showed that complete reduction of heme *a* is coupled to the uptake of ~0.8 proton independent of both these proton uptake pathways [72]. Nevertheless, it is generally agreed that at least 2 protons involved in water formation reach the binuclear center via the D channel, otherwise translocating all pumped protons. This observation immediately raises the question how chemical protons required for water formation are discriminated from pumped protons, directly invoking the requirement for a gating mechanism: protons destined to be pumped must not reach the active site, which would effectively short-circuit the oxidase and abolish any proton pump activity. Elucidating this gating and the elements involved, should be the key to our understanding of the principles of proton transfer within the enzyme, and of the coupling of transmembrane proton translocation to the ET steps.

Inspection of the crystal structure of the *Paracoccus* oxidase already suggested that a conserved glutamate residue, E278, at the upper end of the D pathway could be this branching point, possibly even involving conformational changes of its side chain to achieve this function [5]. FTIR spectroscopy supported the role of E278 as a crucial residue for the redox reaction [73,74]. As direct experimental evidence has been lacking so far, the branching function of E278 is indirectly inferred from several molecular dynamics simulations (see for example [52]). Likewise, it is generally accepted nowadays that in addition to the valve function of the glutamate residue, water molecules play a crucial role for proton access to both destinations [75,76], forming separate H-bonding networks with different proton conduction rates, possibly even providing a kinetic gating mechanism [77]. For the first time, direct evidence for a conformational change of the E278 side chain and changes in the location/orientation of water molecules came from the crystallization of the *Paracoccus* N131D mutant [78]. In addition to the side chain orientation for E278 also found in the wild type (referred to as the downward or input conformation allowing proton uptake from a network of water molecules in the D pathway), a second electron density indicative of an alternative (upward or output) orientation is evident which connects the D pathway to the above networks (see Fig. 3). The routing of protons further on could depend on transiently existing

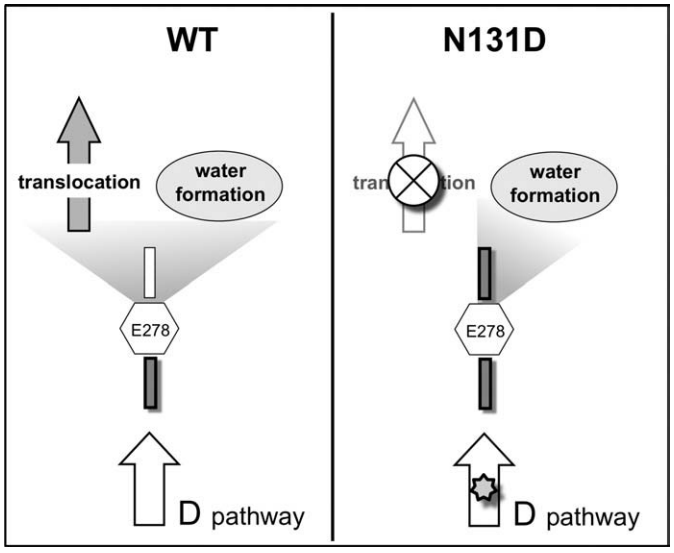

Fig. 3. Highly schematic representation of the side chain mobility of glutamate-278, showing potential protonic routes for "chemical" and "vectorial" protons entering the D pathway. Left, single position of the E278 side chain (filled, shaded stick element) facing downwards in the wild type (WT) crystal structure. Right, double density for the E278 side chain as observed in the N131D mutant structure (denoted by the asterisk), where the mobility of the side chain is restricted such that in the protonic output position, only the binuclear site ("water formation") is served, thus effectively inhibiting proton translocation; for further details, see text.

