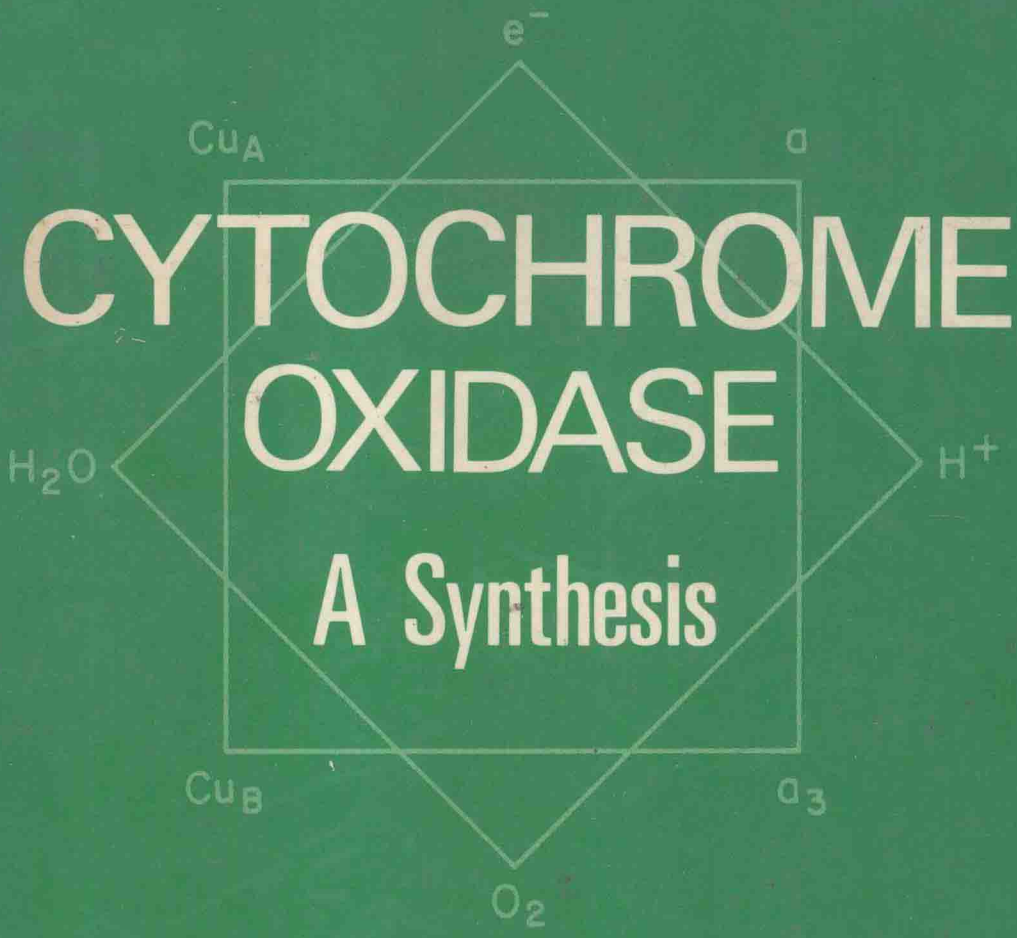

CYTOCHROME OXIDASE

A Synthesis

Mårten Wikström Klaas Krab
Matti Saraste

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Cytochrome Oxidase
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Preface

The intention in this book is to provide a current, integrated and fairly comprehensive insight into the structure and function of cytochrome oxidase, which is, if not the most important, at least one of the most intriguing enzymes of aerobic metabolism. The present work is not a review of the voluminous literature on this subject, at least not in the usual sense. We have called our approach a synthetic one to emphasize our attempt to bring together both temporally and methodologically different research material and concepts, and to construct from this a single picture of this enzyme. Admittedly, our success in this respect is limited. It is clear that there are still considerable gaps of information, which require much future work before they can be filled. However, we have attempted to bridge many of these gaps with working hypotheses and models, which is why part of the presented material is speculative and should be viewed as such. Yet we hope that the book may prove useful also as a source of references. We apologize for the possibility that our approach may have emphasized some research data at the expense of other data of equal or even more importance. However, this is the inevitable price that must be paid in attempting a synthesis, showing, if that price is high, the authors' inadequate judgement.

