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Evolution of the Cytochrome *c* Oxidase Proton Pump

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Abstract. The superfamily of quinol and cytochrome *c* terminal oxidase complexes is related by a homologous subunit containing six positionally conserved histidines that ligate a low-spin heme and a heme–copper dioxygen activating and reduction center. On the basis of the structural similarities of these enzymes, it has been postulated that all members of this superfamily catalyze proton translocation by similar mechanisms and that the Cu_A center found in most cytochrome *c* oxidase complexes serves merely as an electron conduit shuttling electrons from ferrocytochrome *c* into the hydrophobic core of the enzyme. The recent characterization of cytochrome *c* oxidase complexes and structurally similar cytochrome *c:*nitric oxide oxidoreductase complexes without Cu_A centers has strengthened this view. However, recent experimental evidence has shown that there are two ubiquinone(ol) binding sites on the *Escherichia coli* cytochrome bo_3 complex in dynamic equilibrium with the ubiquinone(ol) pool, thereby strengthening the argument for a $Q(H_2)$-loop mechanism of proton translocation [Musser SM et al. (1997) Biochemistry 36:894–902]. In addition, a number of reports suggest that a $Q(H_2)$-loop or another alternate proton translocation mechanism distinct from the mitochondrial aa_3-type proton pump functions in *Sulfolobus acidocaldarius* terminal oxidase complexes. The possibility that a primitive quinol oxidase complex evolved to yield two separate complexes, the cytochrome bc_1 and cytochrome *c* oxidase complexes, is explored here. This idea is the basis for an evolutionary tree constructed using the notion that respiratory complexity and efficiency progressively increased throughout the evolutionary process. The analysis suggests that oxygenic respiration is quite an old process and, in fact, predates nitrogenic respiration as well as reaction-center photosynthesis.

Key words: Cytochrome *c* oxidase — Ubiquinol oxidase — Nitric oxide reductase — Proton pump — $Q(H_2)$-loop — Evolutionary tree — Respiratory efficiency — Respiratory complexity

Introduction

The many similarities between the *Escherichia coli* cytochrome bo_3 complex and the aa_3-type cytochrome *c* oxidase complexes led to the recognition of a superfamily of terminal oxidase complexes related in structure and function. All of the enzymes in this superfamily contain a low-spin, six-coordinate heme which mediates electron input to a heme–copper dioxygen activating and reduction center. This superfamily is divided into two families: the quinol (typically, ubiquinol) oxidase complexes and the cytochrome *c* oxidase complexes (Table 1). The variations within the families are mainly in the number and type of hemes present in the various enzymes. In general, all members of this superfamily of terminal oxidase complexes are thought to translocate protons against a transmembrane potential gradient (although this has not been proven in all cases) and all appear to catalyze dioxygen activation and reduction at a heme–copper binuclear center (García-Horsman et al. 1994a; Musser et al. 1995; van der Oost et al. 1994).

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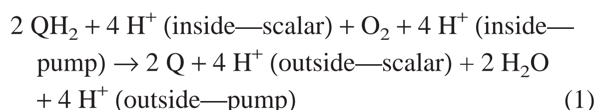
Table 1. Representative members of the superfamily of ubiquinol and cytochrome *c* terminal oxidase complexes[a]

Type	Cu_A present?[b]	Translocates protons?	Hemes *c*?	Species	Reference(s)
				Quinol oxidase complexes	
bo_3[c]	No	Yes	No	*E. coli*	Chepuri et al. 1990; Minghetti et al. 1992; Puustinen et al. 1989, 1991
ba_3	No	No	No	*A. aceti*	Fukaya et al. 1993; Matsushita et al. 1990
	Yes	Yes	No	*S. acidocaldarius*[d]	Lübben et al. 1994; Schäfer et al. 1996b
bb_3	No	Yes	No	*P. denitrificans*	de Gier et al. 1996; Solioz et al. 1982; van der Oost et al. 1991a
	No	ND[e]	No	*B. japonicum*	Surpin et al. 1996
aa_3	No	Yes	No	*B. subtilis*	Lauraeus et al. 1991; Santana et al. 1992
	No	Yes	No	*S. acidocaldarius*[d]	Anemüller et al. 1992; Anemüller and Schäfer 1989, Schäfer 1990; Gleißner et al. 1997; Lübben et al. 1992
				Cytochrome *c* oxidase complexes	
aa_3[f]	Yes	Yes	No		
ba_3	Yes	ND	No	*T. thermophilus*	Zimmerman et al. 1988
caa_3	Yes	Yes	1	*T. thermophilus*	Büse et al. 1989; Fee et al. 1988; Mather et al. 1993
	ND	Yes	1	*Bacillus stearothermophilis*	de Vrij et al. 1989; Sone and Fujiwara 1991
	Yes	Yes	1	Thermophilic *Bacillus* PS3[g]	Ishizuka et al. 1990; Sone and Hinkle 1982; Sone and Yanagita 1984; Sone et al. 1988
	Yes	ND	1	*B. subtilis*	Lauraeus et al. 1991; Saraste et al. 1991; van der Oost et al. 1991b
cbb_3	No	ND	3	*R. capsulatus*	Gray et al. 1994; Thöny-Meyer et al. 1994
	No	ND	3	*R. sphaeroides*	García-Horsman et al. 1994b
	No	Yes	3	*P. denitrificans*	de Gier et al. 1994, 1996
	No	ND	3	*B. japonicum*	Preisig et al. 1993; Zufferey et al. 1996

[a] This table includes the more well-characterized heme–copper oxidase complexes. See García-Horsman and co-workers (1994a) for a more expanded listing.
[b] The presence or absence of a Cu_A site has not been definitively confirmed in all cases. A best guess is made based on the present literature.
[c] This enzyme is expressed as a cytochrome oo_3 complex, with little difference in activity in various overexpressing strains (Puustinen et al. 1992).
[d] There are two *S. acidocaldarius* enzymes; both contain cytochrome *b* homologues, yet only one contains a Cu_A site (see text).
[e] Not determined.
[f] The aa_3-type cytochrome *c* oxidase complex has been isolated from many organisms, from bacteria to mammals. See Musser and co-workers (1995) and García-Horsman and co-workers (1994a) for reviews.
[g] The PS3 enzyme is expressed as a cytochrome cao_3 complex under air-limited conditions (Sone and Fujiwara 1991; Sone et al. 1994).

The structural similarities of the ubiquinol and cytochrome *c* oxidase complexes naturally suggest that both families of enzymes may catalyze proton translocation via similar mechanisms. The aa_3-type cytochrome *c* oxidase complexes have been shown to pump an average of 1 H^+/e^- (for a review see Musser et al. 1995). In contrast, the observed overall H^+/e^- stoichiometry for the cytochrome bo_3 complex has been found to be 2 (Puustinen et al. 1989). Half of the protons translocated most certainly arise from the scalar release of protons from the ubiquinol oxidation chemistry and the uptake of protons from the opposite side of the membrane to balance the dioxygen reduction chemistry electrically. It is possible, then, that a mitochondrial cytochrome *c* oxidase-type proton pump mechanism (hereafter referred to as an ‘‘ion pump mechanism’’) accounts for the remaining protons translocated by the cytochrome bo_3 complex:



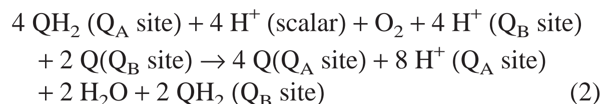
$$2\ QH_2 + 4\ H^+\ (\text{inside—scalar}) + O_2 + 4\ H^+\ (\text{inside—pump}) \rightarrow 2\ Q + 4\ H^+\ (\text{outside—scalar}) + 2\ H_2O + 4\ H^+\ (\text{outside—pump}) \quad (1)$$

Electrons are input to the mitochondrial-type cytochrome *c* oxidase complex at the Cu_A site. This redox site is located in the cytoplasmic domain of the enzyme complex. Additionally, it is noteworthy that the Cu_A center is the only redox site found in subunit II, and cytochrome *c* binding residues have been localized on this subunit (Iwata et al. 1995; Musser et al. 1995; Tsukihara et al. 1995, 1996). Thus, Cu_A is appropriately situated to act as an intermediary acceptor of electrons from aqueous ferrocytochrome *c* molecules, subsequently donating electrons to the hemes and Cu_B in the more hydrophobic recesses of the enzyme in the membrane. In contrast, ubiquinol-8 (UQ_8H_2), the physiological electron donor for the cytochrome bo_3 complex, is localized in the hydrophobic interior of the membrane bilayer. Thus, the binding domain for UQ_8H_2 is expected to be in a different three-dimensional location on the enzyme complex relative to the binding domain for ferrocytochrome *c*. The structural differences needed to accommodate these different substrate binding sites can be easily satisfied by appropriate sequence changes. It is possible that UQ_8H_2, by virtue of its localization within the lipid bilayer, can approach more closely than cytochrome *c* to the hydrophobic core of subunit I containing the hemes and Cu_B.

As a consequence, then, the intermediary electron acceptor, Cu_A, would be unnecessary and therefore is absent from the quinol oxidase complexes. This reasoning implies that the Cu_A site serves merely to funnel electrons into the ''catalytic core'' of the enzyme.

