Crystals and structures of cytochrome *c* oxidases — the end of an arduous road

Crystal structures of cytochrome *c* oxidases, one of which is the largest membrane-bound protein complex crystallized to date in a form suitable for X-ray diffraction, have recently been solved. The information from these accomplishments confirms many of the structural properties known from earlier spectroscopic and analytical studies, and provides a basis for understanding the complex mechanisms of electron transfer and proton pumping.

Chemistry & Biology December 1995, **2**:781–785

In 1931 Otto Warburg received the Nobel Prize in Physiology or Medicine for his demonstration that the photochemical action spectrum of his 'Atmungsferment' (respiratory enzyme, now cytochrome oxidase) was that of a heme protein. With reference to the technique used, he said in his acceptance address: "The procedure resembles . . . the spectral analysis of stars, and indeed the matter of which the enzyme is made up, even though close at hand, is out of reach for us as is that of the stars" [1]. Today, 25 years after Warburg's death, we have identified that matter — unimaginable even for one of the pace-setting and most innovative biochemists of the first half of our century. Almost simultaneously two groups have published the crystal structures of two cytochrome *c* oxidases at high resolution [2,3]. It is particularly fortunate that these two groups used oxidases from organisms that are evolutionarily far apart (cattle and bacteria), so that comparisons between the two structures can be used to identify the essential features of the enzyme.

Respiratory oxidases (O_2 reductases)

Cytochrome *c* oxidase, one of the most complex enzymes of the respiratory chain of mammalian mitochondria, is the largest membrane protein (M_r ~ 200 K) thus far crystallized in a form suitable for X-ray diffraction. It is a member of a large family of terminal oxidases, the cytochrome and quinol oxidases, found in aerobic organisms [4]; its ancestry has been traced back to times before photosynthetically generated oxygen became available [5]. These oxidases all use the basic principle of a bimetallic copper–heme center (usually hemes of the A- or O-type) as an intermediate electron carrier. The cytochrome oxidases have an additional Cu site as an immediate electron acceptor, which is related to that present in 'blue' Cu proteins such as plastocyanin or azurin [6]. This center has only recently been shown to consist of a pair of Cu atoms, which accept electrons from the substrate, cytochrome *c* (cyt *c*) [7].

The enzyme also has the task of converting the energy available from the oxidation that it catalyzes (~550 mV) into a form usable by the organism in which it resides. Cytochrome *c* oxidase is located in eukaryotes in the mitochondrial inner membrane, and in prokaryotes is found in the cytoplasmic membrane. At the same time as transporting electrons, the enzyme transfers protons from the mitochondrial matrix to the cytoplasm in eukaryotes or from the cytoplasm to the periplasmic space in prokaryotes. An electrochemical potential of protons is thus established across the membrane separating these compartments, and this potential drives the synthesis of ATP by the membrane-bound ATP synthase. These coupled functions require an elaborate structure, which has to make contact with substrates (cyt *c*, quinol and O_2), prevent the formation or escape of noxious O_2 reduction products, and channel protons toward the locus of O_2 reduction or through the membrane to the outside. Four protons are required per molecule of O_2 to form H_2O, and up to four protons are simultaneously transferred across the membrane to the outside.

What we knew

The enzyme requires at least two subunits (Su I and Su II) to accomplish its tasks [8]. In most organisms, however, there are at least three to four subunits and in mammals up to 13. Subunits I–III are the largest and are encoded by mitochondrial DNA, whereas the smaller subunits are encoded by nuclear DNA. The functions of subunits other than Su I and II have not yet been clearly defined; they may have to do with location and assembly of the enzyme in the membrane, interaction with other membrane proteins, stabilization or optimization of enzyme function, or organ specificity in higher eukaryotes.