With the exception of Chapters 1 and 2, which describe the scope of our work and give a bird's eye view on the subject, respectively, the book is best read in the numbered chapter sequence.

During the process of writing we have been aided by a great number of colleagues in the form of criticism, access to unpublished data, and discussions. We are particularly indebted to Drs A. Azzi, G. Babcock, C. H. Barlow, G. Buse, R. Capaldi, E. Carafoli, R. P. Casey, B. Chance, M. Clore, K. De Fonseka, L. Ernster, R. Henderson, P. Hinkle, W. J. Ingledew, D. Kell, A. A. Konstantinov, J. S. Leigh, Jr, B. Ludwig, M. W. Makinen, B. G. Malmström, B. D. Nelson, T. Ohnishi, T. Penttilä, R. O. Poyton, G. Schatz, E. Sigel, G. J. Steffens, R. J. P. Williams and E. Yang, whose invaluable help is gratefully acknowledged. In addition, several col-

leagues have provided us with permission to reproduce figures from their original work, which is acknowledged separately in the appropriate context.

We are also grateful for the understanding and sympathetic attitude of Arthur Bourne of Academic Press during the prolonged stages of writing the manuscript. Ms Marja Immonen, Ms Sirkka Rönnholm and Ms Hilkka Vuorenmaa have given us invaluable help in preparation of the manuscript. Our research group has been supported by grants from the Sigrid Jusélius Foundation and the Finnish Academy (Medical Research Council) during preparation of this book, K. K. was supported by a post-doctoral fellowship from EMBO, and by a travel fellowship from the Sigrid Jusélius Foundation.

Helsinki, May 1981

Mårten Wikström
Klaas Krab
Matti Saraste

It is far better to foresee even without certainty than not to foresee at all
(Henri Poincaré)

I keep the subject constantly before me and wait till the first dawnings open little by little into the full light
(Sir Isaac Newton)

Everything should be made as simple as possible but not simpler
(Albert Einstein)

On to a bridge
Suspended over a precipice
Clings an ivy vine
Body and soul together
(Bashō)

Contents

1
Scope

Modern research on cytochrome oxidase is multidisciplinary to say the least. Due to its central position in the energy metabolism of all respiratory organisms, this enzyme has attracted much interest in the fields of bioenergetics and energy metabolism. Its function as an oxidoreductase and as the principal O_2-reducing enzyme makes it an interesting subject in the fields of biological electron transport and oxygen activation mechanisms. As an integral protein in the inner mitochondrial membrane that is assembled from several polypeptide chains, cytochrome oxidase has attracted scientists in the fields of protein structure and topography. The fact that part of the cytochrome oxidase protein is coded for by the mitochondrial genome and synthesized on mitochondrial ribosomes, while part follows the more familiar nuclear-cytoplasmic route, has stimulated much research in the fields of genetics and protein biosynthesis, as well as in mitochondrial biogenesis. The presence of four different redox centres in the cytochrome oxidase molecule (two haems and two coppers) has traditionally interested haemo- and cuproprotein chemists as well as physicists due to the applicability of a variety of spectroscopic and other physical techniques to unravelling the structure of these centres. On top of this truly multidisciplinary attack, research in the cytochrome oxidase field has traditionally been divided into research on the isolated and purified enzyme in detergent solution on one hand, and on the membranous oxidase in mitochondria on the other.

