JOURNAL OF BACTERIOLOGY, Sept. 1994, p. 5587–5600
0021-9193/94/$04.00+0

Vol. 176, No. 18

MINIREVIEW

The Superfamily of Heme-Copper Respiratory Oxidases

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INTRODUCTION

It has been estimated that about 90% of the reduction of molecular oxygen in the biosphere is catalyzed by respiratory oxidases (95). Much of this is accomplished by a wide variety of bacterial oxidases. It has long been recognized that bacterial respiratory systems are branched, having a number of distinct terminal oxidases, rather than the single cytochrome *c* oxidase present in most eukaryotic mitochondrial systems. This multiplicity of oxidases in bacteria has long been a source of confusion, both in terms of the nature of the enzymes themselves and in terms of the rationale for such complex systems. During the past several years, it has been recognized that most of the bacterial oxidases, despite differences in their substrates (quinol versus cytochrome *c*), oxygen affinities, and heme types and metal compositions, are closely related members of a single superfamily called the heme-copper oxidase superfamily. A large number of enzymes which just a few years ago were considered to be totally unrelated are now known to be close relatives. This recognition has provided considerable order to a chaotic field, and the research that has been stimulated in this field has generated new insights into heme biosynthesis and the evolution of respiratory systems.

The heme-copper oxidase superfamily also includes the eukaryotic mitochondrial oxidases. Much of the impetus for pursuing the study of the bacterial oxidases has been to exploit the experimental advantages offered by the prokaryotic systems to learn about the structures and the functional mechanisms common to the members of the superfamily. The mitochondrial cytochrome *c* oxidase (primarily the bovine oxidase) has long been the object of intensive study (for reviews, see references 6, 10, 17, 20, 42, 59, and 86). This enzyme catalyzes the four-electron reduction of molecular oxygen to two molecules of water and utilizes the free energy available from this reaction to pump protons (one per electron) across the mitochondrial inner membrane, thus generating a transmembrane proton electrochemical gradient, or proton motive force (118). Although they typically contain only 3 or 4 subunits, in contrast to the 13-subunit mammalian enzyme, the bacterial oxidases catalyze the reduction of dioxygen and pump protons as efficiently as the mitochondrial oxidases do (39). Studies of several of the bacterial oxidases have made a very significant contribution to our knowledge of both the structures and functional mechanisms of these proton-pumping enzymes (10, 42).

The purpose of this minireview is to provide a brief overview of the current status of this rapidly moving field. For more in-depth studies on particular aspects, numerous recent reviews are available (6, 10, 17, 20, 42, 59, 86).

BRANCHED BACTERIAL RESPIRATORY SYSTEMS

All aerobic bacterial species examined have multiple respiratory oxidases (4, 73). This has been verified by spectroscopic, biochemical, and genetic approaches in several cases. The different respiratory oxidases allow the cells to customize their respiratory systems to meet the demands of a variety of environmental growth conditions. The amount of a particular oxidase present in the bacterial cytoplasmic membrane depends on the growth conditions. For example, *Escherichia coli* has two different respiratory oxidases, cytochrome *bd* and cytochrome bo_3 (5). Cytochrome *bd* has a substantially higher affinity for O_2 than cytochrome bo_3 has and is induced to high levels when the oxygen tension in the growth medium is low (27, 37, 83). The electron flow diagram of the relatively simple aerobic respiratory chain of *E. coli* is illustrated in Fig. 1A. Substrates such as NADH or succinate are oxidized by specific dehydrogenases which, in turn, reduce the quinone pool within the cytoplasmic membrane. The resulting quinol diffuses within the bilayer and is then oxidized by either one of the two oxidases. The partitioning of the electron flux between the two oxidases will depend on the amount of each oxidase present in the membrane and also on their steady-state kinetics parameters (K_m and V_{max}) and the quinol and O_2 concentrations. The replacement of the bo_3-type quinol oxidase by the *bd*-type oxidase at low aeration presents what is probably a common theme in bacterial respiration. Cytochrome *bd* has a higher affinity for O_2 (K_m in whole cells for O_2, 0.02 μM) than does cytochrome bo_3 (K_m in whole cells for O_2, 0.1 μM) (83) and will allow a greater rate of respiration at very low O_2 concentrations (i.e., microaerophilic conditions). However, the price paid is that the coupling efficiency is lower for cytochrome *bd* than for the bo_3-type quinol oxidase (14, 75). Net proton translocation across the membrane by cytochrome *bd* proceeds at a ratio of 1 H^+/e^- (where e^- is an electron), whereas net proton translocation by the bo_3-type oxidase has double the efficiency, 2 H^+/e^- (75).

It is important to realize that it is not necessarily in the best interest of the organism to utilize a respiratory system which has the highest H^+/e^- ratio. Although a primary function of the respiratory system is the generation of the proton motive force, which is utilized for ATP synthesis as well as for a variety of other essential functions, the respiratory system is also necessary to regenerate NAD^+ from NADH and to eliminate excess reducing equivalents. This latter function can be very

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important, especially under growth conditions where the cells have an adequate alternative energy supply, for example, from glycolysis or from photosynthesis. In addition, under some growth conditions, the aerobic respiratory system might be important to reduce the steady-state intracellular concentration of O_2 in order to protect oxygen-sensitive enzymes from damage. This has been suggested, for example, as a function of the *bd*-type oxidase in enteric bacteria (41) and under nitrogen-fixing conditions in *Azotobacter vinelandii* (50). Under such conditions, the rate of respiration can be of greater importance for survival than the efficiency of the proton translocation machinery. In sum, depending on growth conditions, the cell must balance competing needs in fashioning the optimal respiratory chain: (i) maximal H^+/e^- ratio; (ii) rapid removal of excess reducing equivalents; (iii) and oxygen scrubbing.

The *bd*-type quinol oxidase is the only well-characterized bacterial oxidase that is not a member of the heme-copper oxidase superfamily. Although it is found in a number of bacteria (4, 51), the occurrence of the *bd*-type oxidase is by no means universal. In contrast, it appears that the occurrence of heme-copper oxidases in bacteria is universal (Table 1). The bo_3-type oxidase from *E. coli*, for example, is a member of this superfamily. The branched aerobic respiratory system of *E. coli* is somewhat atypical in that it contains no cytochrome *c*, cytochrome *c* reductase (bc_1 complex), or cytochrome *c* oxidase (Fig. 1A) (5). A more representative example is the scheme for the purple nonsulfur bacterium, *Rhodobacter sphaeroides*, shown in Fig. 1B. *R. sphaeroides*, which can be grown aerobically, anaerobically, or photosynthetically, has three distinct respiratory oxidases, two which utilize cytochrome *c* as a substrate (32, 43), and one which is a quinol oxidase (124). The two cytochrome *c* oxidases and (most probably) the quinol oxidase are members of the heme-copper oxidase superfamily, illustrating the variations that nature has devised using the same structural framework. The aa_3-type cytochrome *c* oxidase (43) predominates when the cells are grown aerobically with high O_2 tension. The alternate cytochrome *c* oxidase is the cbb_3-type oxidase (32), which is present under microaerophilic (low O_2) conditions and when the cells are grown photosynthetically. Presumably, the cbb_3-type oxidase has a high affinity for O_2, similar to the *bd*-type oxidase, but this has yet to be demonstrated. The quinol oxidase is sufficient to support aerobic growth in strains which lack the bc_1 complex (Fig. 1B) (125).