Su I is the center of the action. It contains 12 transmembrane helices, the proton channels and the two heme molecules (the Cu–heme O_2 binding site and the heme that transfers electrons to the O_2 reduction site). Su II harbors the cyt *c* binding site and a mixed valence Cu pair (the Cu_A site), which accepts one electron from reduced cyt *c* during one turnover. The electron is then passed to heme *a* in Su I which transfers it to the Cu_B–heme a_3 binuclear O_2 reduction site. (Because of their unique spectral properties, the hemes that occur in the O_2 binding site in various organisms are designated with the suffix 3, for example, a_3 or o_3.) In quinol oxidases, which lack the Cu pair, electrons enter directly into heme *a*. In most cytochrome *c* oxidases, particularly those from

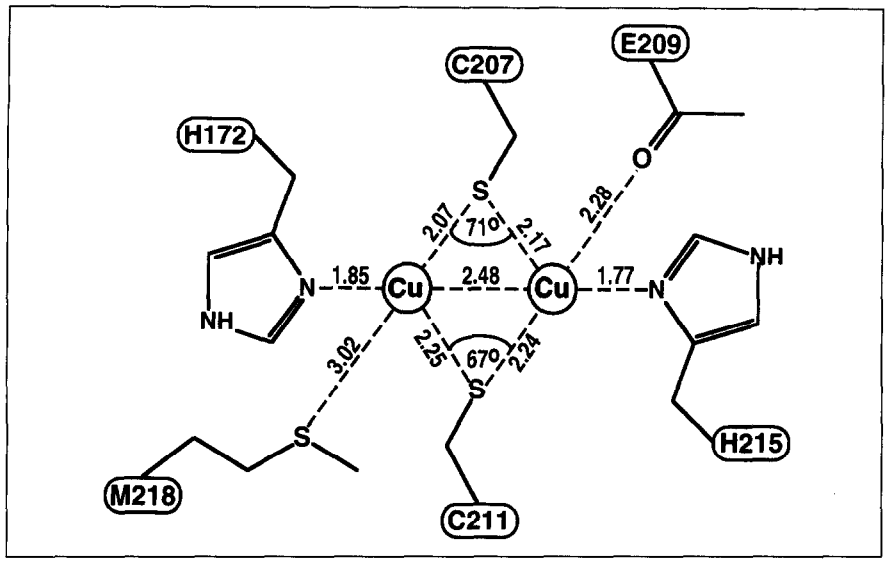

Fig. 1. Schematic view of the Cu_A site in the reconstituted purple fragment from a quinol oxidase (reprinted with permission from [12]).

higher organisms, both heme *a* and heme a_3 are A-type hemes, which contain a formyl group at C8 and a lipophilic hydroxyethylfarnesyl side chain at C2 of the porphyrin ring. Both hemes are bound non-covalently to the protein.

Significance of the structures

All of the above information had been obtained before the crystal structures were known, by chemical analysis, amino acid sequence determination, hydropathy analysis and mutagenesis studies on microbial oxidases [9,10] and by the application of various spectroscopic techniques [11]. In many instances, the information available from spectroscopy concerning certain components of the protein is far more detailed and precise than that now furnished by the crystal structures, but these pieces of information were like lights in a foggy landscape. The structure of the entire molecule now provides the scaffold on which these isolated observations can be ordered. Experiments can now be designed to explore details of the function of the enzyme; spectroscopic approaches should be especially useful. It is particularly gratifying that, with very few exceptions, the information available from methods other than crystallography was confirmed by the structures. Even the Cu pair at the electron entrance site, which had no precedent in known protein structures, had previously been identified by spectroscopy [7].

The keys to success

How was the extraordinary feat of obtaining the crystal structure of cytochrome *c* oxidase achieved? The greatest hurdle was that of obtaining suitable crystals of this membrane protein. Persistence was the main ingredient; it took Shinya Yoshikawa [3] about 20 years to reach this goal with the beef-heart enzyme and Hartmut Michel [2] seven years with the *Paracoccus* oxidase. The vital step in crystallizing the beef-heart enzyme was to find the proper detergent to stabilize the enzyme molecule in aqueous solution, namely decylmaltoside, not dodecyl- or octylmaltoside, and not glucoside or other glycosides. Yoshikawa tried every commercially available non-ionic detergent before identifying this one! The right precipitating agent, polyoxyethylene 4000, and a high protein concentration (9 % w/v) were also important. With the *Paracoccus* enzyme the successful strategy was to produce a conformation-specific monoclonal antibody to the protein — a laborious and time-consuming procedure. The antibody covered enough of the hydrophobic surface of the protein to enhance its solubility and prevent aggregation. In a separate effort, the research group of Matti Saraste [12] constructed, by recombinant techniques, a soluble fragment of Su II from quinol oxidase containing a reconstructed Cu_A site. They started from the soluble periplasmic fragment of Su II of the *bo* quinol oxidase of *Escherichia coli*, which lacks the Cu_A site, and built into it a peptide loop containing a Cu-binding site based on the sequence found in cytochrome *c* oxidases. The resulting protein, an 11-stranded, mostly antiparallel β-sandwich, was purple and contained the typical mixed valence Cu_A site. The crystal structure of this fragment is now also available at a resolution of 2.3 Å. It shows somewhat higher resolution than the other structures, and confirms the findings made with the other enzymes.