transfer pathways which may be modulated by the redox state of the binuclear center metals. This upward orientation of the E278 side chain may only be short lived in the normal reaction cycle of wild type oxidase, and therefore invisible in the respective crystal structures obtained thus far, while populated to a significant amount in the N131D structure due to electrostatic stabilization/trapping. This N131D mutant oxidase displays a rather unique enzymatic property: while retaining full cytochrome *c* oxidation activity, it does no longer pump protons [59,63]. The same loss of proton pumping has later been observed in *R. sphaeroides* as well, and explained by an increase in the apparent pK_a of the glutamate residue corresponding to *Paracoccus* E278 due to long-range electrostatic interactions [79]. Such an interpretation seems less likely considering the long distance of 18 Å [80] and the fact that the same decoupling is also seen for the only weakly acidic N131C side chain [78]. The crystal structure offers an alternative explanation in that the number of observable water molecules in the mutant structure is diminished compared to wild type. Although probably not missing altogether, the disordered water molecules most likely change the environment of E278, thereby provoking the observed effects on the side chain of this residue. Consistently, FTIR spectroscopy indicates a distinct perturbation in the hydrogen-bonding environment of this residue, while the protonation state of E278 is unaltered by the mutations N131D/C [78]. At the moment no definite explanation concerning the basis for the decoupled phenotype can be given, since kinetic as well as conformational arguments or a combination of both seem plausible.

1.3. Paracoccus cytochrome c oxidase — a radical story

Apart from the redox centers mentioned so far (see above and Fig. 2), there is growing evidence that certain amino acid side chains are also redox-active and are intimately involved in the overall catalytic cycle.

Electrons required for breaking the oxygen-oxygen bond are supplied in a concerted fashion, thus avoiding the formation of intermediate reactive oxygen species. While three electrons are provided by the oxidation of binuclear site metal ions, the fourth electron seems to originate from an aromatic amino acid residue

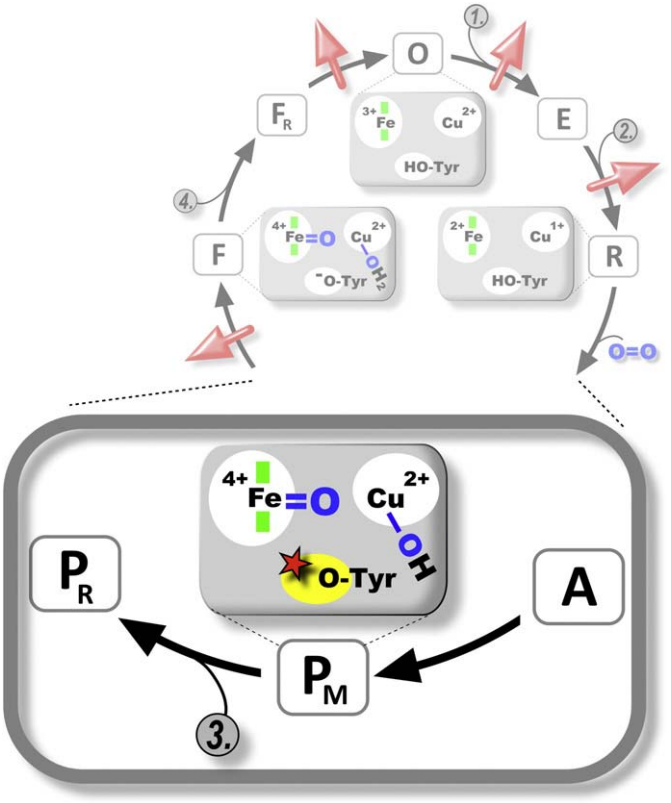

Fig. 4. The P_M state within a full O_2 redox cycle of oxidase, after splitting the dioxygen bond (resulting oxygen atom species in blue), with electronic states of the heme a_3 and Cu_B in the binuclear center; the tyrosine radical state is indicated by a red asterisk; for further details, see text.

which transiently forms a radical species (see Fig. 4). The idea that a tyrosine residue close to the active site may donate this additional redox equivalent during the oxygen reduction cycle is not new (see [81] and references cited therein), although other radical sources (tryptophan, porphyrin species) have been discussed as well [82].