Although the dioxygen chemistry of the ubiquinol and cytochrome *c* oxidase complexes is virtually identical, the electron input mechanisms are necessarily different (Musser et al. 1993). The physiological substrate for the cytochrome *c* oxidase complexes is a water-soluble ferrocytochrome *c* (MW ~12 kDa), a one-electron donor, whereas that for the quinol oxidase complexes is a small, membrane-associated quinol (MW <1 kDa), a two-electron donor. The electron input from ferrocytochrome *c* to the Cu_A site occurs through a simple outer-sphere process. In contrast, the quinol oxidase complexes must stabilize the highly reactive semiquinone intermediate through strong interaction with the enzyme complex since the low-spin heme of these enzymes can accept only one electron. At present, there is little understanding of how the quinol oxidase family is able to couple the two-electron chemistry of the quinol donor to the one-electron-accepting low-spin heme of these enzymes.

Quinone(ol) [$Q(H_2)$] binding proteins often have two $Q(H_2)$ binding sites to facilitate the coupling of one-electron redox chemistry to a two-electron acceptor/donor. Typically, one of these binding sites is responsible primarily for stabilizing the semiquinone radical, whereas the other acts as the electron input/output site. Based in part on this fact, Musser and co-workers (1993) suggested that the cytochrome bo_3 complex has two ubiquinone(ol) [$UQ(H_2)$] binding sites. In support of this prediction, Sato-Watanabe and co-workers (1994) found a tightly bound ubiquinone-8 (UQ_8) molecule in the as-isolated cytochrome bo_3 complex that could not be removed by high concentrations of ubiquinol-1 (UQ_1H_2) or inhibitors. These investigators concluded that the UQ_8 binding site was not the ubiquinol oxidation site and that there were in fact two $UQ(H_2)$ binding sites on the cytochrome bo_3 complex. These data were interpreted to imply that the tightly bound ubiquinone acts as an intermediate electron acceptor from ubiquinol, subsequently passing electrons on to cytochrome *b*. According to this model, then, the electron input reactions of the cytochrome bo_3 complex are similar to the electron output reactions of photosynthetic reaction centers. It has been found, however, that UQ_1, UQ_6, and UQ_8 as well as 2,6-dichloro-4-nitrophenol (an inhibitor) can be reconstituted into the enzyme with an empty UQ_8 binding site (Puustinen et al. 1996; Sato-Watanabe et al. 1994). These data suggest that the UQ_8 binding site is accessible from the solvent. In fact, Musser and co-workers (1997) have recently shown that there are two $UQ(H_2)$ binding sites *in dynamic equilibrium* with the $UQ(H_2)$ pool during enzyme turnover. It is reasonable to conclude from these data that one of the $UQ(H_2)$ binding sites is the ubiquinol oxidation site, whereas the other is the UQ_8 binding site found by Sato-Watanabe and co-workers (1994). These data therefore support a $Q(H_2)$-loop proton translocation mechanism as proposed earlier (Musser et al. 1993). We emphasize that this $Q(H_2)$-loop mechanism fully accounts for the observed proton translocation stoichiometry (Puustinen et al. 1989) and is energetically feasible (Musser et al. 1997):



$$4\ QH_2\ (Q_A\ \text{site}) + 4\ H^+\ (\text{scalar}) + O_2 + 4\ H^+\ (Q_B\ \text{site}) + 2\ Q(Q_B\ \text{site}) \rightarrow 4\ Q(Q_A\ \text{site}) + 8\ H^+\ (Q_A\ \text{site}) + 2\ H_2O + 2\ QH_2\ (Q_B\ \text{site}) \quad (2)$$

In the discussion to follow, attention is focused on the evolutionary relationship between the quinol and the cytochrome *c* terminal oxidase complexes. While it is certainly possible to argue that the bo_3-type ubiquinol oxidase complex arose from a cytochrome *c* oxidase complex that lost a Cu_A site, this viewpoint does not seem tenable from the standpoint of respiratory complexity and efficiency. Rather, the argument is made here that a primitive quinol terminal oxidase complex evolved into the more structurally and functionally complex cytochrome bc_1/cytochrome aa_3 ubiquinol oxidase system.

Phylogenetic Trees Based on Sequence Information

Phylogenetic tree construction from 16S/18S rRNA sequences is generally considered one of the most reliable methods for determining evolutionary relationships because ribosomes are universally distributed across all organisms and the 16S/18S rRNA serves functionally homologous roles in all cases. However, when such a tree is constructed, it is impossible to root the tree such that organisms containing quinol oxidase complexes and those containing cytochrome *c* oxidase complexes compose separate lineages (Fig. 1). All eukaryotes contain a mitochondrial aa_3-type cytochrome *c* oxidase complex. The eubacteria contain either quinol or cytochrome *c* oxidase complexes or both. The archaebacteria are rooted between the eubacteria and the eukaryotes. The archaebacterium *Sulfolobus acidocaldarius* contains two different quinol oxidase complexes and therefore severely complicates the analysis of terminal oxidase complex evolution. It is clear that alternate means must be utilized to construct the terminal oxidase complex evolutionary tree.

There are a number of evolutionary trees in the literature constructed based on amino acid sequence similarities of quinol and cytochrome *c* oxidase complexes (Castresana et al. 1994, 1995; Saraste and Castresana 1994; Schäfer et al. 1996a; van der Oost et al. 1994). These analyses are valid only if it can be safely assumed that the various terminal oxidase complexes are functionally homologous. However, the validity of this assump-

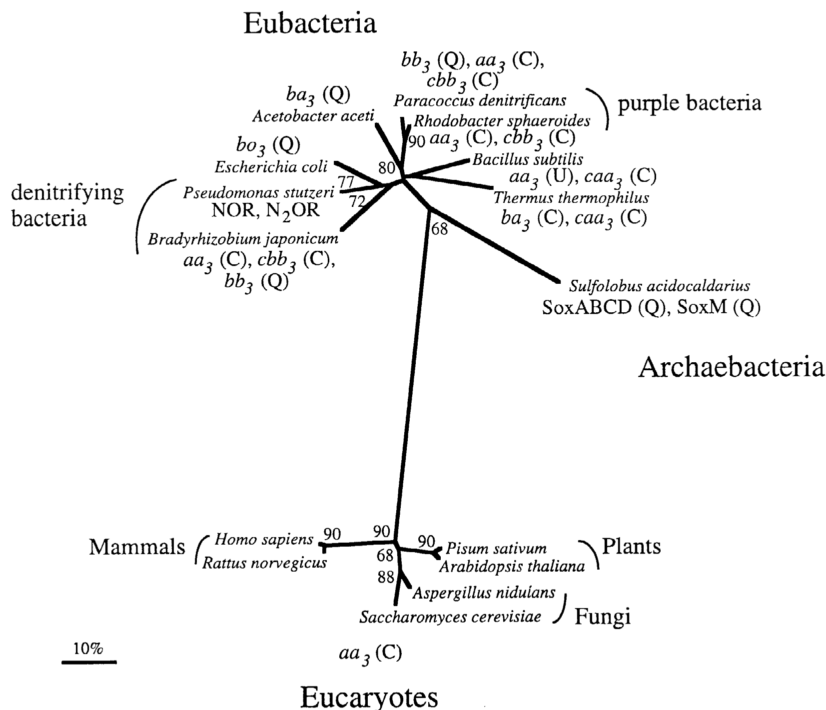
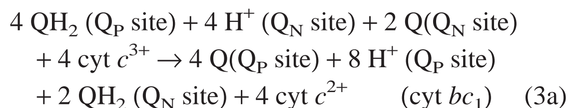


Fig. 1. Unrooted phylogenetic tree constructed using 16S/18S rRNA sequences from the GenBank database. The various types of quinol (Q) and cytochrome *c* (C) oxidase complexes present in the species shown are noted. Only terminal oxidase complexes that are members of the heme–copper superfamily are included. The eubacterial 16S rRNA sequences and the eukaryotic 18S rRNA sequences were first aligned as two separate groups, then aligned together. The archaebacterial *S. acidocaldarius* 16S rRNA sequence was added to the alignment last. The tree was constructed from the distance matrix using the neighbor-joining method (Saitou and Nei 1987). Bootstrap confidence intervals (only those larger than 50% are shown) were calculated from 9,000 samples including gaps and correcting for multiple substitutions (Felsenstein 1985). All of the above calculations were made with the CLUSTAL W 1.7 software package (Thompson et al. 1994); the tree was drawn with TREEVIEW (Page 1996).

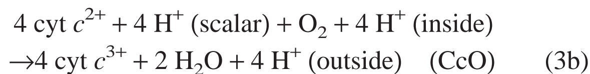
tion is called into question by the different substrates (quinol and cytochrome *c*) and the different total respiratory efficiencies of the respective respiratory chains. We explicitly take into account quaternary structural complexity and respiratory efficiency and use minimal sequence information in the analysis that follows.