From this it is clear that the cytochrome oxidase literature is not only voluminous, but that research has been and still is conducted from a great variety of angles, all of which require a certain degree of specialization. One of the greatest problems is, in our view, the almost total lack of co-ordination of the different approaches. For instance, it is not uncommon that kineticists studying the purified and solubilized enzyme are ignorant of functional characteristics observed only with the membranous enzyme. Analogously, students of mitochondrial energy conservation are often not sufficiently initiated in the kinetic and catalytic properties of the enzyme. These are but a few examples of a situation which has rather obvious causes, and which is by no means uncommon to modern experimental science in general.

Although lack of detailed knowledge from neighbouring disciplines

might not be a hindrance for scientific development up to a certain level, "cross-information" is as a rule essential for further progress both conceptually and experimentally. If an interdisciplinary approach is not taken in time, there is a certain danger of stagnation in the development due to "saturation" with experimental detail.

Our feeling that this situation may be imminent in the research on cytochrome oxidase gave us the first motive to write this book. The voluminous and multifaceted literature on this enzyme has also, in our opinion, prevented researchers from drawing connections on a temporal scale. Modern intense studies, often with sophisticated new techniques, have come into the foreground, but this has sometimes happened at the expense of very useful information gathered some 10–20 years ago, or earlier.

We have called our approach in producing this book a synthetic one. By this we do not mean to claim that we have succeeded in incorporating every piece of experimental information into a singular picture, but synthesis certainly describes the general thrust and direction of our endeavour, which are encapsulated by the first three quotations on page vii. The last quotation beautifully describes our ultimate goal. It is clear, however, that this has not been achieved. Much more information will be required than is presently available to describe the structure and function of cytochrome oxidase in molecular detail so that all the information clings together in perfect harmony. But we hope that our approach might provide a stimulus for more integrated research on this enzyme in the future.

We would like to persuade the reader that the kind of approach taken is often associated with unforeseen and sometimes delightful discoveries, similar to finding a missing piece in a jigsaw puzzle. However, more important than such delights is the fact that such discoveries can and should be put to test by experiment. Experiments that have been suggested in this way are almost unique in the sense that they would not have been designed without the unifying model. It is, perhaps, mainly for this reason that we concur so wholeheartedly with Poincaré, whose statement introduces this book. The danger of "foreseeing" structure and function in terms of models and theories is, we think, compensated for by the secure settlement that can be reached by experimental test. On the other hand, the process of "foreseeing", even at the risk of failure, is not compensated for by anything. This has been our second main motivation for the approach taken in this book.

2
Introduction and general orientation

Cytochrome oxidase is the oxygen-activating enzyme of cellular respiration in eukaryotes (animal, plant and yeast cells) as well as in certain prokaryotes. In the former the enzyme is located in the inner mitochondrial membrane, and in the latter organisms it is part of the cell membrane. Cytochrome oxidase enables these cells to oxidize foodstuffs using molecular oxygen by catalysing electron transfer from cytochrome *c* to O_2.

The utilization of O_2 as the terminal oxidant by all higher forms of life has probably contributed greatly to evolution due to the large energetic advantages over other available oxidants. The essential nature of cytochrome oxidase may be exemplified by the fact that it is probably responsible for more than 90% of the O_2 consumption by living organisms on Earth. The very critical dependence of vital organs such as brain, heart muscle and kidney on aerobic metabolism is another facet of this enzyme's central position in physiology. As succinctly stated by Lemberg (1969) in his already classical review on cytochrome oxidase,

> "the general significance of cytochrome oxidase thus greatly exceeds that of haemoglobin, its much studied and much more completely known chemical relative. Biologically, haemoglobin is only an auxiliary of the process of cell respiration in that it carries the oxygen into the tissues via the bloodstream. This is necessary only in bulky animals, in which diffusion of oxygen from the surface or from a tracheal system is insufficient."

The history of cytochrome oxidase research covers the entire period of modern biochemical research (Table 2.1). Due to the limited space available we cannot give a full historical account here. Such an account may also be unnecessary in view of the eloquent historical reviews available (Slater *et al.*, 1965; Keilin, 1966; Lemberg, 1969; Nicholls and Chance, 1974; Florkin, 1975). Here we limit ourselves to a brief chronological list of "classical" discoveries on which much of our present basic knowledge rests (Table 2.1).