The corresponding tyrosine residue, Y280, which is post-translationally modified to covalently link to one of the Cu_B ligating histidines (H276; Fig. 5; [83]) is thought to contribute the extra (fourth) electron, assuming that the crosslink supports the generation and stabilization of the resulting neutral tyrosine radical. There is experimental evidence that the crosslink secures Cu_B in a precise spatial arrangement, thereby maintaining the structural integrity of the binuclear center [84,85]. On the other hand, this tyrosine residue is also essential for turnover as the Y280H mutant lacks catalytic activity almost completely, while the configuration of the active site is not compromised, allowing for example the binding of O_2 to heme a_3 [84]. Amongst others, FTIR spectroscopy reveals changes of the Y280 properties upon electron transfer and coupled protonation [86]. Recent experiments have refined our understanding of the contribution of Y280 during oxygen catalysis. For the first time, direct assessment of the protonation state of Y280 by FTIR in parallel with electrometric measurements allowed the conclusion that the proton required for scission of the O–O bond originates from this tyrosine residue [87]. In addition it was shown that reprotonation of the tyrosinate only occurs after transfer of the first electron to the fully oxidized enzyme in the course of the next turnover cycle [88].

The experimental situation became more complicated when a set of six *Paracoccus* oxidase variants with tyrosine residues mutated within 20 Å to the binuclear site was treated with H_2O_2 [89]. Even in the Y280H mutant oxidase a radical species could be detected, while the mutant Y167F did not show any radical signal in the respective EPR experiments. This particular mutant oxidase displayed a residual activity of about 60% compared to wild type. An explanation for the seemingly contradictory observations may lie in the nature of this H_2O_2 incubation experiment itself: not the catalytically competent radical is detected, rather the thermodynamically most stable radical is monitored (Y167, Fig. 5), implying that a relay network must exist allowing radicals to migrate from the original site of generation to Y167. Therefore, Y167 is probably not the kinetically relevant radical which leaves the exact nature of the primary radical to be determined.

A very powerful approach for the identification of radicals involved in the kinetic mechanism is rapid quenching of the oxidase reaction after mixing the reduced enzyme with oxygen followed by EPR. The corresponding study lead to the detection of a tryptophan radical [82] assigned to W272 (Fig. 5) and shown to occur after 300–500 μs [90]. While a direct role in oxygen bond splitting (as suggested in [91]) could not be excluded, this residue was considered to contribute an electron (and proton) only later in the catalytic cycle as the reductant of one of the metal centers. Interestingly, the transiently formed tryptophan anion may be part of a proton relay network, eventually directing protons from the cytoplasm to the periplasma [90]; according to this assumption, proton pumping was discussed to be driven by the oxidoreduction of W272 and the strong basicity of the corresponding radical anion.

In line with current experimental results more than a single amino acid derived radical may be involved in the oxygen reaction cycle catalysed by heme/copper cytochrome oxidases. Y280 is still the most likely candidate as the donor of the fourth electron in the oxygen bond splitting reaction, while the redox-active W272 may be of prime importance for proton pumping connecting the driving force of the underlying oxygen chemistry (dioxygen reduction) to proton translocation. Presumably other residues could also form reaction competent radicals as can be seen by the formation of a Y167 radical (see above).

1.4. Biogenesis of cytochrome c oxidase

A number of aspects make the biogenesis of the mitochondrial oxidase a complicated issue: its large number of subunits, its intricate

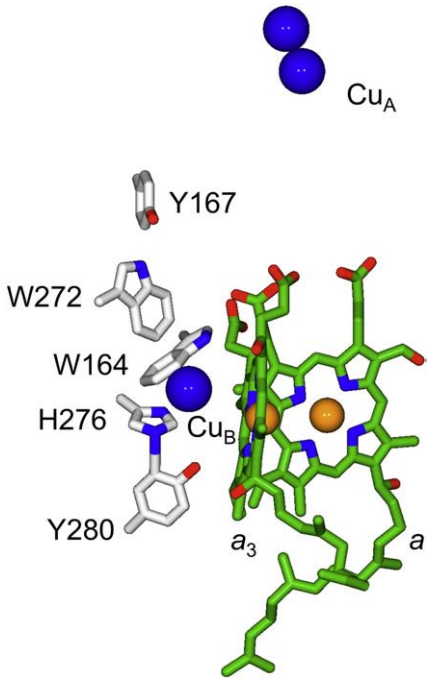

Fig. 5. Redox centers in the *P. denitrificans* aa_3 oxidase, along with the side chain cross-linked (tyr–his) ligand of Cu_B and potential radical residue candidates; hemes in green, iron in orange, copper in blue.