Respiratory Efficiency

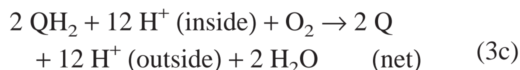
The cytochrome bo_3 complex combines the oxidation–reduction chemistry mediated by the cytochrome bc_1 and cytochrome *c* oxidase complexes. However, a price is paid. The cytochrome bo_3 complex catalyzes the net translocation of 8 H^+/4 e^-, whereas the combination of the cytochrome bc_1 and cytochrome *c* oxidase complexes results in the net translocation of 12 H^+/4 e^-:



$$4\ QH_2\ (Q_P\ \text{site}) + 4\ H^+\ (Q_N\ \text{site}) + 2\ Q(Q_N\ \text{site}) + 4\ \text{cyt}\ c^{3+} \rightarrow 4\ Q(Q_P\ \text{site}) + 8\ H^+\ (Q_P\ \text{site}) + 2\ QH_2\ (Q_N\ \text{site}) + 4\ \text{cyt}\ c^{2+} \qquad (\text{cyt}\ bc_1) \qquad (3a)$$



$$4\ \text{cyt}\ c^{2+} + 4\ H^+\ (\text{scalar}) + O_2 + 4\ H^+\ (\text{inside}) \rightarrow 4\ \text{cyt}\ c^{3+} + 2\ H_2O + 4\ H^+\ (\text{outside}) \qquad (\text{CcO}) \qquad (3b)$$

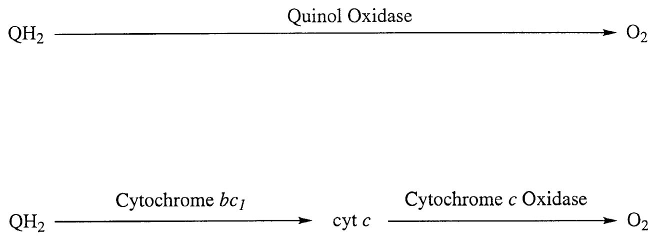


$$2\ QH_2 + 12\ H^+\ (\text{inside}) + O_2 \rightarrow 2\ Q + 12\ H^+\ (\text{outside}) + 2\ H_2O \qquad (\text{net}) \qquad (3c)$$

Clearly, a respiratory chain which contains the latter two enzymes is more efficient in energy transduction. However, a number of bacteria, e.g., *Paracoccus denitrificans, Bacillus subtilis,* and *Bradyrhizobium japonicum,* encode both ubiquinol and cytochrome *c* terminal oxidase complexes in their genome. In this manner, presumably, these bacteria are able to regulate the energy conversion efficiency of their respiratory chain at the genomic level. This regulatory capability is advantageous. When the energy supply is high, for example, ATP synthesis is unnecessary but elimination of excess reducing equivalents (e.g., regeneration of NAD^+ from NADH) may become a necessity in order for other cellular functions to proceed. In contrast, regulation of the eukaryotic cytochrome *c* oxidase proton pump can be achieved through one or more of the many nuclear-encoded subunits associated with these enzymes. For example, the H^+/e^- stoichiometry of the bovine heart cytochrome *c* oxidase complex is linked to the intramitochondrial ATP/ADP ratio through a regulatory process that involves subunit VIa-H (Frank and Kadenbach 1996). This regulation or control at the protein level obviates the need for a bo_3-type ubiquinol oxidase complex.

It is typically assumed that the bo_3-type ubiquinol oxidase complex evolved from a cytochrome *c* oxidase complex that lost its Cu_A site (Castresana et al. 1994; García-Horsman et al. 1994a; van der Oost et al. 1992, 1994). However, it is difficult to rationalize why a primitive bacterium such as *E. coli* would contain this ubiquinol terminal oxidase complex, yet no cytochrome *c* oxidase complex (Trumpower and Gennis 1994), if the former enzyme evolved later. *Acetobacter aceti* contains a ba_3-type ubiquinol terminal oxidase complex very similar (67% identical) to the *E. coli* bo_3-type enzyme; like *E. coli, A. aceti* lacks a cytochrome *c* oxidase complex, although it does contain cytochrome *c* (Fukaya et

Oxygenic Respiration



Nitrogenic Respiration

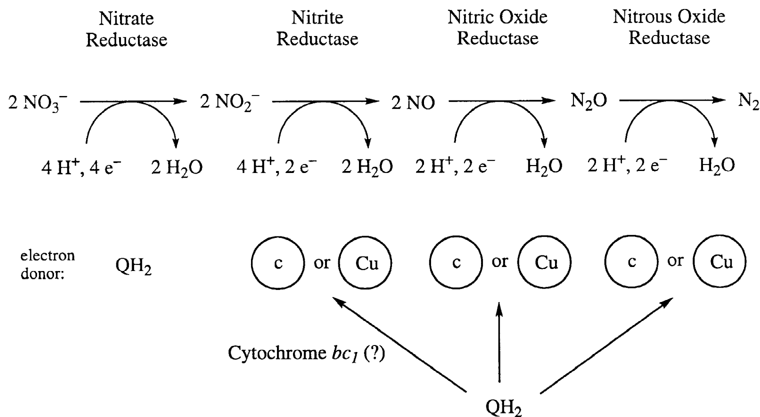


Fig. 2. Summary of the chemistry catalyzed by oxygenic and nitrogenic respiratory chains between quinol (typically ubiquinol) and the terminal electron acceptor. See text for details.

al. 1993; Matsushita et al. 1992a, b). The respiratory efficiency of an organism that contains cytochrome bc_1 and cytochrome *c* oxidase complexes in its respiratory chain can be reduced in two ways: (a) by uncoupling the cytochrome *c* oxidase proton pump (Frank and Kadenbach 1996) and (b) by increasing the proton permeability of the membrane with fatty acids or an uncoupling protein like that found in brown adipocytes (Rehnmark et al. 1992; Ricquier et al. 1991). However, an organism which contains only a quinol terminal oxidase complex cannot *increase* its respiratory efficiency in a simple manner. In light of this argument, an organism would be at a severe selective disadvantage (e.g., during an exponential growth phase) if it had started with both a quinol terminal oxidase complex and cytochrome bc_1 and cytochrome *c* oxidase complexes but then somehow, through evolutionary processes, lost the function of one of the latter two complexes. On the other hand, it makes evolutionary sense that the cytochrome bc_1 and cytochrome *c* oxidase complexes arose from a primitive quinol terminal oxidase complex via a series of beneficial mutations. Unfortunately, this idea has not received the attention it deserves, largely because of the similarities between the cytochrome *c* oxidase complexes and a number of denitrifying enzymes (Saraste and Castresana 1994) and the assumption that nitrogenic respiration existed before dioxygen was plentiful enough to support oxygenic respiration (Castresana and Saraste 1995). However, one of the general rules of evolutionary tree construction is that simplicity leads to complexity. As we elaborate below, it is this basic rule that suggests that the cytochrome bc_1 and cytochrome *c* oxidase complexes evolved from a primitive quinol oxidase complex.

Saraste and Castresana (1994; Castresana and Saraste 1995) have postulated that the aa_3-type cytochrome *c* oxidase complexes evolved from a *bc*-type nitric oxide reductase complex. Considering the complexity of nitrate respiration, we disagree with this postulate. To illustrate this complexity, we summarize in Fig. 2 the respiratory reduction pathway of nitrate to dinitrogen by denitrifying bacteria. Of the four enzymes that make use of nitrogenous electron acceptors in this reduction process, one (the nitrate reductase complex) utilizes ubiquinol as an electron donor, whereas the other three (the nitrite reductase, nitric oxide reductase, and nitrous oxide reductase complexes) utilize a soluble cytochrome *c* or blue copper protein as the electron donor. These cytochrome *c* or blue copper electron donors are thought (in general) to obtain their electrons from a cytochrome bc_1 complex (or a functionally similar enzyme). Thus, regeneration of ubiquinone from ubiquinol requires at least five enzymes (plus the cytochrome *c* or blue copper protein electron

carrier) in nitrogenic respiration. In contrast, the reduction of dioxygen to water with ubiquinol electrons requires a single enzyme (a ubiquinol oxidase complex).

Evolution of the Mitochondrial Cytochrome *c* Oxidase Complex

If the $Q(H_2)$-loop model of proton translocation for the cytochrome bo_3 complex [Eq. (2)] proves to be correct, then it is apparent that not only does the cytochrome bo_3 complex carry out, in effect, the chemistry catalyzed by the cytochrome bc_1 and cytochrome *c* oxidase complexes, but also it contains functional features of both of these enzyme complexes. This intriguing possibility intimates that during evolution, a primitive quinol oxidase complex split into two separate enzyme complexes catalyzing the same chemistry but together more efficient in energy conservation.

