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Some recent advances relating to prokaryotic cytochrome *c* reductases and cytochrome *c* oxidases

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Prokaryotic systems provide excellent experimental opportunities for exploring structure/function relationships for the complex, membrane-bound, multisubunit enzymes responsible for the reduction and subsequent oxidation of *c*-type cytochromes in respiratory or photosynthetic electron transport chains. Two points are made in this mini-review: (1) The eukaryotic and prokaryotic aa_3-type cytochrome *c* oxidases are members of an apparently large superfamily of structurally related respiratory oxidases. This superfamily displays considerable variation in terms of the heme prosthetic groups (*a* or *b*) as well as the substrate oxidized (quinol or cytochrome *c*). The relationships among these enzymes help to facilitate explorations of how they work. (2) Molecular biology techniques can be used to generate intact, redox-active, water-soluble domains of membrane-bound subunits. These soluble domains can be used for detailed examination, including obtaining high resolution structure by NMR techniques or by X-ray crystallography. This approach is being used to study the soluble heme-binding domain of cytochrome c_1 from the bc_1 complex of *Rhodobacter sphaeroides*.

Introduction

Many prokaryotic *c*-type cytochromes are components of aerobic respiratory chains or of photosynthetic systems. In numerous laboratories, the application of molecular genetics techniques is being directed at questions relating to these systems and, in this brief review, two such applications will be discussed. The first relates to the realization that there is a superfamily of respiratory terminal oxidases of which the aa_3-type oxidase is only one member. The second points out that, in some instances, complicated membrane-bound redox enzymes can yield water-soluble, functionally competent domains that are amenable for detailed study. In particular, this approach is being used to examine a water-soluble derivative of cytochrome c_1, obtained by genetic engineering of the bc_1 complex of *Rhodobacter sphaeroides*.

Part I: A superfamily of heme/copper respiratory terminal oxidases

A superfamily comprises a group of proteins which are structurally related but functionally diverse. Well known examples are neurotransmitter receptors (e.g., nicotinic acetylcholine receptor), receptors that activate G proteins (e.g., β-adrenergic receptor) and P-type (E_1E_2) ion-motive ATPases (e.g., Ca^{2+}-ATPase). The recently completed sequence of the cytochrome *o* complex from *Escherichia coli* has revealed that there is also a superfamily of respiratory oxidases [1].

Bacteria have branched respiratory systems and, in most cases, can contain multiple terminal oxidases [2,3]. Whereas all mitochondrial oxidases use cytochrome *c* as a substrate and contain heme *a* [4], considerably more variety is found in bacterial respiratory chains. Presumably, this permits the bacteria to fashion a respiratory chain to suit particular physiological requirements. For example, the number of coupling sites (H^+/e^- ratio) or the K_m for molecular oxygen can be genetically regulated by adjusting the levels of particular terminal oxidases in response to environmental conditions (e.g. Ref. 5).

It has been recognized for many years that bacteria possess both cytochrome *c*-dependent as well as cytochrome *c*-independent respiratory pathways. That is, terminal oxidases can be classified based on whether they utilize a *c*-type cytochrome as a substrate or whether they directly oxidized a quinol [2,3]. Furthermore, it has long been known that both types of terminal oxidase can be further subdivided by heme composition. The sequence of the genes encoding the subunits

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of the *o*-type ubiquinol oxidase from *E. coli* [1] establishes that there is an underlying order beyond the evident classification by substrate or heme composition. Numerous respiratory oxidases appear to be variants of a common structural motif and are members of a heme/copper terminal oxidase superfamily.

The *E. coli* cytochrome *o* complex is a four-subunit enzyme that catalyzes the two-electron oxidation of ubiquinol-8 within the bacterial cytoplasmic membrane and the four-electron reduction of molecular oxygen to water [6,7]. The oxidase contains two heme *b* (protoheme IX) prosthetic groups [6–8] and one copper (Minghetti et al., unpublished data). The two hemes give rise to the cytochrome *b*-562 and cytochrome *o* components of the purified oxidase. The enzyme has been purified to homogeneity and shown to generate a protonmotive force in reconstituted proteoliposomes [7,9,10]. In situ measurements using spheroplasts have established that two protons appear in the periplasm for each electron passing through the enzyme [11]. This is most easily explained by a combination of both scalar and vectorial proton translocation mechanisms. Briefly, one proton per electron arises from the oxidation of ubiquinol near the periplasmic surface, and the second proton arises from a redox-gated proton pump, as demonstrated for both the prokaryotic (see Ref. 12) and eukaryotic (see Refs. 4, 13) aa_3-type cytochrome *c* oxidases.