interplay of two genetic systems encoding these subunits, and the insertion of the heme and copper cofactors into a rather rigid protein scaffold [3]. Bacterial oxidases only share the latter complexity, and should therefore offer a suitable model system for following up this particular assembly step. So far, most information has been collected for the *S. cerevisiae* cytochrome oxidase, which has been well-studied and analyzed mostly on genetic grounds for its assembly components and chaperones required (see [92–94] for recent reviews). In comparison, a comprehensive bioinformatic survey [95] established that bacteria encode only a limited subset of such assembly factors, and in particular *Paracoccus* is restricted to only a few hypothetical genes required for cofactor insertion into both relevant oxidase subunits: (i) *cta*G, (ii) a *surf*1 homologue, and (iii) two copies of *sco*. Interestingly, the two former genes are intimately associated with the *cta* operon, and are briefly introduced below.

CtaG, the Cox11 homologue of yeast, has long been affiliated with a role in the biosynthesis of the binuclear center in subunit I of cytochrome *c* oxidase, but an initial inactivation in *Paracoccus* did not allow a clear distinction of effects on either of the two redox centers in this site, heme a_3 or Cu_B [96]. In line with results from a *R. sphaeroides* deletion strain [97] and yeast Cox11 data [98], a purified *P. denitrificans* soluble CtaG fragment was shown to bind Cu(I) in a 1:1 stoichiometry, and a spectral assay was developed to basically follow the transfer of the ion in-vitro [95]. However, a heterologous coexpression of both this chaperone along with subunit I of oxidase did not yet yield copper insertion. As an alternative approach, in-vitro transcription/translation experiments (e.g. [99]) were initiated to follow the notion that the insertion of an ion into a hydrophobic environment (see Fig. 1) may well proceed in a contranslational step before terminal folding of the 12 transmembrane helices of this subunit [100]; such an "open" expression system easily allows addition of previously purified chaperones and cofactors. Reasonable yields of subunit I have been obtained already using the standard *E. coli* S-30 extract [95], and the translation system should be switched to a *Paracoccus* extract to exploit the full potential of this cell-free method, expressing genes of interest in the presence of homologous membranes obtained from specific deletion strains.

Surf1, in yeast termed Shy1p, has long been recognized as an auxiliary factor acting in the assembly of oxidase, in human genetic defects causing a fatal encephalopathy disorder called Leigh syndrome (see [101,102] and ref. therein). Despite a number of yeast genetic studies and even animal models available, its precise function, however, has remained unclear, with suggestions ranging from translational regulation, subunit and complex stability to heme assembly and oxygen affinity [103–106]. In *Paracoccus*, a challenging situation has been encountered in the identification of two versions of a Surf1 homologue, each physically associated with gene loci encoding either the aa_3 cytochrome *c* oxidase or the ba_3 quinol oxidase subunits [107]. Single deletions (and a double deletion along with their specific complementations) unequivocally show a specificity of each of the two versions for "its" cognate oxidase, lacking any promiscuity in function, despite an identical overall topology and a sequence identity of around 30% for both. The deletion phenotype, e.g. for the Surf1c gene product, is a loss of approx. 60–70% of activity in membranes as well as in the purified aa_3 oxidase, and a defect in spectrally detectable heme *a* (a_3) to a similar level. A corresponding phenotype applies to the Surf1q, again serving specifically only its quinol oxidase, whereas for the third heme–copper oxidase present in *P. denitrificans*, the cbb_3 oxidase lacking heme *a* both in its low- as well as in its high-spin site altogether, apparently no (further) Surf1 homologue is present in the genome [107].