The same basic respiratory scheme (Fig. 1B) appears to apply to *Paracoccus denitrificans* (the quinol oxidase may be a ba_3-type) (23, 58) and with variations to *Rhodobacter capsulatus* (36) and *Bradyrhizobium japonicum* (8, 9, 48, 74, 104). *R. capsulatus* lacks the aa_3-type oxidase but appears to have the two other oxidases shown in Fig. 1B. Genes corresponding to the aa_3-type (8, 30) and cbb_3-type (74) cytochrome *c* oxidases in *B. japonicum* have been characterized. It has been shown that the cbb_3-type oxidase is induced under microaerophilic growth conditions and that this oxidase is required for forming nitrogen-fixing bacteroids (74). In addition, two additional heme-copper oxidases have been identified from cloned genes in *B. japonicum* (9, 104). These two enzymes correspond to a possible third cytochrome *c* oxidase and a quinol oxidase. Hence, the respiratory system of *B. japonicum* appears to have at least four distinct oxidases which are all members of the heme-copper oxidase superfamily (Table 1).

In summary, bacteria contain multiple respiratory oxidases, and in most cases, it is clear that these oxidases are variants within the heme-copper oxidase superfamily. These variations include substrate type, substrate affinity, and heme and copper composition.

THE HEME-COPPER OXIDASE SUPERFAMILY

Membership defined by subunit I. Membership in the heme-copper oxidase superfamily is based on the presence of a subunit homologous to subunit I (the largest subunit) of the mammalian cytochrome *c* oxidase. Approximately 80 sequences of subunit I from a variety of species are listed in GenBank. It is this subunit that contains the unique bimetallic center where O_2 binds and is reduced to water. The bimetallic center, also called the binuclear center, consists of a heme (ligated to a single histidine residue) and a copper, called Cu_B, located within 5 Å (0.5 nm) of the heme iron. Subunit I contains a second heme in addition to the heme that is part of the binuclear center. This second heme is ligated to two histidine residues, and its function appears to be primarily to facilitate the transfer of electrons to the binuclear center. The presence of two hemes and Cu_B within subunit I is common to all members of the superfamily (10, 42).

The mammalian cytochrome *c* oxidase contains 13 subunits, of which 3 are encoded within the mitochondrial genome (subunits I, II, and III) and the remaining 10 are encoded within the nuclear genome (17). Nearly all of the bacterial oxidases contain homologs of subunits II and III, in addition to subunit I. Exceptions are the aa_3-type quinol oxidase (*sox ABCD*) from *Sulfolobus acidocaldarius* (57) and the ba_3-type cytochrome *c* oxidase from *Thermus thermophilus* (25a, 127), which lack subunit III, and the cbb_3-type oxidases (32, 36), which lack both subunits II and III (Table 1). Homologs of the 10 nucleus-encoded subunits of the mammalian oxidases have not been found in bacteria. However, some of the bacterial oxidases contain an additional subunit (subunit IV) which is unrelated to any eukaryotic gene product (45).

Gene organization. The gene clusters encoding the subunits of several bacterial oxidases have been completely sequenced. In most cases, the structural genes are within a single operon and are in the same order: subunit II–subunit I–subunit III. Several examples of this gene organization are shown in Fig. 2. In at least one instance, also shown in Fig. 2, the genes encoding subunits I and III are fused (62). The putative cbb_3-type cytochrome *c* oxidase from *B. japonicum* is also encoded by a single operon (*fixNOQP*) (74).

The genes encoding the subunits of the aa_3-type cytochrome *c* oxidase from *R. sphaeroides* (16, 92) and from *P. denitrificans* (80, 81, 112) are organized differently. In these cases, subunit I is encoded separately from the cluster encoding subunits II and III (Fig. 2).

Associated genes required for heme biosynthesis. As shown in Fig. 2, the gene clusters also contain genes other than those encoding oxidase subunits. Examination of the functions of the genes associated with the bo_3-type quinol oxidase from *E. coli* (84) and with the caa_3-type cytochrome *c* oxidase from *Bacillus subtilis* (105) have indicated that they play a role in heme biosynthesis.

The three types of heme associated with subunit I of the heme-copper oxidases are shown in Fig. 3. The three are heme B (protoheme IX), heme O, and heme A. (Note that the different chemical species shown in Fig. 3 are denoted with uppercase letters, but lowercase letters are used to refer to the protein-bound heme species, e.g., cytochrome *a* or heme *a*.) Heme O differs from heme B by the addition of a hydroxyethyl farnesyl side chain (120) (Fig. 3). Heme B and heme O have very similar absorption properties, and both confer a red color to the oxidases containing them. Heme A has a formyl group in place of a methyl group which is present in both heme B and heme O. This shifts the absorption spectrum so that heme A and the proteins containing it have a green color.

TABLE 1. Bacterial heme-copper oxidases which have been biochemically or genetically characterized[a]

Species	Oxidase type	Electron donor	Subunit I		Subunit II		Proton pumping	Gene organization	Comments and/or reference(s)
			Low-spin heme	High-spin heme	Heme *c*	Cu_A			
Well-characterized examples									
Acetobacter aceti	bo_3	Quinol	B	O	No	No	ND	ND	29, 67
	ba_3	Quinol	B	A	No	No	No	*cyaBACD*	There is a switch from ba_3 to bo_3 from shaking to static cultures (named cyt a_1) (63, 64)
Bacillus sp. strain PS3	caa_3	Cyt *c*	A	A	1	Yes	Yes	*co2134*	101, 102, 103
	cao_3	Cyt *c*	A	O	1	Yes	Yes	*co2134*	Same polypeptides as the caa_3-type oxidase (98, 100)
Bacillus firmus OF4	caa_3	Cyt *c*	A	A	1	ND	ND	*ctaCDEF*	78
Bacillus subtilis W23	aa_3	Menaquinol	A	A	No	ND	ND	ND	56
Bacillus subtilis W168	caa_3	Cyt *c*	A	A	1	Yes	ND	*ctaBCDEF*	54, 89, 115
	aa_3	Quinol	A	A	No	Yes	Yes	*qoxABCD*	54, 85
Bradyrhizobium japonicum	aa_3	Cyt *c*	A	A	No	ND	ND	ND	Subunit I sequence (*coxA*) (8)
	"cbb_3"	ND	ND	ND	ND	ND	ND	*fixNOQP*	No biochemical characterization. Presumed to be cbb_3-type (74)
Escherichia coli	bo_3	Quinol	B	O	No	No	Yes	*cyoABCDE*	12, 13, 22, 33, 75, 76
Paracoccus denitrificans	aa_3	Cyt *c*	A	A	No	Yes	Yes	*ctaDII* and *ctaCBGE*	Split genes. There is subunit I-like gene (*ctaDI*) of unknown function (96, 112)
	ba_3/bb_3	Quinol	B	B/A	No	No	Yes	*cyoABC*	23, 58
Rhodobacter capsulatus	cbb_3	Cyt *c*	B	B	2 or 3	No	ND	ND	Lacks subunits II and III (36)
Rhodobacter sphaeroides	cbb_3	Cyt *c*	B	B	3	No	ND	ND	Lacks subunits II and III (32)
	aa_3	Cyt *c*	A	A	No	Yes	Yes	*ctaD* and *ctaABC*	16, 43, 92
Sulfolobus acidocaldarius	aa_3	Caldariella quinol	A	A	No	No	ND	*soxABC*	Unique. SoxC is a homolog of the cytochrome *b* subunit of the bc_1 complex (2, 3, 11)
Thermus thermophilus	caa_3	Cyt *c*	A	A	1	Yes	Yes	*caaAB*	Fused genes I-c(*caaA*) and I-III (*caaB*) (11, 61, 62)
	ba_3	Cyt *c*	B	A	No	Yes	ND	ND	35
Less well-characterized examples									
Acetobacter methanolicus	"*co*"	Cyt *c*	ND	O(?)	Yes	ND	ND	ND	68
	"*bo*"	Quinol	B	O(?)	No	ND	ND	ND	19
Azotobacter vinelandii	"*co*"	ND	ND	O(?)	Yes	ND	ND	ND	46
Bacillus sp. strain PS3	bb_3	Cyt *c*	B	B	No	ND	No	ND	99
Bacillus sp. strain YN2000	"*cao*"	Cyt *c*	A	O(?)	Yes	ND	ND	ND	79
Bacillus cereus	aa_3	Cyt *c*	A	A	No	ND	ND	ND	Low turnover. Probably a quinol oxidase (31)
	caa_3	Cyt *c*	A	A	Yes	ND	ND	ND	31
Bacillus stearothermophilus	caa_3	Cyt *c*	A	A	Yes	ND	Yes	ND	97
	"*bo*"	Quinol	B	O(?)	ND	ND	ND	ND	97
Bradyrhizobium japonicum	"bb_3"	ND	ND	ND	ND	ND	ND	*coxMNOP*	No biochemical characterization. Probably a quinol oxidase (9)
	ND	ND	ND	ND	ND	ND	ND	*coxX*	No biochemical characterization (104)
Gluconobacter suboxidans	"*bo*"	Quinol	B	O(?)	No	ND	ND	ND	66
Halobacterium halobium	aa_3	Cyt *c*	A	A	No	ND	ND	ND	Subunit I sequence (24, 28)
Nitrobacter agilis	aa_3	Cyt *c*	ND	ND	No	ND	ND	ND	121
Pseudomonas aeroginosa	"*co*"	Cyt *c*	ND	O(?)	Yes	ND	ND	ND	65, 122
Rhizobium meliloti	"cbb_3"	ND	ND	ND	ND	ND	ND	*fixNOQP*	No biochemical characterization. Presumed to be cbb_3-type oxidase (7)
Rhodospirillum rubrum	"*bo*"	Quinol	B	O(?)	No	No	ND	ND	90
Synechococcus vulcanus	ND	ND	ND	ND	ND	ND	ND	*coII,I,III*	No biochemical characterization (106)
Synechocystis sp. strain PCC6803	aa_3	Cyt *c*	A	A	ND	ND	ND	*coxBAC*	No biochemical characterization (1)
Thiobacillus ferroxidans	aa_3	Cyt *c*	A	A	ND	Yes	ND	ND	3 subunits (47)
Thiobacillus novellus	"aa_3"	Cyt *c*	A	A	No	Yes	ND	ND	Unusual heme stoichiometry and behavior (94)
Vibrio alginolyticus	*bo*	Quinol	B	O	No	No	ND	ND	71
Vitreoscilla species	"*bo*"	Quinol	B	O(?)	No	ND	Yes	ND	Claimed to be a sodium pump (25, 34)