Sulfolobus acidocaldarius is a typical sulfur-dependent archaebacterium which grows at 70–80°C and at pH 2.0–3.5 and thus appears to be a living fossil of the early biotic soup (Brock et al. 1972). This bacterium contains two similar caldariellaquinol terminal oxidase complexes of intriguing composition. One of these caldariellaquinol oxidase complexes (termed SoxABCD) contains four different polypeptides. This enzyme contains homologues of cytochrome *b* of the cytochrome bc_1 complex (SoxC) as well as of cytochrome *c* oxidase subunits I (SoxB) and II (SoxA), respectively, yet the Cu_A site is lacking. The fourth polypeptide has no known homologue (Castresana et al. 1995; Lübben et al. 1992). The four hemes of this enzyme are all hemes A_s (modified hemes A) (Lübben and Morand 1994). This enzyme has been postulated to translocate protons via $Q(H_2)$-loop mechanism (Lübben et al. 1992) similar (in principle) to that described for the *E. coli* cytochrome bo_3 complex [Eq. (2)]. More recent data indicate the presence of a tightly bound semiquinone in the as-isolated enzyme and a proton translocation stoichiometry clearly in excess of 1 H^+/e^- (1.2 H^+/e^- found, on average), supporting the $Q(H_2)$-loop hypothesis (Gleißner et al. 1997). The latter finding (>1 pumped H^+/e^-) indicates that the ion pump mechanism (1 H^+/e^-) is insufficient to explain the observed proton translocation stoichiometry (Gleißner et al. 1997). The other caldariellaquinol oxidase complex (termed SoxM) contains at least four polypeptides. One of these polypeptides is homologous to a fusion between cytochrome *c* oxidase subunit I and cytochrome *c* oxidase subunit III (SoxM), one polypeptide is a cytochrome *b* homologue (SoxG) distinct from that found in SoxABCD, and the third and fourth polypeptides contain a Rieske-type iron–sulfur center (SoxF) and a Cu_A center (SoxH), respectively. This enzyme also contains four hemes: three hemes A_s and one heme B (Castresana et al. 1995; Lübben et al. 1994). The possibility exists that sulfocyanin, encoded in the same gene cluster, substitutes for cytochrome *c* as an electron donor to the SoxM complex (Castresana et al. 1995). It is not known for certain whether proton translocation occurs via a $Q(H_2)$-loop or the ion pump mechanism (or both, since elements of both translocation mechanisms are present in this supercomplex) but it has been suggested that a $Q(H_2)$-loop mechanism is the operational proton translocation mechanism (Schäfer et al. 1996b). The electron transfer pathways in these enzymes have not been worked out, especially with regard to the extra hemes and the iron–sulfur center. However, it is quite apparent that these caldariellaquinol terminal oxidase complexes are more complicated structurally and functionally than the cytochrome bo_3 complex and contain essential structural features of both the mitochondrial cytochrome bc_1 and the cytochrome *c* oxidase complexes.

A ubiquinol supercomplex containing both the cytochrome bc_1 and the cytochrome *c* oxidase complexes have been isolated from *P. denitrificans* and the thermophilic bacterium PS3. The *Paracoccus* enzyme consists of normal mitochondrial-type cytochrome bc_1 and cytochrome *c* oxidase complexes as well as an extra membrane-bound cytochrome *c* (cytochrome c_{552}) sandwiched between the two larger complexes (Berry and Trumpower 1985). *Paracoccus* also contains a soluble cytochrome *c* (cytochrome c_{550}) that is analogous to the mitochondrial cytochrome *c*. The electron transfer path from cytochrome c_{552} to the cytochrome *c* oxidase complex is apparently different from that for the soluble cytochrome c_{550}, indicating an important role for both of these cytochromes *c* (Stowell et al. 1993). The ubiquinol oxidase supercomplex isolated from PS3 is very similar to the *Paracoccus* supercomplex; the major difference is the presence of a cytochrome *c* domain at the C terminus of cytochrome *c* oxidase subunit II (Sone et al. 1987). These supercomplexes are clearly highly advanced versions of a primitive quinol oxidase complex, as they contain all of the redox centers found in the mitochondrial respiratory chain. Analogous membrane-bound cytochromes *c* have been found in *B. subtilis* and *Nitrobacter winogradskyi* as well (Nomoto et al. 1993; von Wachenfeldt and Hederstedt 1990). The membrane-bound cytochromes *c* were possibly initially necessary for efficient electron transfer chemistry. Since a soluble cytochrome *c* was feasible only after bacteria acquired a second membrane, the cytochrome *c* mediating electron transfer between the cytochrome bc_1 and the cytochrome *c* oxidase complexes had to have remained membrane-bound if this membrane had not been acquired at the time these supercomplexes evolved.

A number of cytochrome caa_3 complexes have been found in bacteria. All of these complexes are similar to the PS3 cytochrome caa_3 complex mentioned above—the cytochrome *c* domain is a C-terminal extension on subunit II of the enzyme—although they have been iso-

lated without attached cytochrome bc_1 polypeptides (Table 1). All of these bacteria have a soluble cytochrome *c* that serves to shuttle electrons from the cytochrome bc_1 complex to the cytochrome caa_3 complex. The functional role of the covalently attached cytochrome *c* is a mystery, as it appears to serve as an intermediate electron transfer site between the soluble cytochrome *c* and the Cu_A site, and as the mitochondrial cytochrome *c* oxidase complex exemplifies, this additional electron transfer site is clearly not necessary. This extra cytochrome *c* domain was most likely lost when mitochondria evolved and the mitochondrial genes were transferred to the nuclear genome.

The cbb_3-Type Cytochrome *c* Oxidase Complexes

There is one class of cytochrome *c* oxidase complexes found in a number of bacteria (*B. japonicum, P. denitrificans, R. sphaeroides,* and *R. capsulatus*) that must have arisen within a different evolutionary branch than that which gave rise to the mitochondrial cytochrome *c* oxidase complex. This class of enzymes, the cbb_3-type cytochrome *c* oxidase complexes, contains a low-spin heme and a high-spin heme–Cu_B binuclear center in subunit I of the enzyme as is common for all cytochrome *c* oxidase complexes. However, electron input from a soluble cytochrome *c* occurs through a series of three hemes in extramembranous domains of two other subunits. Notably, none of these enzymes has a Cu_A center (de Gier et al. 1994, 1996; García-Horsman et al. 1994b; Gray et al. 1994; Preisig et al. 1993; Thöny-Meyer et al. 1994). The *P. denitrificans* cbb_3-type oxidase complex translocates protons, although the mechanism appears to be different from that for the mitochondrial aa_3-type enzymes, as residues in the latter enzymes which are important for the translocation process are lacking in the *Paracoccus* cbb_3-type enzyme (see below).

Denitrification Enzymes

The nitric oxide reductase complex from *Pseudomonas stutzeri* is clearly related to the cbb_3-type cytochrome *c* oxidase complexes. The largest subunit of this nitric oxide reductase complex is homologous to subunit I of the heme–copper terminal oxidase superfamily and the redox centers in this subunit are ligated by the six invariant histidines common to this family of enzymes. At first, it was thought that a heme–copper binuclear center is responsible for the reduction chemistry, but recent work suggests that a nonheme iron ion plays the role of Cu_B (Zumft and Körner 1997). In addition to the two hemes B in this subunit, this nitric oxide reductase complex contains a heme C in an associated subunit (hence, this enzyme is termed a *bc*-type nitric oxide reductase complex) which appears to be the primary acceptor of electrons from a soluble cytochrome *c* (Saraste and Castresana 1994; Zumft et al. 1994). There is no evidence that this enzyme catalyzes proton translocation (van der Oost et al. 1994). *P. stutzeri* also contains a *nitrous* oxide reductase complex. This soluble enzyme contains a binuclear copper site that is very similar structurally to the Cu_A site of the cytochrome *c* oxidase complexes (Antholine et al. 1992; Farrar et al. 1991; Jin et al. 1989; Scott et al. 1989). Thus, there are at least two evolutionary links between the nitrogenic and the oxygenic respiratory pathways. As a consequence, which respiratory pathway was utilized first during evolution is certainly a provocative question.

Proton Channels

The two X-ray structures for the aa_3-type cytochrome *c* oxidase complex, one for the 4-subunit *Paracoccus* enzyme (Iwata et al. 1995) and one for the 13-subunit bovine enzyme (Tsukihara et al. 1995, 1996), have allowed more explicit interpretation of mutagenesis results. One of the findings of this more informed analysis is that there are apparently at least two proton transfer paths within these aa_3-type enzymes: the K channel (so named for a conserved lysine residue) and the D channel (so named for a conserved aspartate residue) (Konstantinov et al. 1997). Interestingly, the SoxABCD complex lacks residues in the D channel; this finding is not too surprising, as the proton translocation stoichiometry ($H^+/e^- > 1$) is incompatible with a pure ion pump mechanism (Gleißner et al. 1997), therefore intimating an alternate proton translocation mechanism. In contrast, the SoxM complex contains the ''pump'' residues (Schäfer et al. 1996b), and therefore, based on sequence comparison alone, this enzyme is predicted to translocate protons via an ion pump mechanism. The *P. denitrificans* cbb_3-type cytochrome *c* oxidase complex lacks presumably important residues in each channel and yet translocates protons. This finding argues for an alternate proton translocating machinery in the cbb_3-type enzymes (de Gier et al. 1996). With the present uncertainty in the actual proton transfer pathways, however, it is still too early to theorize on the evolutionary development of these pathways from the primary structure.