The structures

The beef-heart enzyme, in its oxidized form without any tight-binding ligand, crystallized as a dimer. There was no indication of strong interaction between any metal site of one monomer with any metal site of the other. Of 1803 residues, 1763 have been assigned. The ten smaller subunits surround Su I–III on the outside of the assembly. Viewed along the plane of the membrane, the center of the protein is embedded in the membrane (48 Å of its total height), with parts of the molecule protruding into the matrix space (32 Å) and into the cytoplasm (37 Å). Details of the structures of Su IV–XIII are not yet published, and the authors have mainly concentrated on the overall shape of the molecule and on the immediate environments and mutual relationships of the metal sites [3]. In confirmation of previous analytical work [9], the beef-heart enzyme contains seven metal ions: two Fe, three Cu, one Mg, and one Zn (the bacterial enzyme

contains only six, lacking Zn, and the quinol oxidases contain only one Cu, that in the O_2 reduction site, called Cu_B). The heme molecules in Su I are both perpendicular to the membrane plane and are oriented toward each other — which had not been expected — at an angle of 104°. Su I also contains Cu_B, which, like the hemes, is located ~13 Å below the membrane surface.

Heme *a*, at the electron-acceptor site, has two axial His ligands, and is therefore of low spin, whereas heme a_3, at the O_2 binding site, has only one axial His ligand and is high spin. The iron atom of heme a_3 is ~ 1 Å out of the plane of the heme. The distance between this iron atom and Cu_B is 4.5 Å, and no bridging ligand has been observed. This has been a point of uncertainty for some years, as extended X-ray absorption fine structure (EXAFS) studies had indicated that a sulfur or Cl^- ligand might be present. It still seems possible, however, that a solvent-derived ligand may be present, which is not in a unique location and thus shows no discrete electron density. The *Paracoccus* enzyme was crystallized from an azide-containing solution; azide ions might bridge the two metal ions in this structure [2]. The magnetic properties of the heme a_3–Cu_B site [13] indicate that a bridging ligand would be required to produce the relatively strong antiferromagnetic interaction between heme a_3 and Cu_B at the observed distance (4.5 Å in the beef heart enzyme; 5.2 Å in that of *Paracoccus*).

Su II contains two transmembrane helices, which interact with the transmembrane helices of Su I. It also has a globular domain with β-sheet structure, which protrudes into the cytoplasmic space. This portion is clearly analogous to the Cu-binding site of blue Cu proteins. The cyt *c*-binding site is also located in this part of Su II, with the Cu pair (Cu_A) close to it. Each of the two Cu atoms is ligated by two Cys residues and one His residue (Fig. 1). Both of the Cu atoms have an additional ligand — either a methionine sulfur or a carbonyl from a Glu residue. The Cu atoms and the Cys sulfurs lie in one plane; the imidazole rings of the two histidines share a second plane, which is tilted by 15° with respect to the Cu–Cu axis [12]. The thiolate ligands (Cys) and the more distant additional ligands (Met and Glu) are in *trans* with respect to the Cu–Cu axis; the latter produce some asymmetry in the structure. Each Cu atom of the Cu_A site is coordinated in a distorted tetrahedral configuration. The Cu–Cu distance is 2.5 Å and the Cu–S distance is 2.2 Å [12]. According to electron paramagnetic resonance (EPR) studies on the beef-heart enzyme, however, the spin coupling between the two Cu atoms indicates nearly perfect symmetry and high covalency in this metal complex. This issue has recently been the subject of intense debate [14–17]. The features of this structure (Fig. 1) are probably best resolved in the soluble, reconstructed fragment from the quinol oxidase [12]; this shows clearly that there are four Cys–Cu bonds, made by two bridging Cys residues, in this structure. Some spectroscopic data, however, indicate that the Cu_A site in the reconstructed protein is somewhat less symmetrical or less homogeneous than in the native proteins.