[a] In many of these cases, it is not known whether the oxidase contains heme B or heme O in the high-spin site. The traditional name, indicated in quotations (e.g. "*bo*") merely indicates that the CO-binding heme component is protoheme-like (heme B or heme O). The question mark (?) in the high-spin heme column also indicates this uncertainty. In many instances, gene sequences are known, but there is little or no biochemical characterization to support the inferences by analogy based on sequence similarities with other oxidases. There are additional sequences of putative heme-copper oxidases that are not included in this table. ND, not determined; Cyt *c*, cytochrome *c*.

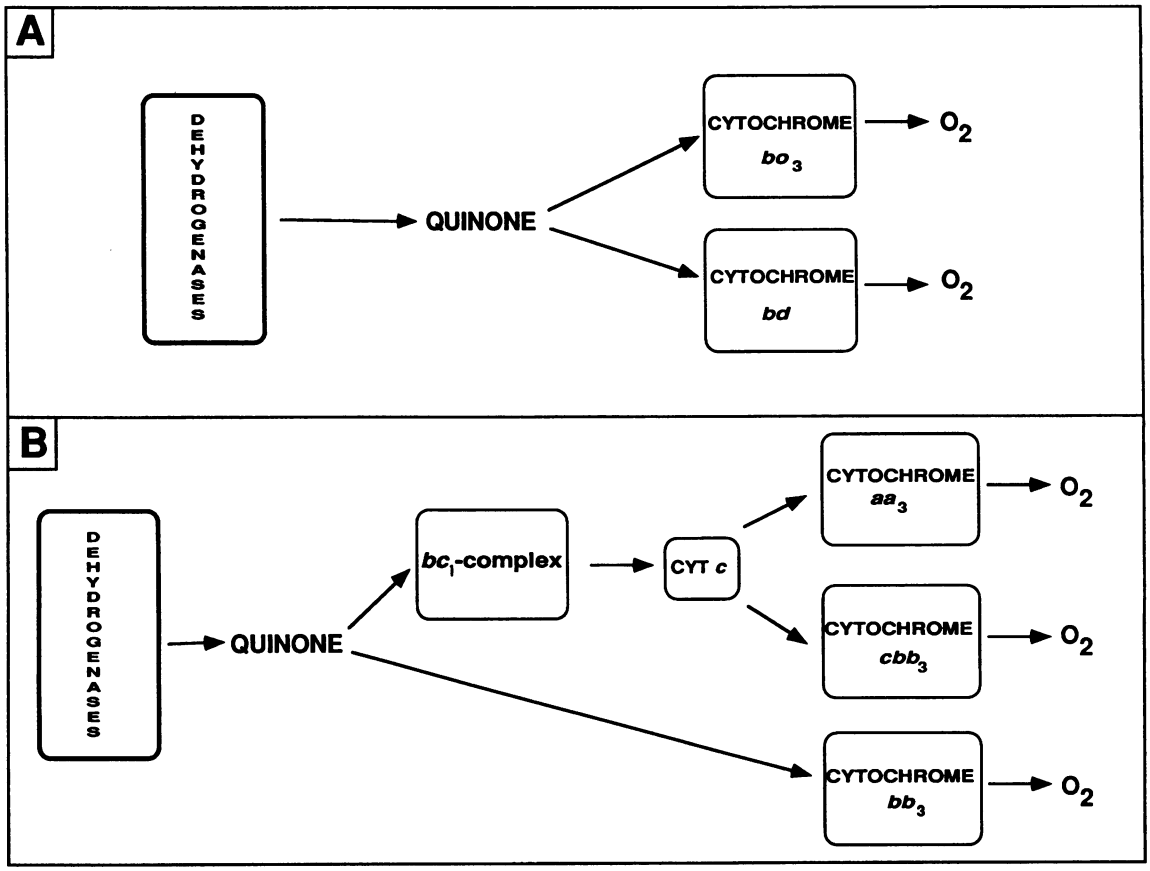


FIG. 1. Schematic of the respiratory chains of *E. coli* (A) and *R. sphaeroides* (B). Both of the cytochrome *c* oxidases in *R. sphaeroides* require the bc_1 complex for function in vivo, but their preferences for different cytochromes *c* are not known. The bb_3-type quinol oxidase of *R. sphaeroides* has not been purified. CYT *c*, cytochrome *c*.

It has been definitively demonstrated (84) that the *cyoE* gene product (Fig. 2) is the farnesyl transferase that is responsible for the conversion of heme B to heme O in *E. coli* (Fig. 3). Heme O is normally found in *E. coli* uniquely as a component of the binuclear center of the bo_3-type oxidase. The conversion of heme O to heme A, indicated in Fig. 3, does not occur in wild-type *E. coli*. However, it has been demonstrated that the expression in *E. coli* of the cloned *ctaA* gene from *B. subtilis* results in the accumulation of heme A in the *E. coli* membrane (105), indicating that the *ctaA* gene plays a role in heme A biosynthesis from heme O. Not surprisingly, *B. subtilis* contains a homolog of the *E. coli cyoE* farnesyl transferase, which is the *ctaB* gene (117) (Fig. 2). Unlike *E. coli*, however, heme O is not normally detected in the membranes of *B. subtilis*, but virtually all of the heme O is apparently converted to heme A (105). The same appears to be true in other bacterial species, including *R. sphaeroides* (31a) and *P. denitrificans* (23). However, in these cases the homolog of the *ctaA* gene has not been found yet. Finally, it should be noted that several genes have been implicated in the biogenesis of the aa_3-type cytochrome *c* oxidase from *Saccharomyces cerevisiae* (72, 109). One of these genes, *COX10*, is a homolog of *cyoE/ctaB* and is undoubtedly required for the conversion of heme B to heme O in *S. cerevisiae* (72).