In the following sections we will present a condensed orientation and survey of cytochrome oxidase as it is known today. This is to aid readers who may not be familiar with this enzyme and the different aspects of its study. To save space, most sections include only a minimum number of

Table 2.1 Chronological list of classical events in the research on cytochrome oxidase.

1884–87	McMunn reported on the four-banded spectrum of histo- or myohaematin in several tissues.
1924	Warburg proposed that cellular oxygen consumption is catalysed by an iron-containing enzyme, *der Atmungsferment*, ferric iron being reduced by foodstuffs and reoxidized by oxygen.
1925	Keilin rediscovered McMunn's pigments and identified them as three species, the *cytochromes a*, *b* and *c*.
1926–33	Warburg *et al.* showed that a haem-containing enzyme is essential for cellular respiration using cyanide and CO as respiratory inhibitors. The photosensitivity of CO inhibition was used to obtain the "photochemical action spectrum" of *der Atmungsferment*. Keilin considered an oxidase separate from the cytochromes, which he suggested might be a copper enzyme.
1929	Dixon proposed the name cytochrome oxidase.
1938	Keilin and Hartree demonstrated the essential role of cytochrome *c* as electron donor to the terminal oxidase, which they called cytochrome *c* oxidase.
1939	Keilin and Hartree showed using their microspectroscope and with the aid of several inhibitors that their previous "cytochrome *a*" was, in fact, composed of two different species, only one of which (called cytochrome a_3) reacted with ligands. The remainder of the original "cytochrome *a*" retained this name.
1953	Chance *et al.* showed that the CO–ferrocytochrome a_3 is photodissociable with a dissociation spectrum identical to Warburg's "photochemical action spectrum". This was the final proof for the co-identity of *der Atmungsferment* and cytochrome a_3.
1954	Maley and Lardy and Lehninger showed that oxidation of cytochrome *c* by O_2 is coupled to oxidative phosphorylation.
1958–61	Okunuki *et al.*, Hatefi *et al.* and Griffiths and Wharton developed the methods for isolation and purification of cytochrome oxidase (which by now was the name for the cytochrome aa_3 entity).
1959–60	Although the presence of copper had previously been noted by several groups, Sands and Beinert provided the first proof for its functional role in cytochrome oxidase.

references. Only Section III.C is different in this regard since basic aspects of the proton pump are discussed, which will not be dealt with any further in subsequent chapters. Relevant information with complete quotations on material presented in this chapter, and indeed in the whole book, may be obtained from one or several of the following review articles or symposium volumes: Falk *et al.* (1961), King *et al.* (1965, 1979), Lemberg (1969), Malmström (1973, 1979), Nicholls and Chance (1974), Caughey *et al.* (1976), Capaldi and Briggs (1976), Wikström *et al.* (1976, 1981), Dutton *et al.* (1978), Erecińska and Wilson (1978), Azzi and Casey (1979), Wikström and Krab (1979*a*), Azzi (1980).

I. Metal centres

A. Nomenclature and chemistry

Cytochrome oxidase (ferrocytochrome *c* : O_2 oxidoreductase; EC 1.9.3.1), also called cytochrome *c* oxidase (sometimes cytochrome aa_3), contains two haem groups and two protein-bound copper ions per minimum catalytic unit, i.e. the aa_3 monomer. On extraction of the non-covalently bound haem from the protein, only haem A is found (Fig. 2.1). Typical features of haem A are the carbonyl group in position 8 and the long isoprenoid chain in position 2 of the porphyrin ring. Haem iron may be further liganded by two (fifth and sixth) co-ordination bonds in the axial direction, perpendicular to the plane of the ring. The haem is a planar disk with a side *c*. 8.5 Å long and *c*. 4.5 Å thick.