Proposal for a Protein Evolutionary Tree

As Nature very rarely builds a new protein but rather builds with the material already at hand, it is quite unlikely that the nitric oxide and nitrous oxide reductase complexes and the cytochrome *c*/quinol oxidase complexes evolved independently to yield very similar structural motifs. Thus, the similar structural elements of

these nitrogenic respiratory enzymes and the cytochrome *c*/quinol oxidase complexes imply that these proteins have a common ancestor. However, it is not so simple to decipher which group of enzymes evolved first, as arguments support both possibilities. The notion that denitrifying and sulfur-reducing bacteria were present on the early earth when the availability of atmospheric dioxygen was low is an attractive one. The average temperature of the early earth was much hotter, to be sure (Bengston 1994), and the many bacteria found only in hot springs or near deep-sea thermal vents today do appear to be living fossils. However, quite a few thermophilic bacteria have quinol and/or cytochrome *c* oxidase complexes in their respiratory chains (e.g., *S. acidocaldarius,* PS3, *Thermus thermophilus;* see Table 1), suggesting that dioxygen was indeed sufficiently abundant wherever and whenever life began. In fact, it is considered possible that localized ''oxygen oases'' existed on the early earth despite the anoxic atmosphere as a result of water and CO_2 photolysis in aqueous basins with low concentrations of reduced substances (such as H_2, CO, H_2S, and Fe^{2+}) (Kasting 1993; Schopf and Klein 1992). The appearance of superoxide dismutases in prokaryotes more primitive than cyanobacteria argues for an early presence of adequate supplies of dioxygen, for ''it is difficult to understand the evolution of an enzyme designed to protect an organism from something that supposedly did not exist'' (Bengston 1994). The many ubiquinol and cytochrome *c* oxidase complexes expressed only under conditions of low oxygen tension indicate that high concentrations of dioxygen are not required for these enzymes to function efficiently (Castresana et al. 1994). Thus, the argument that denitrifying enzymes evolved before terminal oxidase complexes because dioxygen was essentially unavailable on the early earth is not very convincing.

Natural selection works quite rapidly to eliminate mutant organisms that cannot compete or enzymes that do not provide a selective advantage. An organism that has both cytochrome bc_1 and cytochrome *c* oxidase complexes as well as a quinol terminal oxidase complex is not expected to be able to compete successfully with other members of its species if it loses the function of one of the former complexes (e.g., due to a single-site mutation or gene deletion). The loss in respiratory efficiency will quite powerfully select against the mutant organism (e.g., during an exponential growth phase). On the other hand, a cell that is able to split its quinol oxidase activity into two complexes that yield a better respiratory efficiency can certainly father the takeover of its niche by a new species. *E. coli* do not contain cytochrome bc_1 or cytochrome *c* oxidase complexes, or anything resembling these complexes, yet this species does contain the cytochrome bo_3 ubiquinol oxidase complex (García-Horsman et al. 1994a). This discussion suggests, therefore, that a quinol terminal oxidase complex existed before a cytochrome *c* oxidase complex and that *E. coli* does not belong to the same evolutionary branch as that in which the cytochrome *c* oxidase complex arose. So far in this discussion, we have not mentioned the cytochrome *bd* complex, an alternative but structurally unrelated ubiquinol oxidase complex also found in *E. coli* (Trumpower and Gennis 1994). The most likely explanation for this second ubiquinol terminal oxidase complex in *E. coli* is convergent evolution. The cytochrome bo_3 and *bd* complexes probably evolved approximately simultaneously in two physically separated primitive cell populations. Most likely, a gene transfer event between the two populations resulted in a species with greater adaptability: the cytochrome *bd* complex has a higher oxygen affinity and is preferentially expressed under low dioxygen tension, whereas the cytochrome bo_3 complex putatively translocates twice as many protons and is preferentially expressed under high oxygen tension (Trumpower and Gennis 1994). It is interesting to note that the cytochrome *bd* complex exhibits substrate inhibition similar to that found for the cytochrome bo_3 complex (Musser et al. 1997; Sakamoto et al. 1996), therefore raising the possibility that the former enzyme also utilizes a $Q(H_2)$-loop during turnover.

The increase in respiratory complexity throughout evolution as proposed here is summarized in Fig. 3. As every enzyme observed today is the result of approximately 4.5 billion years of evolution, it is quite incorrect to state, for example, that the *E. coli* cytochrome bo_3 complex evolved to yield the mitochondrial cytochrome bc_1 and cytochrome *c* oxidase complexes. Therefore, each of the complexes in Fig. 3 is shown at the end of a branch. However, the present-day enzymes certainly yield clues to the common ancestor of all the enzymes included in this scheme: a primitive quinol oxidase complex. Of the quinol oxidizing systems depicted in Fig. 3, the cytochrome bo_3 complex **(1)** is the simplest structurally and functionally and so is placed at the end of the first branch. Of the two *Sulfolobus* enzymes (**2** and **3**), SoxM is considered the more complex because of the Rieske protein and Cu_A binding domain. The progression from the cytochrome $bc_1/(c)aa_3$ supercomplex **(4)** to separate cytochrome bc_1 and aa_3 complexes **(5)** to the mitochondrial ubiquinol oxidizing system **(6)** is straightforward. There are no indications that the *bc*-type nitric oxide reductase complex (**7**) pumps protons, despite sufficient redox energy to support this function (van der Oost et al. 1994). As this enzyme requires the cytochrome bc_1 complex, it is placed on a branch earlier than **4** but later than **3,** which lacks a fully developed cytochrome bc_1 complex. The nitrous oxide reductase complex **(8),** which requires the Cu_A site, probably arose at about the same time as **7,** as similar evolutionary pressures would select for both of these enzymes. The cytochrome cbb_3 complexes **(9)** catalyze proton translocation, but apparently not by the same ion pump mech-