Subunit composition of the minimal functional unit. Most of the bacterial heme-copper oxidases which have been biochemically characterized are three-subunit or four-subunit species (Table 1). In numerous cases, these enzymes have been demonstrated to be functional proton pumps. Hence, it is easily concluded that the essence of the catalytic mechanism, including proton pumping, is contained within the three-subunit species. The cytochrome *c* oxidases from *R. sphaeroides* (43) and *P. denitrificans* (39), for example, are functional proton pumps but consist of only the three subunits corresponding to the three mitochondrially encoded subunits (I, II, and III) of the eukaryotic oxidases. Furthermore, subunit III can be removed from the purified cytochrome *c* oxidase from *P. denitrificans* and the remaining two-subunit oxidase has been shown to be an efficient proton pump (38, 39, 96). Hence, the minimal functional unit appears to consist of subunits I and II.

One exception to this appears to be the cbb_3-type oxidases, recently isolated from *R. sphaeroides* (32) and *R. capsulatus* (36), and genetically characterized in *B. japonicum* as the *fixNOQP* oxidase (74). This unusual cytochrome *c* oxidase has a homolog of subunit I (*fixN*) that lacks many of the residues present in all the other variants (23, 87, 111). The cbb_3-type oxidases lack the equivalents of the eukaryotic subunits II and III and have, instead, two membrane-bound cytochromes *c* as subunits. Recent data indicate that this oxidase can pump protons, suggesting that subunit I alone is sufficient for this function (23, 82).

With the exception of the cbb_3-type oxidases, all of the metal prosthetic groups of the heme-copper oxidases are contained within subunits I and II.

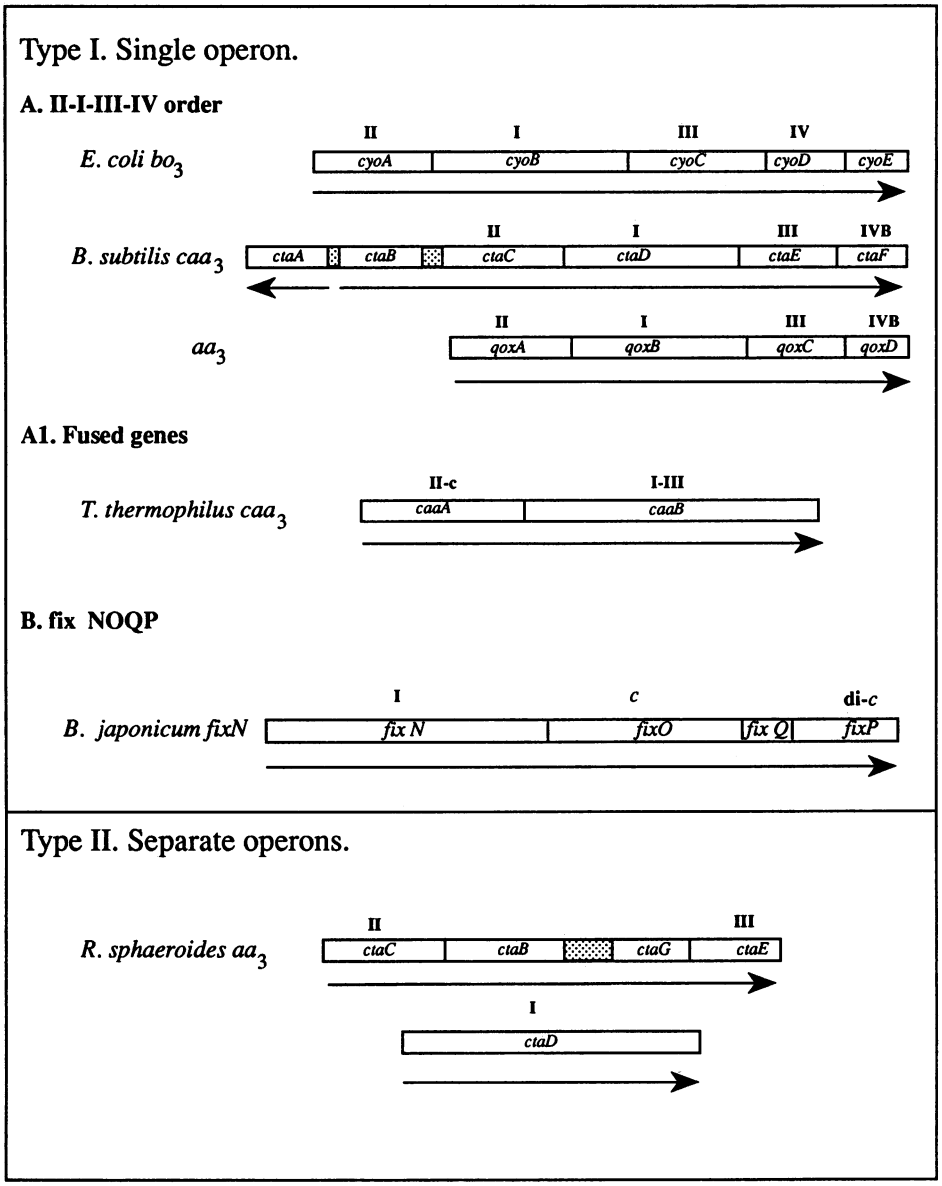


FIG. 2. Diagrams illustrating the gene organizations of the subunits of the indicated heme-copper oxidases. The symbol above each gene indicates the subunit which is encoded by that gene. Subunits I, II, and III are homologs of the mitochondrion-encoded subunits of the eukaryotic oxidases. Subunit IV, present in some, but not all, bacterial oxidases is not homologous to any known eukaryotic gene product. The *ctaA* and *ctaB* (*cyoE*) genes have been implicated in heme biosynthesis (see text).

Subunit I, the oxidase active site (variations on a theme). Subunit I is the most highly conserved subunit in the superfamily (86). For example, the sequence of subunit I of the bo_3-type quinol oxidase from *E. coli* is 40% identical to the sequence of the bovine aa_3-type cytochrome *c* oxidase (22). Figure 4A shows the proposed two-dimensional topography of subunit I from a "generic" heme-copper oxidase. The hydropathy analyses suggest that most subunit I variants contain 12 transmembrane helical spans, although several variants are longer and have additional spans. Gene fusion experiments with the *E. coli* cyo operon (21) confirm the topology shown in Fig. 4.

As already pointed out, subunit I contains the ligands for two hemes and Cu_B, which is part of the heme-copper center (10, 42). The heme which is part of the binuclear center is coordinated to a single histidine and has an available coordination position providing the oxygen-binding site. This heme is called the high-spin heme component of the oxidase because of its spectroscopic properties. The second heme is coordinated to two histidines and is called the low-spin heme center. It is the low-spin heme that is responsible for most of the absorption in the visible region of the spectrum and, thus, is responsible for the color of the various oxidase species. Among the totally conserved residues in all subunit I variants (Fig. 4) are six histidines (87, 111) which have been implicated as the ligands to the two hemes and Cu_B (12, 15, 55, 69, 93). These are located within four of the transmembrane helices: helix II (H-102), helix VI (H-284), helix VII (H-333 and H-334) and helix X (H-419 and H-421), using *R. sphaeroides* numbering.