The sequence of the *cyo* operon has clearly shown a phylogenetic relationship between the heme *b*-containing ubiquinol oxidase from *E. coli* and the aa_3-type family of cytochrome *c* oxidases [1]. The three mitochondrially encoded subunits of the eukaryotic cytochrome *c* oxidases (i.e., subunits I, II, III) each have a homologue in the quinol oxidase. Most striking is the comparison between the largest subunits (subunit I). An alignment of this subunit from the *E. coli* oxidase with the bovine heart subunit I reveals 40% identical residues. These include most of the residues that are fully conserved in all known subunit I sequences from aa_3-type cytochrome *c* oxidases, of which there are over 20 examples [4,14]. This is particularly interesting, since subunit I almost certainly contains the residues ligating cytochrome *a* as well as the cytochrome a_3-Cu_B binuclear center in the aa_3-type oxidases [4,13,15]. The heme/copper binuclear center is the site where oxygen is activated and reduced to water. Such a binuclear center is the heart of this superfamily of heme-copper oxidases.

Numerous biophysical studies, both published and in progress, have confirmed the similarity of the redox centers in the *o*-type ubiquinol oxidase and in the aa_3-type cytochrome *c* oxidases (e.g. Ref. 16). Cytochrome *b*-562 corresponds to the cytochrome *a* component of the cytochrome *c* oxidases. This is a 6-coordinate, low spin component and in the *E. coli* oxidase it is presumably at the site of ubiquinol oxidation near the periplasmic surface of the membrane. Cytochrome *o* (or *b*-555) is a 5-coordinate heme component which is part of a heme-copper binuclear center. Cytochrome *o* corresponds to cytochrome a_3 in the cytochrome *c* oxidases. This is the component that binds CO and cyanide. A major experimental effort is now underway to use site-directed mutagenesis to define the amino acid residues within subunit I which are the heme iron and copper ligands in the cytochrome *o* complex. Clearly, these results will be directly applicable to the aa_3-type oxidases as well.

Subunit II in the cytochrome *c* oxidases has been implicated both in the binding to cytochrome *c* and in being the binding site for a second copper, Cu_A (see Refs. 4, 13). The Cu_A center is EPR-detectable, unlike

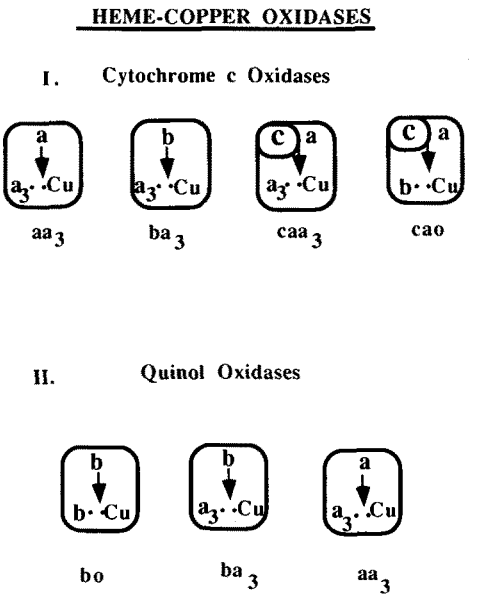

Fig. 1. A schematic illustration of some possible members of the heme-copper superfamily of respiratory oxidases. In all cases, the presence of the indicated hemes and copper are confirmed. The arrangements shown in the figure, however, are in most cases speculated. Also, in most cases there are no sequence data available to confirm that these enzyme are phylogenetically related. The heme(s) at the top of each figure represents a site of either cytochrome *c* or quinol oxidation. The presence or absence of Cu_A is not indicated or known in most cases. Note that a *b*-type cytochrome that binds to carbon monoxide and is part of an oxidase is usually called cytochrome *o*. The possible variants of the heme-copper binuclear center are shown at the lower position of each figure. This, again, is not proven in all cases. These oxidases represent the following:

Cytochrome *c* oxidases:

(1) aa_3-type from *Paracoccus denitrificans* [28] or *Rhodobacter sphaeroides* [29].

(2) ba_3-type oxidase from *Thermus thermophilus* [30].

(3) (*c*)aa_3-type oxidase from *Thermus thermophilus* or *Bacillus PS3* [14,18,31].

(4) (*c*)*ao*-type oxidase from alkalophilic *Bacillus*. YN-2000 [32].

Quinol Oxidases:

(1) *bo*-type oxidase from *E. coli* [17].

(2) ba_3-(or *ba*)type oxidase from *Acetobacter aceti* [33].