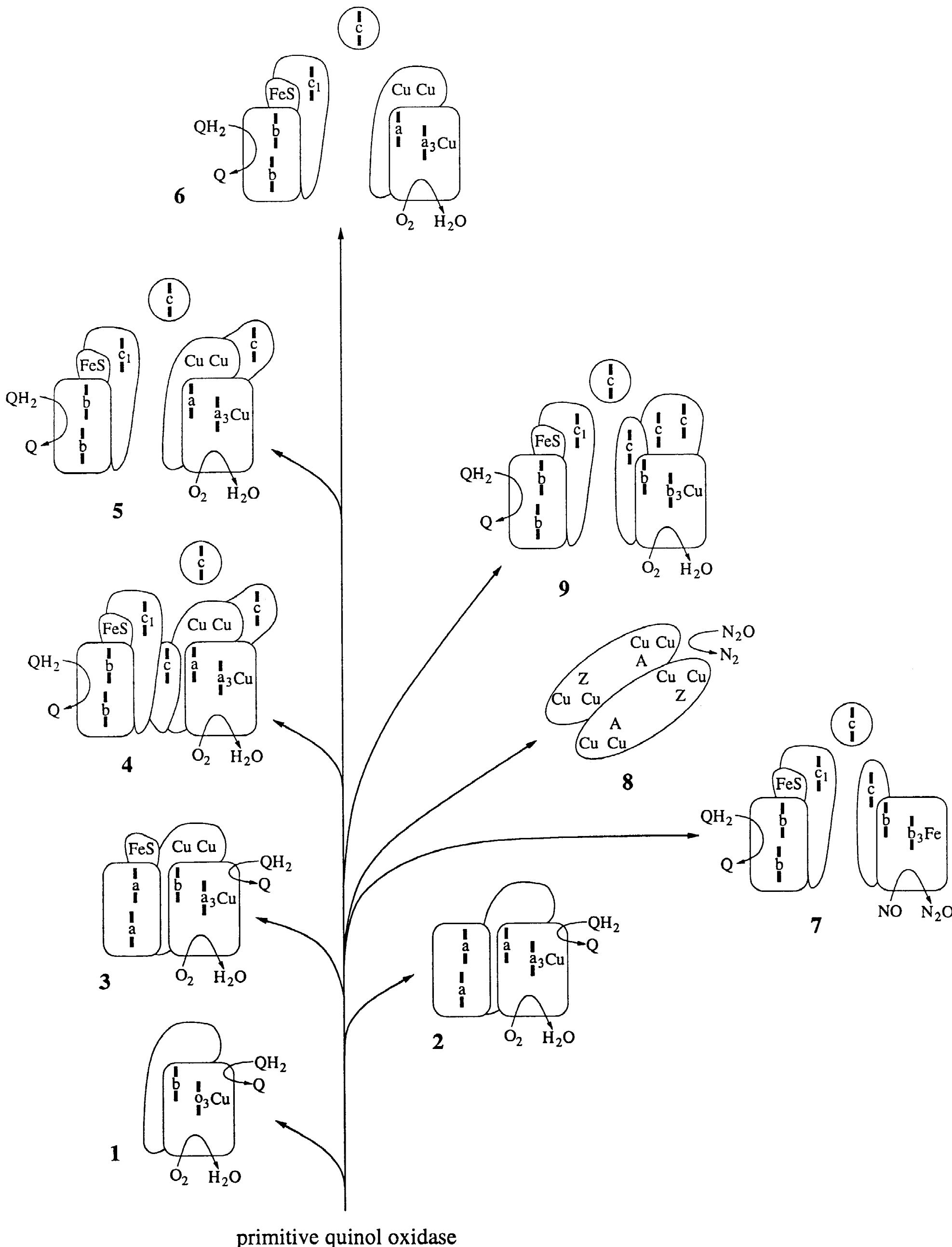


Fig. 3. An evolutionary tree depicting the increase in complexity of respiratory proteins as evidenced by present-day enzyme complexes. Lengths of branches are *not* scaled to evolutionary distance. **(1)** The *E. coli* cytochrome bo_3 ubiquinol oxidase complex. Subunit II contains the same cupredoxin fold found in the Cu_A-binding domain of cytochrome *c* oxidase and nitrous oxide reductase complexes. **(2)** The *S. acidocaldarius* caldariellaquinol terminal oxidase complex (SoxABCD) containing homologues of cytochrome *b* (of the cytochrome bc_1 complex) and cytochrome *c* oxidase subunits I and II. **(3)** The *S. acidocaldarius* caldariellaquinol terminal oxidase complex (SoxM) containing homologues of cytochrome *b* (of the cytochrome bc_1 complex) and a fusion protein of cytochrome *c* oxidase subunits I and III. The third and fourth subunits contain a Rieske-type iron–sulfur center and a Cu_A center, respectively. For **1, 2,** and **3,** proton translocation is postulated to occur via a $Q(H_2)$-loop mechanism (see text). **(4)** The cytochrome bc_1/cytochrome *c*/cytochrome *c* oxidase supercomplex found in *P. denitrificans* and the thermophilic bacterium PS3. The C-terminal extension on subunit II of the cytochrome *c* oxidase complex (*shaded*) is found in the PS3 enzyme. **(5)** A cytochrome caa_3 complex has been found in a number of bacteria (Table 1). It is not known if a cytochrome bc_1/cytochrome *c*/cytochrome *c* oxidase supercomplex exists under physiological conditions in all cases. An active cytochrome *c* oxidase complex from *P. denitrificans* and the thermophilic bacterium PS3 can be isolated alone or as part of a supercomplex as in **4. (6)** The mitochondrial cytochrome bc_1 and cytochrome aa_3 (cytochrome *c* oxidase) complexes. For **4, 5,** and **6,** proton translocation occurs via a $Q(H_2)$-loop mechanism (cytochrome bc_1 complex) and the cytochrome *c* oxidase ion pump mechanism. **(7)** The cytochrome bc_1 and nitric oxide reductase complexes found in *P. stutzeri.* The nitric oxide reductase complex has not been found to catalyze proton translocation. **(8)** The periplasmic nitrous oxide reductase complex found in *P. stutzeri* contains two binuclear copper centers: a Cu_A-type center, thought to function as an electron transfer site, and a Z center, thought to be the catalytic site. **(9)** The cytochrome bc_1 and cytochrome cbb_3 complexes found in *B. japonicum, P. denitrificans, R. sphaeroides,* and *R. capsulatus.* The cytochrome cbb_3 complexes catalyze proton translocation but likely by a mechanism different from that of the mitochondrial-type cytochrome *c* oxidase complexes. The terminal oxidase complexes at the *top* and on the *left* side of the *central line* contain both the ''catalytic'' and the ''pump'' channel residues (see text), whereas those on the *right* lack residues in at least one of these channels. See the text for references and further details.

anism as the mitochondrial aa_3-type enzymes (de Gier et al. 1996), and therefore are depicted in an earlier branch than **4** but later than the nonpumping **7.** The positioning of **7, 8,** and **9** in this tree are clearly the most difficult. According to Musser and co-workers (1995; Musser and Chan 1995), the Cu_A center of the cytochrome *c* oxidase complexes is intimately involved in the proton pumping reactions of these enzymes. In contrast, recent data support a $Q(H_2)$-loop proton translocation mechanism for the cytochrome bo_3 complex (Musser et al. 1997). Consequently, it is proposed that a primitive quinol oxidase complex translocated protons via a $Q(H_2)$-loop mechanism. This proton translocation mechanism has been retained by **1, 2,** and **3.** When the evolving quinol oxidase complex split into two complexes, the $Q(H_2)$-loop mechanism was retained by the ubiquinol oxidation component (the cytochrome bc_1 complex), whereas a new proton translocation mechanism, the Cu_A-based proton pump mechanism, developed in the dioxygen reduction component (the cytochrome *c* oxidase complex). This Cu_A-based proton pump mechanism developed too late to be of use to **7** and **9.** It is certainly possible for a bona fide Cu_A site to be present in **3** yet not be coupled to proton translocation. After all, the cytochrome *b* homologue in this enzyme complex behaves differently than its mitochondrial counterpart. Zickermann and coworkers (1997) discuss the interesting proposal that the progenitor of the Cu_A site was a blue copper site; in this mononuclear form, the copper site could have easily been required solely for electron transfer—the proton pump function arose well after the site became binuclear.

If it is accepted that the ancestral bo_3-type quinol oxidase complex was the progenitor of the cytochrome *c* oxidase complexes, the *bc*-type nitric oxide reductase complexes must have evolved after oxygenic respiration—an obvious implication of Fig. 3. This evolution scenario makes sense from the standpoint of complexity (Fig. 2). Clearly, when a cytochrome *c* oxidase complex existed, a cytochrome bc_1 complex must also have existed. Similarly, the ferrocytochrome *c* arising from cytochrome bc_1 turnover is required for membrane-bound nitrous oxide reductase activity (Ferguson 1994; van der Oost et al. 1994). Once a *bc*-type nitric oxide reductase complex existed, the other enzymes involved in nitrate/nitrite respiration (nitrite and nitrous oxide reductase complexes) could evolve. Note that there is a nitrate reductase in *E. coli* but the nitrite produced is excreted by a nitrate/nitrite antiporter (Kaldorf et al. 1993) or converted directly to ammonia by a soluble, NADH-dependent, siroheme-containing nitrite reductase or a hexaheme nitrite reductase (Brittain et al. 1992). Attempts to find a *bc*-type nitric oxide reductase complex in *E. coli* have been unsuccessful (Kaldorf et al. 1993). In the context of this discussion, the absence of a nitric oxide reductase complex in *E. coli* is not surprising, as the enzyme required for generating reducing equivalents, the cytochrome bc_1 complex, is also absent from this species. Thus, according to this discussion, oxygenic respiratory complexity proceeded from a quinol terminal oxidase complex (one enzyme) to cytochrome bc_1 and cytochrome *c* oxidase complexes (two enzymes). Along the way, nitrogenic respiration was invented involving cytochrome bc_1, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase complexes (four enzymes) (note that the electron carriers between the various complexes also contribute to the increased complexity). At each evolutionary step, many pieces of the new enzymes clearly arise from structural motifs already present. In all cases, complexity and adaptability increase but respiratory efficiency never decreases. Note that this evolutionary picture does not rule out the possibility that the progenitor of the bo_3-type ubiquinol oxidase complex was a bo_3-type ubiquinol:nitric oxide oxidoreductase. Although such an enzyme has not been found in a present-day species, it could have been useful during the anoxic conditions of the early earth.

The Terminal Oxidase Complex Tree Versus the rRNA Tree

The fact that many bacteria contain multiple-terminal oxidase complexes implies that the protein evolutionary tree shown in Fig. 3 *cannot* be converted into a phylogenetic tree. Further, as mentioned above, the 16S/18S rRNA phylogenetic tree (Fig. 1) cannot be rooted such that organisms with ubiquinol oxidase complexes and those with cytochrome *c* oxidase complexes compose separate lineages. Fortunately, there is an interpretation that dispels the apparent incongruence between the phylogenetic and the protein trees—namely, the transfer of genetic material between evolving bacteria. While such genetic transfer events most certainly were rare and resulted from an unusual set of conditions, it had to happen only once to alter dramatically the evolutionary direction of a particular lineage. Considering the evolutionary time scale, such an explanation is certainly plausible. Thus, we conclude that the terminal oxidase complex tree depicts the progression of a few particular enzymes' *appearance on earth,* whereas the rRNA tree depicts these enzymes' *appearance in distinct lineages.*

Concluding Remarks

The greater functional complexity of the cytochrome bc_1/cytochrome *c* oxidase segment of the mitochondrial respiratory chain relative to the bo_3-type ubiquinol oxidase complex strongly argues that the common ancestor of these enzymes was a quinol oxidase complex, and an attempt has been made here to rationalize how the evolution of respiratory processes might have occurred. The basic assumption that an organism with a quinol terminal oxidase complex as well as cytochrome bc_1 and cytochrome *c* oxidase complexes in its respiratory chain would never be able to compete successfully with other members of its species if it lost the function of one of the

latter enzymes can be experimentally tested, for example, by integrating the required genes for cytochrome bc_1/cytochrome *c* oxidase respiration into the *E. coli* genome and determining if wild-type *E. coli* can still survive in the presence of this mutant. In an alternative, simpler experiment, one could determine which of two *P. denitrificans* deletion strains, one lacking ubiquinol terminal oxidase activity and the other lacking cytochrome bc_1/cytochrome *c* oxidase activity [e.g., strains of de Gier and co-workers (1996)], takes over the growth medium.

More importantly, though, an evolutionary tree has been constructed on the basis of enzyme function rather than by comparing amino acid sequence information. It is considered that such an approach is required in light of the problem tackled because natural selection acts on function, not structure. If the functions of the proteins compared are sufficiently similar, an analysis of sequence alignments is expected to yield a fairly accurate estimate of the evolutionary distance between the proteins (e.g., comparison of ribosomal proteins or mitochondrial cytochromes *c*). However, if the functions of the proteins differ substantially, a comparison of primary structures for the sake of calculating evolutionary distance is fairly meaningless when little is known about the differential selective pressures on the amino acid residues of the polypeptides. The $Q(H_2)$-loop and ion pump proton translocation mechanisms of the bo_3-type ubiquinol oxidase and cytochrome *c* oxidase complexes, respectively, are dramatically different means whereby the same function is achieved. In addition, the fact that the *bc*-type nitric oxide reductase complex does not pump protons, whereas the structurally similar cytochrome *c* oxidase complexes do indicates that these enzymes are dramatically different functionally as well. Thus, alternative means have been utilized here to decipher the evolutionary relationship between these enzymes, namely, structural and functional complexity. From this discussion, it is apparent that oxygenic respiration is quite an old process, and, in fact, predates nitrogenic respiration as well as reaction-center photosynthesis, since both of these processes require the cytochrome bc_1 complex (or a functionally similar and homologous enzyme), which arose in the later stages of oxygenic respiratory evolution.