Subunit I is the one common feature in all five subclasses of

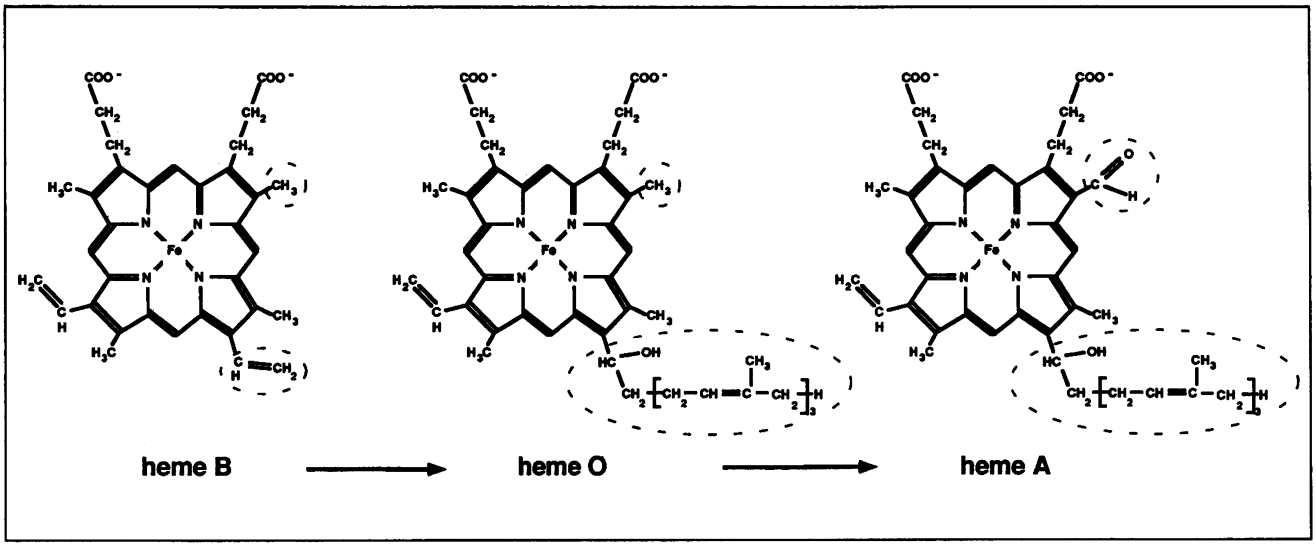


FIG. 3. Structures of heme B, heme O, and heme A. The biosynthesis proceeds as indicated (see text).

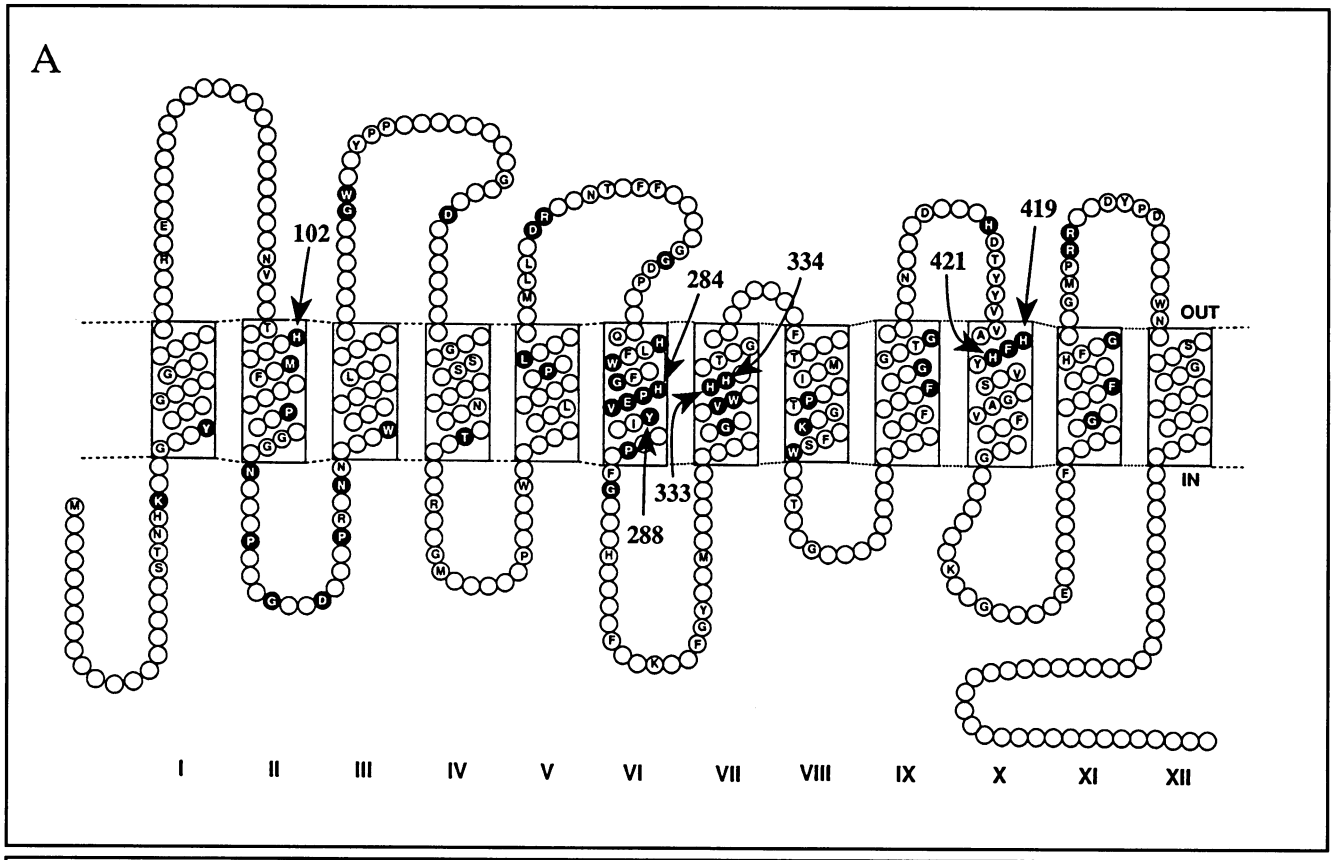
the heme-copper oxidases, of which three are cytochrome *c* oxidases and two are quinol oxidases. These classes are illustrated schematically in Fig. 5. The low-spin heme and the heme-copper binuclear center in subunit I are shown in all cases, and the proton-conducting channel is also shown as being contained within subunit I. Much of the variation observed in the heme-copper oxidases derives from the fact that individual oxidases have different combinations of hemes A, O, and B associated with subunit I. For example, the mitochondrial oxidase has heme A (Fig. 3) in both the low-spin and high-spin sites in subunit I. For historical reasons, the subscript 3 is used to denote the O_2-binding (and CO-binding) high-spin heme, so this enzyme is referred to as cytochrome aa_3, or an aa_3-type oxidase, illustrated in Fig. 5A. However, if heme B is in the low-spin site, as is the case for the cytochrome *c* oxidase from *T. thermophilus* (127), the resulting oxidase is called a ba_3-type oxidase. It is clear from the list of variants (Table 1 and Fig. 5) that heme B, heme O, and heme A can participate in the binuclear center and that either heme B or heme A can be found in the low-spin site. There are no examples in which heme O is normally found in the low-spin site, but under certain conditions, heme O can occupy the low-spin site in the *E. coli* quinol oxidase, and the resulting oo_3-type oxidase is fully functional (77). There is no correlation between the substrate specificity or other functional characteristics of these oxidases and the heme type associated with subunit I. It has been suggested in two bacterial species, *Acetobacter aceti* (64) and *Bacillus* sp. strain PS3 (98), that heme O will replace heme A in the binuclear center of the same oxidase when the cells are grown with low O_2 rather than high O_2. The functional significance of this apparent heme switch requires further exploration. Possibly, one factor could be a requirement for high O_2 for the synthesis of heme A.