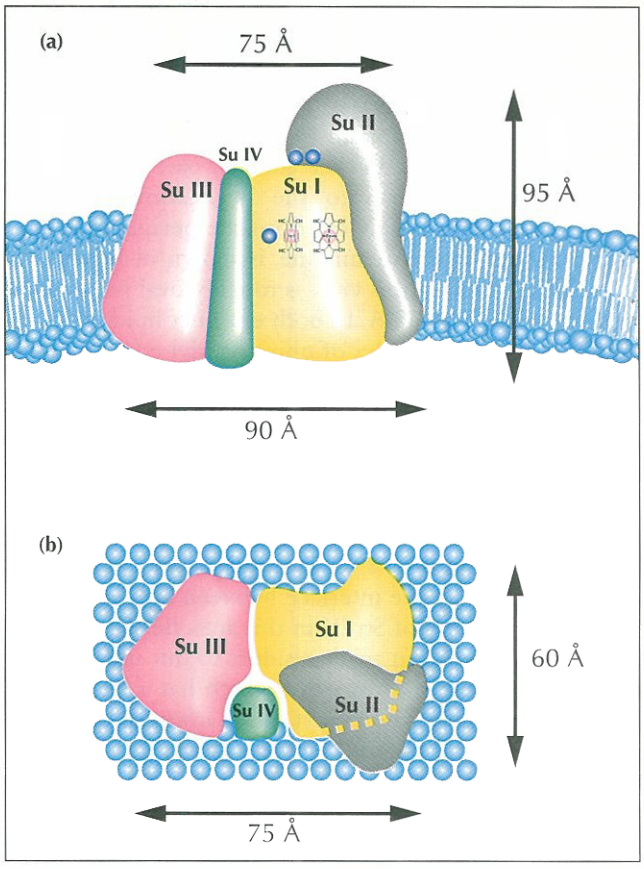

Fig. 2. Schematic drawings of cytochrome *c* oxidase from *Paracoccus*. (**a**) View of the enzyme through the membrane bilayer. The trapezoidal shape of the enzyme is shown. Su I contains 12 transmembrane helices, Su II contains 2, Su III contains 7 and Su IV has a single transmembrane helix. The binuclear Cu_A site (copper atoms depicted in dark blue) in Su II is shown. Su I contains the two heme molecules (heme *a*, left, and heme a_3, right) and the Cu_B site which forms a binuclear center with heme a_3. (**b**) Schematic drawing of the *Paracoccus* enzyme viewed from the periplasmic side of the plasma membrane showing the ovoid shape of the multisubunit complex. Adapted with permission from [2].

The single Zn atom present in the beef-heart enzyme is located on the matrix side of the enzyme in Su VIa and is ligated by four Cys residues. It is far removed from any other metal site and its function is not known. The Mg atom, however, is located at the interface of Su I and Su II, close to the probable path that electrons are thought to take from Cu_A to heme *a*, and may well be important in electron transfer.

The structure of the *Paracoccus* enzyme [2] is described in considerably more detail than the beef-heart enzyme [3]. Where comparison is possible, the essential features of the two enzymes are the same within the resolution achieved (2.8 Å). The major differences are in the crystal form of the protein; it occurs as a monomer in the crystal, with the antibody used for crystallization attached to the site where Su II protrudes from the membrane into the cytoplasm. All four subunits of the enzyme are shown in beautiful detail with 22 mainly

helical transmembrane structures. A view along the membrane plane shows the enzyme as trapezoidal because of tilting of the helices. The longer dimension (~90 Å) faces the cytoplasm (inside) and the shorter one (~75 Å) faces the periplasm (see Fig. 2a). The height of the trapezoid is ~55 Å and the globular part of Su II protrudes ~40 Å further into the periplasmic space. A schematic drawing of a view from the periplasmic side, perpendicular to the membrane, is shown in Figure 2b. The overall shape in this view is roughly oval with cross-sections of ~60 and 75 Å. In both enzymes lipid has been found — three molecules of phosphatidyl ethanolamine per monomer in Su III of the beef-heart enzyme, and electron density, which could be modeled as phosphatidylcholine, in a cleft between the helices of Su III of the *Paracoccus* enzyme.