It is established that the two haems A of the monomer are *a priori* in very different environments. Thus the terms haems *a* and a_3 are clearly motivated. It is possible that the two haem groups are attached to different polypeptide chains. Although this has not been established unequivocally, "cytochromes *a* and a_3" is a very commonly used terminology. The main difference between the haems *a* and a_3 is that the latter is usually of high

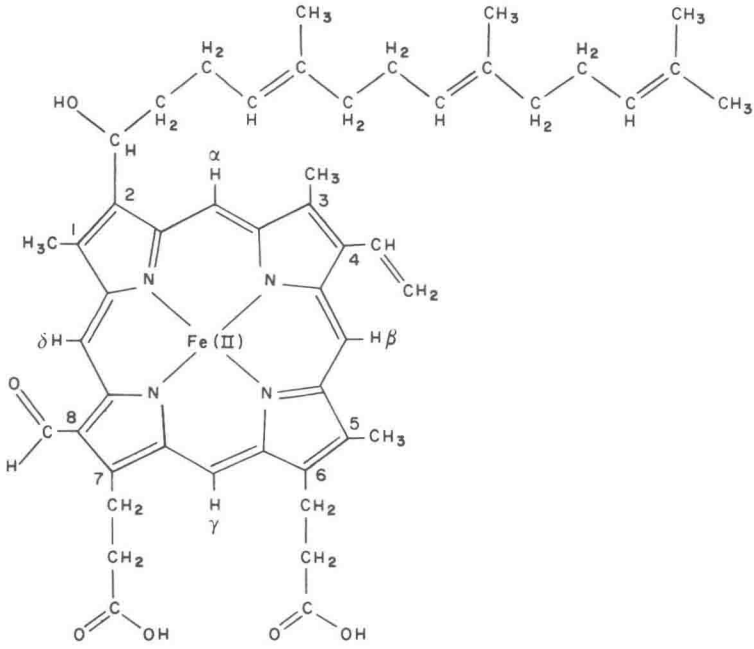

Fig. 2.1 The structure of haem A.

spin and reacts with various ligands, whereas the former is usually of low spin and does not. In fact, this is the classical definition of cytochromes *a* and a_3, of which the latter binds O_2, CO, etc., in the ferrous state, and HCN, HN_3, H_2S, etc., in the ferric state (Keilin and Hartree, 1939). These ligands are bound to the sixth axial position of haem a_3.

Similarly to the haems, the two copper atoms are also in very different environments. This is revealed mainly by spectroscopic and magnetochemical studies. The two copper atoms have been named in a variety of ways in the literature, e.g. Cu_{vis} and Cu_{invis} on the basis of "visibility" and "invisibility" by EPR spectroscopy, or Cu_a and Cu_{a3} on the basis of their assumed functional and structural associations to the two haems. In this book we will use the more neutral terms Cu_A and Cu_B, of which the former is the easily EPR-detectable copper, which appears to be in rapid redox equilibrium with haem *a*. Cu_B is the usually EPR-indetectable copper, which is in close functional and physical contact with the haem of cytochrome a_3. The co-ordination of the two coppers is largely unknown, although some proposals have been made on the basis of EPR spectroscopy and sequence data.

B. Spectroscopy

Various spectroscopic methods have proved very useful in studies of cytochrome oxidase. The most commonly used method is optical spectrophotometry, by which oxidoreduction of the haems, in particular, may be monitored. Figure 2.2 shows the absolute optical spectra of reduced and oxidized cytochrome oxidase. In addition to the bands shown, fully oxidized oxidase exhibits a band at 820–840 nm (about 2 mM^{-1} cm^{-1} per aa_3 unit), which to at least 85% is due to Cu_A^{II} (Wharton and Tzagoloff, 1964; Boelens and Wever, 1980; Beinert *et al.*, 1980). The fully oxidized enzyme also shows a weak band at 655 nm, which has been attributed to ferric haem a_3 in its particular linkage with Cu_B.