As shown in Fig. 5, the most evident structural correlations with function are confined to subunit II.

Subunit II, bringing electrons to subunit I (variations on a theme). All of the cytochrome *c* oxidases, with the exception of the cbb_3-type oxidases, contain a variant of subunit II which contains a second copper-containing redox center called Cu_A (Fig. 5A and B). In these cytochrome *c* oxidases, the binding site for cytochrome *c* has been localized to subunit II (17, 86), and Cu_A is the initial electron acceptor from reduced cytochrome *c* (40). Residues in subunit II that are conserved in all these cytochrome *c* oxidases have been implicated in either the binding of cytochrome *c* or the ligation of Cu_A (86). Recent evidence indicates that the Cu_A center is actually composed of two Cu atoms (49, 52, 60, 116). All of the quinol oxidases (Fig. 5D and E) also contain variants of subunit II, but the residues implicated in either cytochrome *c* or Cu_A binding are absent. Furthermore, it has been shown that the quinol oxidases lack the Cu_A redox center, as expected (29, 53, 67, 70). Recent studies using photoreactive quinol analogs strongly suggest that subunit II forms at least part of the quinol binding site in the bo_3-type quinol oxidase from *E. coli* (117a). Hence, the major role of subunit II may be to provide the substrate-binding site, either for quinol or cytochrome *c*. Exceptions are the cbb_3-type cytochrome *c* oxidases (32, 36) (Fig. 5C) and, possibly, the *soxABCD* quinol oxidase from the archaebacterium *S. acidocaldarius*. The *soxABCD* oxidase is unique in that it is associated with a third subunit that is a homolog of the cytochrome *b* subunit of the bc_1 complex (57). This third subunit is associated with two variants of heme *a*, in addition to the two heme *a* species in subunit I. The biochemical mechanism of this oxidase is likely to have some unique features, distinct from the other members of the superfamily.

A two-dimensional topological model of subunit II from a

FIG. 4. Proposed two-dimensional topology of subunit I (A) and subunit II (B) of a generic cytochrome *c* oxidase (category A in Fig. 5). The numbering used is that for the aa_3-type oxidase from *R. sphaeroides* (16, 92). (The inside corresponds to the bacterial cytoplasm.) Highly conserved residues (not necessarily fully conserved) are indicated (solid circles) for both subunits. For subunit I, residues that are slightly less conserved are indicated by open circles with the amino acid identified. The proposed metal ligands within subunit I are indicated by the arrows. The residues thought to be Cu_A ligands in subunit II are indicated by asterisks. Note that many of the residues in subunit I that are indicated as being highly conserved are not present in the cbb_3-type oxidases and that the cbb_3-type oxidases totally lack subunit II.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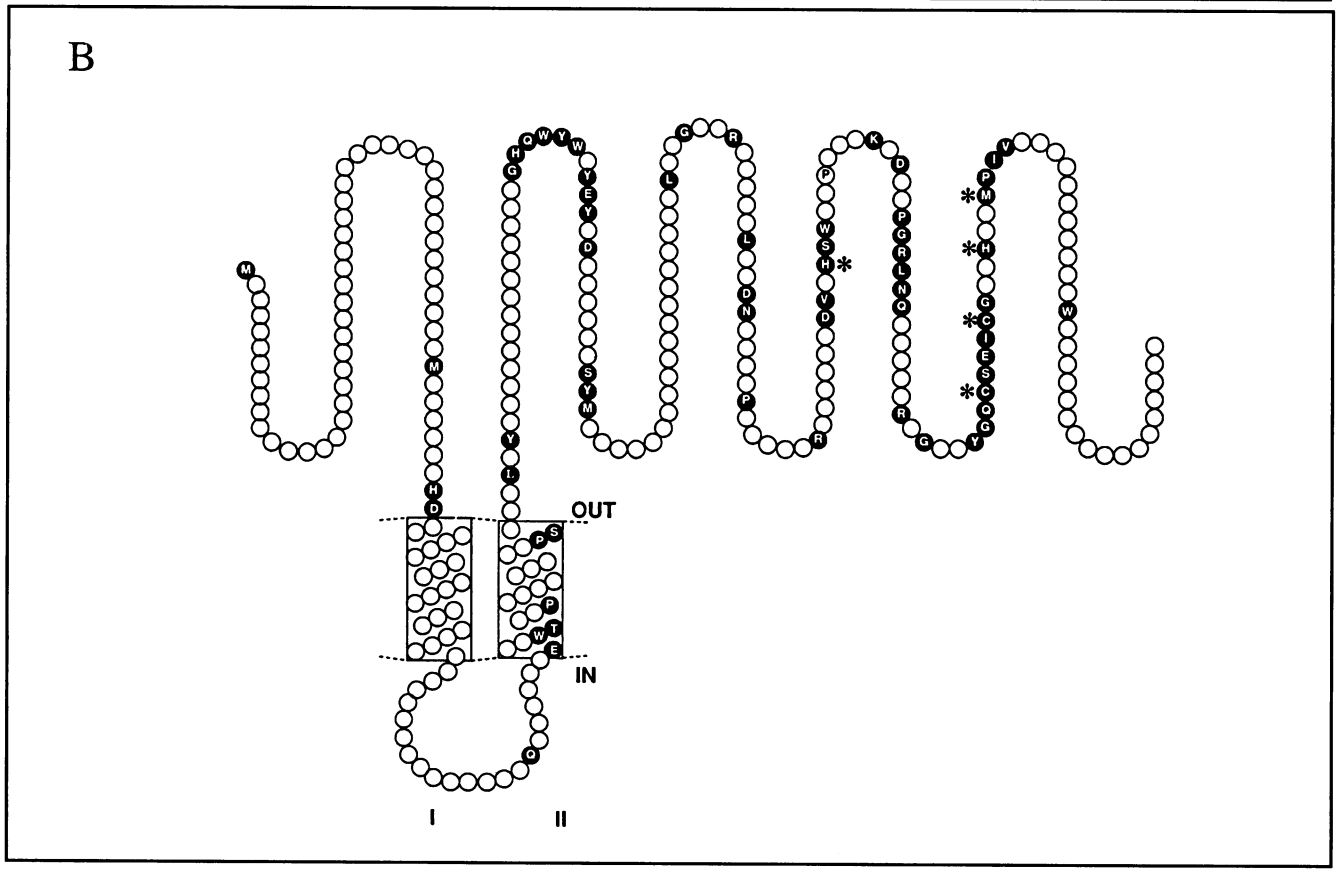
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typical cytochrome *c* oxidase is shown in Fig. 4B. A variety of data indicate that in most cases subunit II has two transmembrane-spanning helices (17, 86), and a large hydrophilic domain that faces the bacterial periplasm (or mitochondrial intermembrane space). The residues that have been implicated in the Cu_A-binding site, located within this hydrophilic domain, are highlighted in the model in Fig. 4B. These residues are missing in the quinol oxidases (22, 29, 85), but recent work has demonstrated that the Cu_A site can be generated within subunit II of the *E. coli* bo_3-type quinol oxidase by using site-directed mutagenesis to restore the "missing" ligands in the subunit (49, 114). This experiment suggests that the three-dimensional structures of subunit II of the cytochrome *c* oxidases and of the quinol oxidases are very similar, despite relatively low sequence conservation. The protein structure in the vicinity of the metal appears to be similar to the cupredoxin fold present in small blue copper proteins (114).