What is the genuine role of either Surf1 copy? The bacterial model system has yielded a clear-cut answer on characterizing both purified proteins expressed either in the homologous or a heterologous host (F. Bundschuh, A. Hannappel et al., ms in preparation): Surf1 proteins bind, probably in a transient fashion, the heme-A cofactor to deliver it to the respective oxidase subunit I, and a direct receiving pathway for the cofactor from the last enzyme of heme-A biosynthesis, heme-A synthase, to the heme chaperone Surf1 appears likely. All available data suggest that this protein is not strictly required for inserting heme into both its target oxidase redox sites, but seems to primarily act as a facilitator chaperone, in line also with the Leigh syndrome human phenotype.

2. Cytochrome bc_1 complexes in *Paracoccus* and in *Thermus*: Supramolecular organization and cytochrome *c* interactions

Complex-III follows a drastically different design in mechanistic terms, when compared to oxidase, and has spurred interest, just to name a few aspects, for its elaborately tuned interaction sites with quinones (both reduced and oxidized) as described in the Q-cycle mechanism of energy transduction, its unusual major domain movement of the FeS subunit to allow internal ET to the cytochrome c_1 heme, and its "forced" dimer structure by functional intertwining of the Rieske FeS subunits, membrane-anchored in the respective other monomer. Altogether, a wealth of information on the cytochrome bc_1 complex is available, which is not covered in detail here (see [108–110] for recent reviews). This section is restricted to just a few aspects of this respiratory complex, again from a bacterial point of view: its higher structural organization and potential functional interactions between monomeric units in *Paracoccus* membranes, the description of a *T. thermophilus* respiratory complex of functional analogy, and some studies on the different interaction scenarios of complex-III of either bacterium with *c*-type cytochromes.

Only those three subunits that carry the essential redox centers, make up the *P. denitrificans* complex-III, the two-heme cytochrome *b*, the FeS subunit and the cytochrome c_1. Even though a 3-D structure of this bacterial complex is not available, sequence homologies (together with several functional analogies, e.g. [111–116]) leave no doubt that the gross subunit topology is identical to the ones solved by X-ray diffraction. With this information in mind, it came as a surprise to find the molecular mass of complex-III isolated from *P. denitrificans* not as that of a dimer (as predominantly observed in an earlier sedimentation equilibrium experiment using detergent matching [117]), but as a tetrameric complex [118]. By a newly developed mass spectrometry technique (LILBID, laser-induced liquid bead ion desorption), purified membrane protein complexes in detergent are desorbed from micro droplets by a tunable infrared laser pulse irradiation, and their masses determined. Low laser intensities leave subunit complexes and higher associations intact,

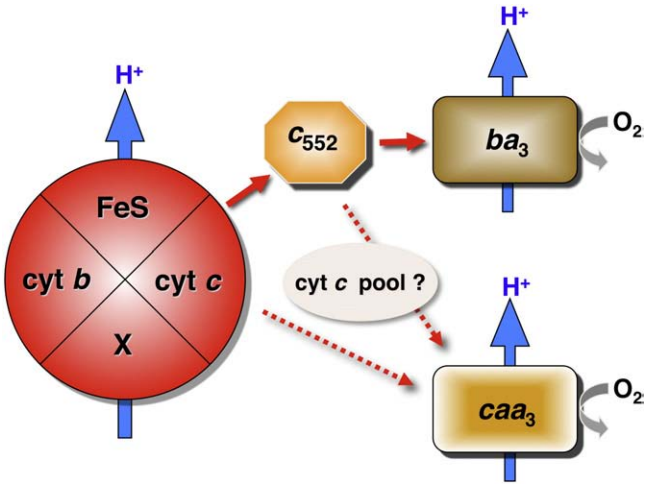

Fig. 6. Schematic representation of the terminal section of the *Thermus thermophilus* electron transport chain, with routes connecting the *bc* complex to the two energy-transducing terminal oxidases.

while harsher conditions lead to disintegration and appearance of subunit mass peaks [118]. A tetrameric structure of complex-III is best explained by the presence of two dimers, each of which most likely is arranged in a FeS subunit-intertwined assembly. As such, a tetramer confirms the well-defined stoichiometry of a *P. denitrificans* supercomplex separated by blue-native gel electrophoresis and by column chromatography [46], consisting of complexes I, III, and IV in a 1:4:4 copy number ratio (see above). However, the overall topology of two such bc_1 dimers, and their further interaction in a supercomplex with the other ET donor and acceptor complexes, remain highly speculative.