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References

Anemüller S, Schäfer G (1989) Cytochrome aa_3 from the thermoacidophile archaebacterium *Sulfolobus acidocaldarius.* FEBS Lett 244: 451–455

Anemüller S, Schäfer G (1990) Cytochrome aa_3 from *Sulfolobus acidocaldarius:* a single-subunit, quinol-oxidizing archaebacterial terminal oxidase. Eur J Biochem 191:297–305

Anemüller S, Bill E, Schäfer G, Trautwein AX, Teixeira M (1992) EPR studies of cytochrome aa_3 from *Sulfolobus acidocaldarius.* Eur J Biochem 210:133–138

Antholine WE, Kastrau DHW, Steffens GCM, Büse G, Zumft WG, Kroneck PMH (1992) A comparative EPR investigation of the multicopper proteins nitrous-oxide reductase and cytochrome *c* oxidase. Eur J Biochem 209:875–881

Bengston S (ed) (1994) Early life on earth. Columbia University Press, New York

Berry EA, Trumpower BL (1985) Isolation of ubiquinol oxidase from *Paracoccus denitrificans* and resolution into the cytochrome bc_1 and cytochrome *c*-aa_3 complexes. J Biol Chem 260:2458–2467

Brittain T, Blackmore R, Greenwood C, Thompson AJ (1992) Bacterial nitrite-reducing enzymes. Eur J Biochem 209:793–802

Brock TD, Brock KM, Belly RT, Weiss RL (1972) *Sulfolobus:* a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. Arch Mikrobiol 84:54–68

Büse G, Hensel S, Fee JA (1989) Evidence for cytochrome oxidase subunit I and cytochrome *c*-subunit II fused protein in the cytochrome c_1aa_3 of *Thermus thermophilus.* Eur J Biochem 181:261–268

Castresana J, Saraste M (1995) Evolution of energetic metabolism: the respiration-early hypothesis. Trends Biochem Sci 20:443–448

Castresana J, Lübben M, Saraste M, Higgens DG (1994) Evolution of cytochrome oxidase, an enzyme older than atmospheric oxygen. EMBO J 13:2516–2525

Castresana J, Lübben M, Saraste M (1995) New archaebacterial genes coding for redox proteins: implications for the evolution of aerobic metabolism. J Mol Biol 250:202–210

Chepuri V, Lemieux L, Au DC-T, Gennis RB (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

de Gier J-WL, Lübben M, Reijnders WNM, Tipker CA, Slotboom D-J, van Spanning RJM, Stouthamer AH, van der Oost J (1994) The terminal oxidases of *Paracoccus denitrificans.* Mol Microbiol 13: 183–196

de Gier JL, Schepper M, Reijnders WNM, van Dyck SJ, Slotboom DJ, Warne A, Saraste M, Krab K, Finel M, Stouthamer AH, van Spanning RJM, van der Oost J (1996) Structural and functional analysis of aa_3-type and cbb_3-type cytochrome *c* oxidases of *Paracoccus denitrificans* reveals significant differences in proton-pump design. Mol Microbiol 20:1247–1260

de Vrij W, Heyne RIR, Konings WN (1989) Characterization and application of a thermostable primary transport system: cytochrome *c* oxidase from *Bacillus stearothermophilus.* Eur J Biochem 178: 763–770

Farrar JA, Thompson AJ, Cheesman MR, Dooley DM, Zumft WG (1991) A model of the copper centers of nitrous oxide reductase (*Pseudomonas stutzeri*): evidence from optical, EPR and MCD spectroscopy. FEBS Lett 294:11–15

Fee JA, Zimmerman BH, Nitsche CI, Rusnak F, Münck E (1988) The cytochrome caa_3 from *Thermus thermophilus.* Chem Scripta 28A: 75–78

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Ferguson SJ (1994) Denitrification and its control. Antonie Van Leeuwenhoek 66:89–110

Frank V, Kadenbach B (1996) Regulation of the H^+/e^- stoichiometry of the cytochrome *c* oxidase from bovine heart by intramitochondrial ATP/ADP ratios. FEBS Lett 382:121–124

Fukaya M, Tayama K, Tamaki T, Egisuya H, Okumura Y, Kawamura Y, Horinouchi S, Beppu T (1993) Characterization of a cytochrome a_1 that functions as a ubiquinol oxidase in *Acetobacter aceti.* J Bacteriol 175:4307–4314

García-Horsman JA, Barquera B, Rumbley J, Ma J, Gennis RB (1994a) The superfamily of heme-copper respiratory oxidases. J Bacteriol 176:5587–5600

García-Horsman JA, Berry E, Shapleigh JP, Alben JO, Gennis RB (1994b) A novel cytochrome *c* oxidase from *Rhodobacter sphaeroides* that lacks Cu_A. Biochemistry 33:3113–3119

Gleißner M, Kaiser U, Antonopoulos E, Schäfer G (1997) The archael SoxABCD complex is a proton pump in *Sulfolobus acidocaldarius.* J Biol Chem 272:8417–8426

Gray KA, Grooms M, Myllykallio H, Moomaw C, Slaughter C, Daldal F (1994) *Rhodobacter capsulatus* contains a novel *cb*-type cytochrome *c* oxidase without a Cu_A center. Biochemistry 33:3120–3127

Ishizuka M, Machida K, Shimada S, Mogi A, Tsuchiza T, Ohmori T, Souma Y, Gonda M, Sone N (1990) Nucleotide sequence of the gene coding for four subunits of cytochrome *c* oxidase from the thermophilic bacterium PS3. J Biochem 108:866–873

Iwata S, Ostermeier C, Ludwig B, Michel H (1995) Structure at 2.8 Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans.* Nature 376:660–669

Jin H, Thomann H, Coyle CL, Zumft WG (1989) Copper coordination in nitrous oxide reductase from *Pseudomonas stutzeri.* J Am Chem Soc 111:4262–4269

Kaldorf M, von Berg K-HL, Meier U, Servos U, Bothe H (1993) The reduction of nitrous oxide to dinitrogen by *Escherichia coli.* Arch Microbiol 160:432–439

Kasting JF (1993) Earth's early atmosphere. Science 259:920–926

Konstantinov A, Siletsky S, Mitchell D, Kaulen A, Gennis RB (1997) The roles of the two proton input channels in cytochrome *c* oxidase from *Rhodobacter sphaeroides* probed by the effects of site-directed mutations on time-resolved electrogenic intraprotein proton transfer. Proc Natl Acad Sci USA 94:9085–9090

Lauraeus M, Haltia T, Saraste M, Wikström M (1991) *Bacillus subtilis* expresses two kinds of haem A-containing terminal oxidases. Eur J Biochem 197:699–705

Lübben M, Morand K (1994) Novel prenylated hemes as cofactors of cytochrome oxidases: archaea have modified hemes A and O. J Biol Chem 269:21473–21479

Lübben M, Kolmerer B, Saraste M (1992) An archaebacterial terminal oxidase combines core structures of two mitochondrial respiratory complexes. EMBO J 11:805–812

Lübben M, Arnaud S, Castresana J, Warne A, Albracht SPJ, Saraste M (1994) A second terminal oxidase in *Sulfolobus acidocaldarius.* Eur J Biochem 224:151–159

Mather MW, Springer P, Hensel S, Buse G, Fee JA (1993) Cytochrome oxidase genes from *Thermus thermophilus:* nucleotide sequence of the fused gene and analysis of the deduced primary structure for subunits I and II of cytochrome caa_3. J Biol Chem 268:5395–5408.