Pathways of electron and proton transport

The cyt *c*-binding site is on the cytoplasmic face of the protein, formed by the interface between Su I and Su II. There is also a loop of Su III in this area. Between them, Su I–III contribute ten acidic residues to this site, offering a suitable docking site for cyt *c*, which has a lysine-rich patch on its surface. The closest metal atoms to the cytoplasmic side of the enzyme are those at the Cu_A site. It is therefore now generally thought that Cu_A is the entrance port of electrons from cyt *c* [11,18]. A Cu_A ligand (His224 of the *Paracoccus* Su II) might be involved in electron transfer to heme *a*, as it can interact with residues in Su I which in turn are in contact with the propionate groups of that heme. Three possible paths of electron transfer from heme *a* to heme a_3 in the beef-heart enzyme have been proposed by Iwata *et al.* [2]. Direct heme-to-heme transfer is one possibility, as the edges of the heme molecules are only 4.7 Å apart at their closest point.

Other interesting features with implications for the later steps in the electron-transport chain have been observed in the crystal structure of the *Paracoccus* enzyme [2,19]. A link between heme *a* and heme a_3 may be provided by one of the His ligands of heme *a*, which is located in the same helix as the axial His ligand for heme a_3, with only one residue separating them. A tyrosine residue, which may form an H-bond to the OH of the hydroxyethylfarnesyl side chain of heme a_3, is close to one of the three His ligands of Cu_B, suggesting a link between a_3 and Cu_B. In view of these relationships, which may provide electron transfer paths or conformational links, it was suggested that one may have to consider the metal site in Su I as a trinuclear center of heme *a*, heme a_3 and Cu_B [19].

Now that the structure of cytochrome *c* oxidase is known, the main emphasis will probably shift from electron-transfer paths to proton-transfer paths, which must be yet more complex. Iwata *et al* [2] devote a sizeable section of their paper to proposals for possible proton-transfer paths in the *Paracoccus* structure. Su I shows approximate three-fold symmetry, with its 12 helices arranged in three semicircular arcs. This structure has three pore-like features, which are blocked by heme a_3, aromatic residues, and the farnesyl side chain of heme *a*, respectively. A His residue located at what may be a pivotal point for proton transport does not show defined electron density for its side chain; it is thus possible that this residue may alternate between two conformations, as one might expect of a gating device.

On the basis of these and other observations, Iwata *et al.* propose paths both for the scalar protons (used in H_2O formation), which must eventually continue as pathways for H_2O, and also for the vectorial, 'pumped' protons that have to cross the entire membrane space. Their tentative proposal of a mechanism for the whole process integrates previously suggested mechanisms [20] with the new information gained from the crystal structure. The authors are well aware, however, that there may be other reasons for some of their observations. They also point out — and this is well known to most who have dealt with this enzyme — that cytochrome oxidase has been found by spectroscopy to occur in many different states, depending on conditions (e.g., the history of the preparation or mode of storage, ionic environment, pH, oxidation state of the individual metal sites and presence of dissolved gases). Thus, the details of the structure may vary depending on such conditions and, of course, on the conditions of crystallization. There are bound to be further inquiries along these lines, but they may be limited by the need for suitable crystals. This, however, should not detract from the impressive accomplishment of all those who have contributed to giving us an exciting picture of this complicated assembly of proteins. The intense interest provoked in the scientific community is evident in the number of comments [19,21–23] — such as the present one — written by colleagues who are or have been close to the field of cytochrome oxidase research. They are likely, according to their specific interests, to emphasize and comment on varied aspects of the progress that this work has brought, thus providing a wider perspective of the achievement. Those who do not have the time or the stamina to wade through the small print of the original accounts may want to take advantage of the different points of view provided by such comments.

Acknowledgements: I thank Drs Harmut Michel and Matthias Wilmanns for permission to adapt or use illustrations from their publications [2,12], and Dr Robert A. Scott for comments on an earlier draft of this manuscript and for a preprint of [22].

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