What governs the interaction of complex-III with its downstream ET substrate, cytochrome *c*? Early evidence was presented for the mitochondrial ET chain that in analogy to the oxidase interaction, the highly basic mitochondrial cytochrome *c* interacts with the acidic c_1 subunit by long-range electrostatic forces [23], offering almost the same interface, centered around its heme cleft, to either ET partner complex. Current direct co-crystal data for the yeast complex [119] define the specific interaction site between the two *c*-type cytochromes as rather small, hydrophobic in nature, with a prominent cation–π interaction, and lacking any ion pairs to cope with the transient nature of this encounter. For the bacterial ET chain, a (minimal) quinol oxidase supercomplex in *Paracoccus* [120] suggested that the membrane-bound c_{552} is the mediator of ET between complex-III and oxidase (see also above). Taking the same approach for interaction studies as for cytochrome *c* oxidase, soluble modules were prepared and the ET reaction followed between a hydrophilic *c*-heme domain derived from the cytochrome c_1 subunit, and the two acceptor cytochromes, c_{552} (see above) and cytochrome c_{550} [121,122]. ET reactions turned out to be very fast, on the limit of diffusion-controlled processes, and a careful ionic strength analysis revealed that, much like in the oxidase reactivity of the c_{552} cytochrome (outlined above), between 2–3 charges of opposite sign are required for a long-range pre-orientation of either protein molecule. Using a Ru-derivative of the c_{552}, laser flash photoexcitation allowed to assess the even faster reaction time window at lower ionic strength [122], and a two-step model for partner protein interaction was derived in which the initial pre-orientation of both proteins governed by opposite surface potentials is followed by a very fast hydrophobic diffusion process, largely independent of ionic strength, to reach the final encounter (and productive ET) orientation.

How is the quinol-oxidizing part of the *Thermus* ET chain organized in terms of a complex-III subunit structure and cytochrome *c* interaction? Recently, the composition of a *bc* complex was unravelled both on its genetic and its protein subunit level ([123,124], and see Fig. 6): four subunits, all encoded in a putative operon structure, constitute the detergent-purified, menaquinol-oxidizing complex of high thermostability. Three of its subunits, cytochrome *b*, the FeS subunit, and a *c*-type cytochrome, are bona fide subunits of *bc* complexes, with the latter showing an unexpected topology with a C-terminal hydophilic domain carrying the heme and an extended N-terminal domain, separated by a putative central transmembrane helix. The fourth subunit, of high hydrophobicity and a predicted four transmembrane helix topology, shares no sequence identity to any other complex-III subunit, and its functional role in the complex remains obscure [124].

How are the two terminal oxidases encountered in the *T. thermophilus* respiratory chain served with electrons from the *bc* complex? Analyzing the interaction of the soluble heme *c* domain derived from complex-III with the c_{552} of *Thermus* by stopped flow spectroscopy [123], for this step in the ET chain as well, mostly hydrophobic contacts were found responsible for interaction, with a K_{eq} of around unity. As outlined in Fig. 6, the flow of electrons would then be mainly directed to the ba_3 oxidase (as also discussed above), but based on kinetic grounds [45], may also reach the *c*-domain of the caa_3 oxidase, or may redox-equilibrate with other still hypothetical components of a cytochrome *c* pool. Interestingly, the same approach using soluble modules has also provided evidence for a kinetically preferred direct interaction of complex-III and the caa_3 terminal oxidase [45], and it may be speculated that these two large membrane protein complexes are members of a *Thermus* supercomplex anyway, thus avoiding collisonal encounters altogether.

Acknowledgements

Work from our own group has been funded by long-term support from Deutsche Forschungsgemeinschaft (SFB 472 and CEF-MC, Cluster of Excellence Frankfurt "Macromolecular Complexes", EXC 115). We are indebted to all group members, present as well as past, and appreciate long-standing, successful collaborations within the Collaborative Research Center "Molecular Bioenergetics".

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