It is interesting to note that there are clusters of highly conserved residues in the hydrophilic domain of subunit II of the quinol oxidases (not including the unique *soxABCD* oxidase) that are not conserved in the cytochrome *c* oxidase (not shown). If the quinol-binding site is located, at least partially, in subunit II, it is possible that these residues may be involved in this binding site. Importantly, the hydrophilic carboxyl-terminal domain of subunit II of the *E. coli* oxidase has been crystallized (113), and a high-resolution structure is eagerly awaited.

The hydrophilic carboxyl-terminal domain in several bacterial cytochrome *c* oxidases has an extension containing covalently bound heme C (26, 45, 89), as indicated in Fig. 5B. None of the quinol oxidases contain covalently bound heme. This appears to be a relatively minor variation among the cytochrome *c* oxidases. There are no known functional differences, between those oxidases that contain the heme *c* addition and those that lack it (e.g., caa_3-type versus aa_3-type oxidases).

MODELLING SUBUNIT I

The combination of the application of site-directed mutagenesis along with the powerful spectroscopic techniques available for characterizing each of the metal centers in the oxidases has proven to be of great value in recent years. Most of the efforts have focussed on subunit I, with the intention of identifying the specific residues ligating to each of the metals and of locating other residues of functional importance. Mutants of both the *E. coli* bo_3-type quinol oxidase and of the *R. sphaeroides* aa_3-type cytochrome *c* oxidase have been characterized. Most of the site-directed mutants have been directed at residues that are highly conserved in the superfamily, and the results from examination of the two different oxidases are consistent. The data indicate that H-102 and H-421 (*R. sphaeroides* numbering [Fig. 4]) are the ligands to the low-spin heme (55, 69, 93), H-419 is the ligand to the high-spin heme (15), and H-333, H-334, and probably also H-284 and Y-288 are ligands to Cu_B (12, 110). These assignments have been discussed in recent reviews (10, 42) and are illustrated in the top-down model showing the helical wheel representation of several of the transmembrane spans in subunit I (Fig. 6). There are two important points to notice. (i) All of the metal ligands are predicted to be near the periplasmic side of the membrane. (ii) The two hemes are ligated to residues on opposite sides of helix X at approximately the same depth in the membrane. The consequence of the first point is that all the oxygen chemistry catalyzed by the enzyme must occur near the outer surface of the membrane. However, the protons required for the formation of water are known to originate from the opposite side of the membrane (i.e., the bacterial cytoplasm). Hence, a proton-conducting channel is required through the body of the enzyme in order to form water, in addition to the pathway required for the protons that are pumped across the membrane. It is possible that the protons required for chemistry and those that are pumped share portions of the same pathway (as pictured in Fig. 5), but this remains unknown. The implication of the second point is that electron transfer between the hemes is expected to be fast and to essentially be parallel to the plane of the membrane. Hence, transmembrane voltage generation must be in part dependent on proton translocation processes that accompany the electron transfer reactions.

More than 60 different positions within subunit I have been subjected to mutagenesis, and remarkably few appear to be essential for function. Several regions of interest have emerged from the mutagenesis studies that will be the focus of future efforts. Helix VIII is conserved in the superfamily as an amphipathic helix, and the polar residues within this helix may be important for facilitating the movement of protons from the bacterial cytoplasm to the site where oxygen is reduced to water (107). Mutations in the interhelical connection between helix II and III have been shown to specifically decouple the electron transfer reactions from proton pumping (108). Finally, the interhelical connection between helices IX and X appears to serve as a cap over the three metal centers in the enzyme (13, 44). This region may also be involved in the association of subunit I and the hydrophilic domain of subunit II.

The results of the mutagenesis studies have provided a limited list of residues that appear to be functionally important and a structural framework which is useful as a guide both for future experimental design and for speculation concerning the functional roles of specific residues.

THE EVOLUTION OF RESPIRATORY SYSTEMS

Relationship to the dentrification enzymes. It is clear that heme-copper respiratory oxidases are present both in eubacteria and in archaebacteria (2, 57) (e.g., *Halobacterium halobium* and *S. acidocaldarius*). The development of the proton-pumping heme-copper oxidases would seem to be sufficiently complex to rule out multiple and independent phylogenetic origins (i.e., convergent evolution). Since the split between the archae and eubacteria is thought to have occurred long before the development of oxygenic photosynthesis (119), it is likely that the first oxidases were present when the atmospheric oxygen concentration was very low, prior to the release of oxygen from water by photosynthesis (approximately 2.5 billion years ago). The recently recognized relationship between the heme-copper oxidases and the enzymes involved in denitrification (87, 111) has stimulated further elaboration of a model based on a single phylogenetic origin of the heme-copper respiratory oxidases prior to the development of oxygenic photosynthesis (18, 87, 88).

The conversion of nitrate to dinitrogen is catalyzed by a series of enzymes that utilize nitrite, nitric oxide, and nitrous oxide as free intermediates (128). The nitric oxide (NO) reductase is a two-subunit, membrane-bound enzyme (129). In *Pseudomonas stutzeri*, the subunits are encoded by the *norB* and *norC* genes (129). The NorC protein is a membrane-anchored cytochrome *c*, and the NorB subunit contains heme B and has recently been shown to be homologous to subunit I of the heme-copper oxidases. The NorB protein is predicted to have 12 transmembrane spans (128), and the sequence alignment reveals all six of the totally conserved histidines shown in