Matsushita K, Shinagawa E, Adachi O, Ameyama M (1990) Cytochrome a_1 of *Acetobacter aceti* is a cytochrome *ba* functioning as ubiquinol oxidase. Proc Natl Acad Sci USA 87:9863–9867

Matsushita K, Ebisuya H, Ameyama M, Adachi O (1992a) Change of the terminal oxidase from cytochrome a_1 in shaking cultures to cytochrome *o* in static cultures of *Acetobacter aceti.* J Bacteriol 174:122–129

Matsushita K, Takaki Y, Shinagawa E, Ameyama M, Adachi O (1992b) Ethanol oxidase respiratory chain of acetic acid bacteria. Reactivity with ubiquinone of pyrroloquinoline quinone-dependent alcohol dehydrogenases purified from *Acetobacter aceti* and *Gluconobacter suboxydans.* Biosci Biotech Biochem 56:304–310

Minghetti KC, Goswitz VC, Gabriel NE, Hill JJ, Barassi CA, Georgiou CD, Chan SI, Gennis RB (1992) Modified, large-scale purification of the cytochrome *o* complex (*bo*-type oxidase) of *Escherichia coli* yields a two heme/one copper terminal oxidase with high specific activity. Biochemistry 31:6917–6924

Musser SM, Chan SI (1995) Understanding the cytochrome *c* oxidase proton pump: the thermodynamics of redox linkage. Biophys J 68:2543–2555

Musser SM, Stowell MHB, Chan SI (1993) Comparison of ubiquinol and cytochrome *c* terminal oxidases. FEBS Lett 327:131–136

Musser SM, Stowell MHB, Chan SI (1995) Cytochrome *c* oxidase: chemistry of a molecular machine. In: Meister A (ed) Advances in enzymology and related areas of molecular biology, Vol 71. John Wiley and Sons, New York, pp 79–208

Musser SM, Stowell MHB, Lee HK, Rumbley JN, Chan SI (1997) Uncompetitive substrate inhibition and noncompetitive inhibition by 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) and 2-*n*-nonyl-4-hydroxyquinoline-N-oxide (NQNO) is observed for the cytochrome bo_3 complex: implications for a $Q(H_2)$-loop proton translocation mechanism. Biochemistry 36:894–902

Nomoto T, Fukumori Y, Yamanaka T (1993) Membrane-bound cytochrome *c* is an alternative electron donor for cytochrome aa_3 in *Nitrobacter winogradskyi.* J Bacteriol 175:4400–4404

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. Comp Appl Biosci 12:357–358

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 USA 90:3309–3313

Puustinen A, Finel M, Virkki M, Wikström MKF (1989) Cytochrome *o* (*bo*) is a proton pump in *Paracoccus denitrificans* and *Escherichia coli.* FEBS Lett 249:163–167

Puustinen A, Finel M, Haltia T, Gennis RB, Wikström M (1991) Properties of the two terminal oxidases of *Escherichia coli.* Biochemistry 30:3936–3942

Puustinen A, Morgan JE, Verkhovsky M, Thomas JM, Gennis RB, Wikström M (1992) The low-spin heme site of cytochrome *o* from *Echerichia coli* is promiscuous with respect to heme type. Biochemistry 31:10363–10369

Puustinen A, Verkhovsky MI, Morgan JE, Belevich NP, Wikström M (1996) Reaction of the *E. coli* quinol oxidase cytochrome bo_3 with dioxygen—the role of a bound ubiquinone molecule. Proc Natl Acad Sci USA 93:1545–1548

Rehnmark S, Bianco AC, Kieffer JD, Silva JE (1992) transcriptional and posttranscriptional mechanisms in uncoupling protein mRNA response to cold. Am J Physiol 262:E58–E67

Ricquier D, Casteilla L, Bouillaud F (1991) Molecular studies of the uncoupling protein. FASEB J 5:2237–2242

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sakamoto K, Miyoshi H, Takegami K, Mogi T, Anraku Y, Iwamura H (1996) Probing substrate binding site of the *Escherichia coli* quinol oxidases using synthetic ubiquinol analogues. J Biol Chem 271: 29897–29902

Santana M, Kunst F, Hullo MF, 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, Castresana J (1994) Cytochrome oxidase evolved by tinkering with denitrification enzymes. FEBS Lett 341:1–4

Saraste M, Metso T, Nakari T, Jalli T, Lauraeus M, van der Oost J (1991) The *Bacillus subtilis* cytochrome *c* oxidase. Variations on a conserved protein theme. Eur J Biochem 195:517–525

Sato-Watanabe M, Mogi T, Ogura T, Kitagawa T, Miyoshi H, Iwamura H, Anraku Y (1994) Identification of a novel quinone-binding site in the cytochrome *bo* complex from *Escherichia coli.* J Biol Chem 269:28908–28912

Schäfer G, Purschke W, Schmidt CL (1996a) On the origin of respiration: electron transport proteins from archaea to man. FEMS Micro Rev 18:173–188

Schäfer G, Purschke WG, Gleißner M, Schmidt CL (1996b) Respiratory chains of archaea and extremophiles. Biochim Biophys Acta 1275:16–20

Schopf JW, Klein C (eds) (1992) The proterozoic biosphere: a multidisciplinary approach. Cambridge University Press, New York

Scott RA, Zumft WG, Coyle CL, Dooley DM (1989) *Pseudomonas stutzeri* N_2O reductase contains Cu_A-type sites. Proc Natl Acad Sci USA 86:4082–4086

Solioz M, Carafoli E, Ludwig B (1982) The cytochrome *c* oxidase of

Paracoccus denitrificans pumps protons in a reconstituted system. J Biol Chem 257:1579–1582

Sone N, Fujiwara Y (1991) Effects of aeration during growth of *Bacillus stearothermophilus* on proton pumping activity and change of terminal oxidases. J Biochem 110:1016–1021

Sone N, Hinkle PC (1982) Proton transport by cytochrome *c* oxidase from the thermophilic bacterium PS3 reconstituted into liposomes. J Biol Chem 257:12600–12604

Sone N, Yanagita Y (1984) High vectorial proton stoichiometry by cytochrome *c* oxidase from the thermophilic bacterium PS3 reconstituted in liposomes. J Biol Chem 259:1405–1408

Sone N, Sekimachi M, Kutoh E (1987) Identification and properties of a quinol oxidase supercomplex composed of a bc_1 complex and cytochrome oxidase in the thermophilic bacterium PS3. J Biol Chem 262:15386–15391

Sone N, Yokoi F, Fu T, Ohta S, Metso T, Raitio M, Saraste M (1988) Nucleotide sequence of the gene coding for cytochrome oxidase subunit I from the thermophilic bacterium PS3. J Biochem 103: 606–610

Sone N, Ogura T, Noguchi S, Kitagawa T (1994) Proton pumping activity and visible absorption and resonance Raman spectra of a *cao*-type cytochrome *c* oxidase isolated from the thermophilic bacterium PS3. Biochemistry 33:849–855

Stowell MHB, Larsen RW, Winkler JR, Rees DC, Chan SI (1993) Transient electron-transfer studies on the two-subunit cytochrome *c* oxidase from *Paracoccus denitrificans.* J Phys Chem 97:3054–3057

Surpin MA, Lübben M, Maier RJ (1996) The *Bradyrhizobium japonicum coxWXYZ* gene cluster encodes a bb_3-type ubiquinol oxidase. Gene 183:201–206

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Thöny-Meyer L, Beck C, Preisig O, Hennecke H (1994) The *cccNOQP* gene cluster codes for a *cb*-type cytochrome oxidase that functions in aerobic respiration of *Rhodobacter capsulatus.* Mol Microbiol 14:705–716

Trumpower BL, Gennis RB (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

Tsukihara T, Aoyama H, Yamashita E, Tomizaki T, Yamaguchi H, Shinzawa-Itoh K, Nakashima R, Yaono R, Yoshikawa S (1995) Structure of metal sites of oxidized bovine heart cytochrome *c* oxidase at 2.8 Å. Science 269:1069–1974

Tsukihara T, Aoyama H, Yamashita E, Tomizaki T, Yamaguchi H, Shinzawa-Itoh K, Nakashima R, Yaono R, Yoshikawa S (1996) The whole structure of the 13-subunit oxidized cytochrome *c* oxidase at 2.8 Å. Science 272:1136–1144

van der Oost J, Haltia T, Raitio M, Saraste M (1991a) Genes coding for cytochrome *c* oxidase in *Paracoccus denitrificans.* J Bioenerg Biomembr 23:257–267

van der Oost J, von Wachenfeld C, Hederstedt L, Saraste M (1991b) *Bacillus subtilis* cytochrome oxidase mutants: biochemical analysis and genetic evidence for two aa_3-type oxidases. Mol Microbiol 5:2063–2072

van der Oost J, Lappalainen P, Musacchio A, Warne A, Lemieux L, Rumbley J, Gennis RB, Aasa R, Pascher T, Malmström BG, 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

van der Oost J, de Boer APN, de Gier J-WL, Zumft WG, Stouthamer AH, van Spanning RJM (1994) The heme-copper oxidase family consists of three distinct types of terminal oxidases and is related to nitric oxide reductase. FEMS Micro Biol Lett 121:1–10

von Wachenfeldt C, Hederstedt L (1990) *Bacillus subtilis* 13-kilodalton cytochrome *c*-550 encoded by *cccA* consists of a membrane-anchor and a heme domain. J Biol Chem 265:13939–13948

Zickermann V, Wittershagen A, Kolbesen BO, Ludwig B (1997) Transformation of the Cu_A redox site in cytochrome *c* oxidase into a mononuclear copper center. Biochemistry 36:3232–3236

Zimmerman BH, Nitsche CI, Fee JA, Rusnak F, Münck E (1988) Properties of a copper-containing cytochrome ba_3: a second terminal oxidase from the extreme thermophile *Thermus thermophilus.* Proc Natl Acad Sci USA 85:5779–5783

Zufferey R, Thöny-Meyer L, Hennecke H (1996) Histidine 131, not histidine 43, of the *Bradyrhizobium japonicum* FixN protein is exposed towards the periplasm and essential for the function of the cbb_3-type cytochrome oxidase. FEBS Lett 394:349–352

Zumft WG, Körner H (1997) Enzyme diversity and mosaic gene organization in denitrification. Antonie Van Leeuwenhoek 71:43–58

Zumft WG, Braun C, Cuypers H (1994) Nitric oxide reductase from *Pseudomonas stutzeri:* primary structure and gene organization of a novel bacterial cytochrome *bc* complex. Eur J Biochem 219:481–490