(3) aa_3-type oxidase from *Sulfolobus acidocaldarius* [34].

Cu_B, and it is presumably involved in assisting in the oxidation of cytochrome *c*. It has also been suggested to be directly involved in gating the proton pumping activity of the cytochrome *c* oxidases [13]. However, Cu_A is not present in the *E. coli o*-type ubiquinol oxidase, and none of the amino acid residues purported to be Cu_A ligands or to be involved in cytochrome *c* binding is conserved in the sequence of subunit II from the *E. coli* oxidase [17].

Circumstantial evidence suggests that the *E. coli* quinol oxidase and the aa_3-type cytochrome *c* oxidase represent just two variants of a broad range of enzymes. It is already clear that one variation is the caa_3-type cytochrome *c* oxidases found in *Bacillus* PS3, *Bacillus subtilis* and *Thermus thermophilus* [14,18]. In this case, the Cu_A-containing subunit II is fused to an additional domain which contains a *c*-type cytochrome. A number of cytochrome *c* or quinol oxidases have now been characterized from bacteria which contain a variety of combinations of heme *b* and/or heme *a* plus copper (see Fig. 1). It is premature to conclude that these are all members of the heme/copper superfamily as defined here, since sequence data are not available in most cases. It is also premature to conclude as a rule that all quinol oxidases lack Cu_A, since in some cases more than one copper has been found. Nevertheless, the pictorial summary in Fig. 1 is indicative of how inclusive this heme/copper superfamily might be. Note that this figure is somewhat speculative and many more data are necessary to characterize the enzymes indicated before they are properly classed together. As sequences of the subunits become available, the breadth of this superfamily will become more evident. Also, it will be of interest to see how closely related are those respiratory oxidases that are not members of this superfamily (e.g., *bd*-type ubiquinol oxidases) [3,19,20].

Part II: A water-soluble form of cytochrome c_1

There is no substitute for a high-resolution structure if one is interested in protein biochemistry or enzymology. Certainly, the kinetics and spectroscopic studies of the bc_1 complexes (ubiquinol : cytochrome *c* oxidoreductases) have resulted in a rather clear general picture of how the enzyme works (see Ref. 21). However, many questions can only be resolved if we know how the polypeptide chains interact with each other and with the prosthetic groups. In the absence of suitable quality crystals of the entire enzyme, it is possible to examine smaller water-soluble domains which are more tractable for structural studies, either by X-ray crystallography or by modern solution NMR techniques. This is not a new approach to biochemical experimentation in general, and has even been applied previously to examining components of the bc_1 complex from *Neurospora crassa*. Water-soluble domains that contain the Rieske Fe-S cluster [22,23] or the cytochrome c_1 [24] component of the bc_1 complex have been generated by proteolysis and examined. High-resolution structural data, however, have not resulted [25].

An alternative approach to obtaining such soluble domains is to utilize molecular genetics and alter the genes in such a way to direct the synthesis of independent, soluble domains rather than membrane-bound subunits. By introducing a stop codon in the *fbc*C gene, the polypeptide is produced in *Rb. sphaeroides* without its C-terminal hydrophobic anchoring segment. One would predict that a soluble version of cytochrome c_1 would result and would be localized in the periplasm. This is, indeed, what occurs when such a site-directed mutant is made (Konishi, Van Doren, Crofts and Gennis, unpublished data). The bc_1 complex is not functional when cytochrome c_1 is made without its C-terminal membrane anchor. Similar data were obtained previously with mutants of yeast cytochrome c_1 [26,27].

A combination of gel-filtration chromatography and DEAE ion-exchange chromatography have been used to obtain a homogeneous preparation of the soluble cytochrome c_1 starting with an osmotic shock lysate of *Rb. sphaeroides*. The amino-terminal sequence of the soluble cytochrome is identical to that of authentic cytochrome c_1, and the spectroscopic and electrochemical properties also appear to be very similar to the membrane-bound cytochrome c_1 within the bc_1 complex. Studies with intact cells have demonstrated that the soluble cytochrome c_1 can be oxidized by cytochrome c_2, its natural redox partner.

In summary, it has been demonstrated that a water-soluble domain of the *Rb. sphaeroides* cytochrome c_1 can be generated by genetic engineering. The optical and electrochemical properties of the covalently-bound heme *c* indicate that the conformation must be very similar to that in the native enzyme. This is confirmed by the demonstrated interaction in whole cells with cytochrome c_2. The challenge now is to optimize the production of this soluble cytochrome c_1 to obtain a high-resolution structure and to examine the nature of its interactions with its natural redox partners.

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