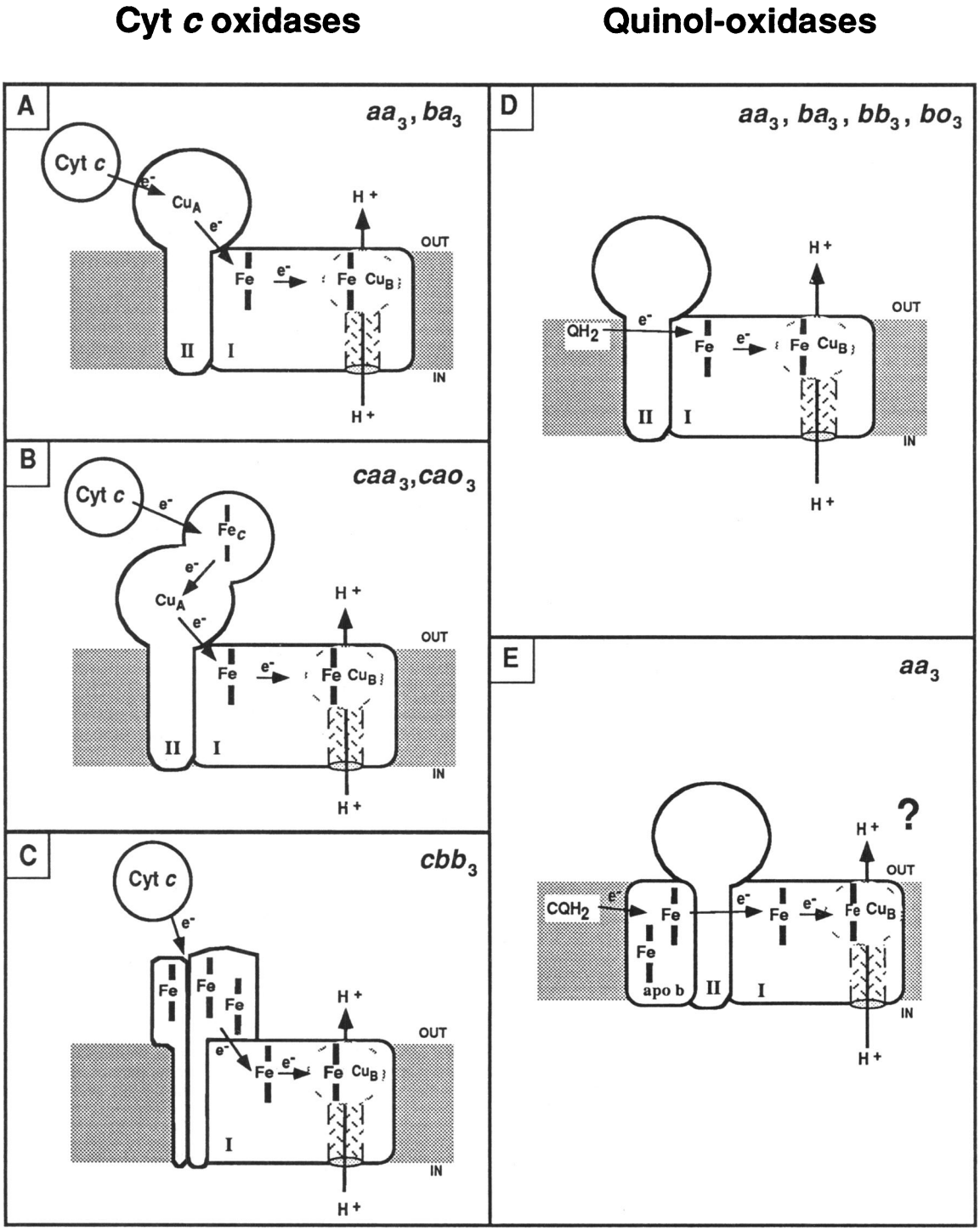

FIG. 5. Schematic illustrating the similarities and differences of five subclasses of the heme-copper oxidase superfamily. Panels A, B, and C depict cytochrome *c* oxidases, and panels D and E depict quinol oxidases. Subunits homologous to the mitochondrially encoded subunits I and II of the eukaryotic oxidases are indicated by Roman numerals. Subunits other than these two are not shown unless they contain additional prosthetic groups, as shown for the cbb_3-type oxidases in panel C and the *S. acidocaldarius* aa_3-type quinol oxidase in panel E. Indicated in each panel is the variety of heme combinations found within each subclass. The notation ba_3, for example, indicates that heme B occupies the low-spin site shown on the left side in subunit I and that heme A is in the binuclear center shown on the right side in subunit I. It is not yet known if the aa_3-type oxidase from *S. acidocaldarius* is a true proton pump, but it appears that the oxidases of each of the other subclasses can pump protons. Note that the site of interaction of the substrate cytochrome *c* on the cbb_3-type oxidases is not known. Cyt *c*, cytochrome *c*; apo b, homolog of the cytochrome *b* subunit of the bc_1 complex.

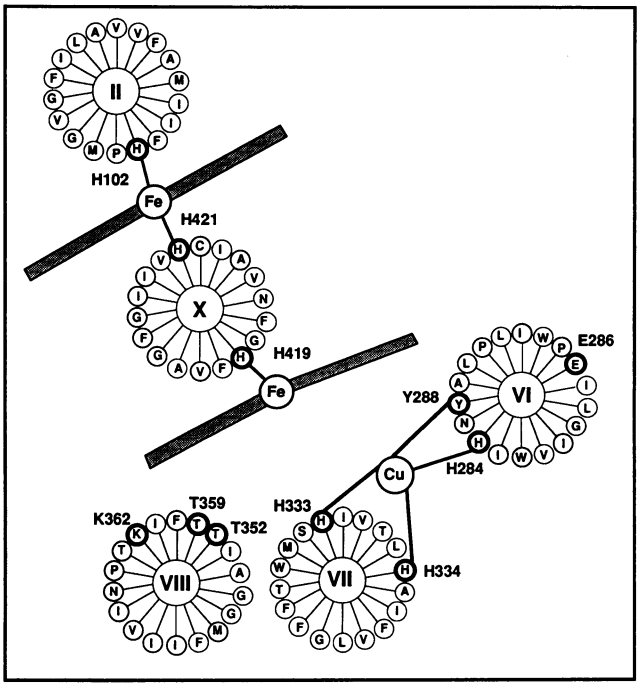

FIG. 6. A view from the periplasmic side of subunit I of the *R. sphaeroides* aa_3-type oxidase. Shown are helical wheel projections of 5 of the 12 putative transmembrane helices, along with heme *a* (between helices II and X), heme a_3 (ligated to H-419 in helix X), and Cu_B (ligated to residues in helices VI and VII). All the metals are near the periplasmic surface of the membrane. The polar residues highlighted in helix VIII are proposed to be involved in facilitating the movement of protons from the cytoplasm to the binuclear center.

Fig. 4A for the heme-copper oxidases (87, 111). The active site of NO reductase is thought to be facing the periplasm, but unlike the respiratory oxidases, the protons consumed during the reduction of NO come from the external side of the membrane, and there is no transmembrane voltage generated concomitant with the electron transfer reaction (128). Hence, NO reductase is not a proton pump and does not generate a charge separation across the membrane.

Sequence comparisons have been used to support a plausible argument that the respiratory oxidases evolved from NO reductase (18, 87, 88). The oxidase that most closely resembles NO reductase is the cbb_3-type cytochrome *c* oxidase (Fig. 5C), both in terms of the sequence of subunit I (FixN) and the subunit composition. It is speculated that this oxidase may be most closely related to the putative primitive oxidase. The acquisition of subunit II and its associated Cu_A center may also involve the denitrifying system. The nitrous oxide (N_2O) reductase contains a domain that has sequence similarity to the portion of the sequence in subunit II that is thought to bind to copper (Cu_A) (114, 130). Furthermore, N_2O reductase contains a copper redox center that has spectroscopic properties very similar to those of Cu_A (91).

The cytochrome *c* oxidases came first. According to the analysis by Saraste and colleagues, several gene duplication events and a lateral transfer between bacterial species are necessary to rationalize the phylogenetic relationships that are deduced from the sequence relatedness patterns of subunit I of the oxidases (18, 87, 88). The quinol oxidase from the archaebacterium *S. acidocaldarius* (Fig. 5E) forms a separate group along with the ba_3-type cytochrome oxidase from *T. thermophilus* (an eubacterium), presumably deriving from a branch originating prior to the split of the two bacterial kingdoms. The other quinol oxidases (Fig. 5D) appear to have evolved separately within the bacilli, according to this analysis and to have then been transferred laterally to gram-negative proteobacterial species such as *E. coli*. The sequence analysis and gene organization of these quinol oxidases very closely resemble those of the cytochrome *c* oxidases in the bacilli (18, 88). If this analysis is correct, then the quinol oxidases evolved from the cytochrome *c* oxidases.

PERSPECTIVES

The substantial progress which has taken place over the last several years provides a framework for addressing major questions that have been left unanswered. The roles of the different oxidases in the branched bacterial respiratory chains and their impact on microbial physiology remains to be addressed in a quantitative and definitive manner. This will require knowledge of the enzymology of the various oxidase species and how they function in the cell. The genetic regulation of the numerous respiratory components and their mutual interactions also need much additional study. Heme biosynthesis and the mechanism of how they are transported and assembled in these enzymes are other areas of research at a point where rapid progress should be possible.

Certainly the question of how the heme-copper oxidases couple the redox chemistry to pumping protons across the membrane remains a high priority in many laboratories. The bacterial systems, available in sufficient quantity, amenable to all the techniques of molecular genetics, and offering a rich variety of spectroscopic handles, will continue to be exploited in these studies. Certainly, one approach that will receive increasing attention in the future will be to obtain a high-resolution structure of one of the heme-copper oxidases. Although crystals of the mammalian oxidase have been reported (123, 126), no crystals suitable for high-resolution structural determination appear to have been obtained. The availability of suitable amounts of pure protein from a variety of bacterial species should enhance the chances of success in future efforts.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Institutes of Health (HL16101) and the Department of Energy (DEFG02-87ER13716).

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