All bands in Fig. 2.2 are attributable to haem transitions. Interpretation of these spectra in terms of cytochromes *a* and a_3 has been the subject of much controversy and ambiguity (see Chapter 4). However, there is presently strong evidence in favour of the original proposal (Keilin and Hartree, 1939) that the 605 nm band of the reduced enzyme is mainly due to ferrous haem *a*, whereas the band at 445 nm is due to both *a* and a_3 in roughly equal proportions.

The EPR spectrum of oxidized "resting" cytochrome oxidase, as isolated, is shown in Fig. 2.3(a). It reveals only two clearly defined components, viz. a low spin ferric haem with resonances centred at *c*. $g = 3, g = 2$ and $g = 1.5$, and a signal with $g = 2$, which is attributed to Cu_A^{II}. Quanti-

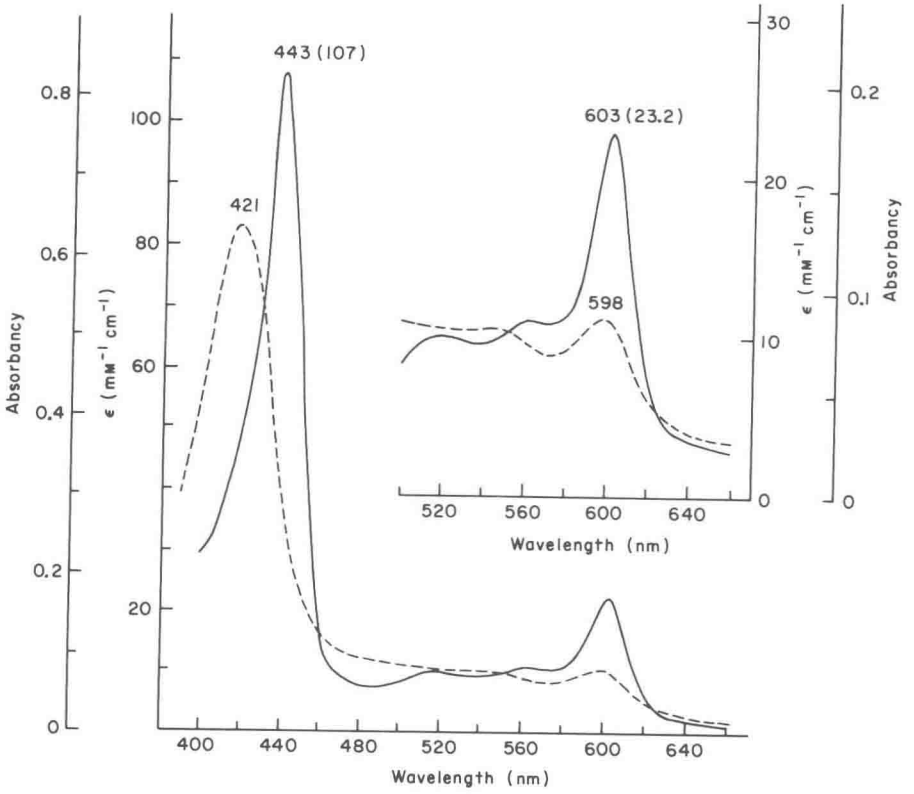

Fig. 2.2 Absolute spectra of fully reduced (——) and fully oxidized ("resting") (----) cytochrome oxidase. Extinction coefficients are on a haem A basis (should be multiplied by two to get the extinction on an aa_3 basis). From Vanneste (1966) with permission.

tation of the EPR signals reveals that the low spin haem represents only some 50% of the total haem present, and that the $g = 2$ signal due to copper accounts for only 40% of the copper that is intrinsic to the enzyme. Extraneous copper with well defined EPR characteristics is often associated to the isolated enzyme, but may be removed by dialysis against EDTA.

As shown in Fig. 2.3(b), the EPR spectrum changes dramatically on partial reduction. The low spin haem resonances disappear and are replaced by high spin ferric haem signals in the $g = 6$ region. All EPR resonances disappear on full reduction of the enzyme.

Also the EPR data have been difficult to interpret in terms of assigning