

CYTOCHROME OXIDASE

A Synthesis

Mårten Wikström Klaas Krab
Matti Saraste



ACADEMIC PRESS

A Subsidiary of Harcourt Brace Jovanovich, Publishers
London New York Toronto Sydney San Francisco

2 /

Cytochrome Oxidase

A Synthesis /

① MÅRTEN WIKSTRÖM, ^{et al} KLAAS KRAB
and MATTI SARASTE

University of Helsinki, Finland

1981



ACADEMIC PRESS

A Subsidiary of Harcourt Brace Jovanovich, Publishers

London • New York • Toronto • Sydney • San Francisco

ACADEMIC PRESS INC. (LONDON) LTD
24-28 Oval Road
London NW1

United States Edition published by
ACADEMIC PRESS INC.
111 Fifth Avenue
New York, New York 10003

Copyright © 1981 by
ACADEMIC PRESS INC. (LONDON) LTD

All Rights Reserved

No part of this book may be reproduced in any form by photostat, microfilm, or any other means, without written permission from the publishers

British Library Cataloguing in Publication Data

Wikström, M.
Cytochrome oxidase.
I. Cytochrome oxidase
I. Title II. Krab, K III. Saraste, M.
574.19'258 QP603.C85

ISBN 0-12-752020-1
LCCCN 81-67888

Typeset by Preface Ltd., Salisbury, Wilts., and printed in Great Britain by
Thomson Litho Ltd., East Kilbride, Scotland

Cytochrome Oxidase
A Synthesis

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 Fonseca, 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 Rönholm and Ms Hilikka 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 dawns 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

Preface, v

Chapter 1. Scope, 1

Chapter 2. Introduction and general orientation, 3

- I. Metal centres, 5
 - A. Nomenclature and chemistry, 5
 - B. Spectroscopy, 6
 - C. Oxidoreduction properties, 8
- II. Structure and organization in the mitochondrial membrane, 9
- III. Energetics and catalytic functions, 10
 - A. Basic energetic considerations, 10
 - B. Basic features of electron transfer and O_2 reduction, 11
 - C. Generation of the electrochemical proton gradient, 11

Chapter 3. Structure and topography, 14

- I. Introduction, 14
- II. Purification of cytochrome oxidase, 14
 - A. Methods of purification, 15
 - B. Some pitfalls of purification, 17
- III. The subunits of cytochrome oxidase, 23
 - A. Nomenclature and number of different subunits, 24
 - B. The stoichiometry of the subunits, 27
 - C. The chemistry of the subunits in different organisms, 32
 - D. Binding of prosthetic groups and other functions of subunits, 34
- IV. Quaternary structure of cytochrome oxidase, 38
 - A. Minimal molecular weight: the haem aa_3 unit mass, 38
 - B. The size of the functional unit, 41
 - C. Resolution of cytochrome oxidase into subdomains, 43
- V. Structural arrangement of cytochrome oxidase in the membrane, 44
 - A. Subunit relationships in the isolated enzyme, 44
 - B. Subunit topography in the membranous enzyme, 46
 - C. The binding of cytochrome *c*, 48
 - D. Three-dimensional structure, 49
- VI. Interaction with phospholipids and enzyme mobility, 51
 - A. Effect of phospholipid on enzymic activity, 51
 - B. Mobility of cytochrome oxidase in the membrane, 52
- VII. Conclusion, 53

Chapter 4. Physical properties, configuration and topography of the redox centres, 55

- I. Introduction, 55
- II. Structure and physical properties of the redox centres, 56

- A. The $g = 3$ EPR signal and cytochrome a , 56
- B. The haem a_3/Cu_B centre, 57
- C. Spectroscopy and configuration of the coppers, 60
- D. The reduced enzyme, 61
- E. Further information from Mössbauer and resonance Raman spectroscopy, 62
- III. Transient forms of the fully and partially oxidized enzyme, 63
 - A. "Oxygenated" and "pulsed" oxidase, 63
 - B. Transient states exhibiting EPR signals from haem a_3 , 65
- IV. The binding of ligands to cytochrome oxidase, 67
 - A. Ligand binding to partially and fully oxidized enzyme, 67
 - B. The effect of NO on oxidized cytochrome oxidase, 70
 - C. Ligand binding to the reduced enzyme, 72
- V. Haem/haem interactions and interpretation of optical spectra, 74
 - A. Optical spectra of cytochromes a and a_3 , 75
 - B. CO dissociation and the interpretation of optical spectra, 77
 - C. Other evidence for haem/haem interaction, 80
- VI. Topography of the redox centres, 83
- VII. Conclusion, 86

Chapter 5. Oxidoreduction properties of the redox centres, 88

- I. Introduction, 88
- II. Redox titrations and their interpretation, 88
 - A. The requirement of redox equilibrium, 88
 - B. Redox potential dependence of EPR resonances at equilibrium, 91
 - C. Redox potential dependence and interpretation of optical spectra, 91
 - D. The redox properties of Cu_B , 93
 - E. The basic models for interpretation of EPR spectroscopy, 94
- III. Analysis of EPR data according to the neoclassical model, 96
 - A. The assignment of EPR signals, 97
 - B. The notion of a high spin form of haem a , 99
 - C. Simulation, 100
- IV. The effect of ligands on the redox properties of the haems, 102
 - A. General remarks, 102
 - B. Method for calculation and simulation, 103
 - C. Titrations of the 605 nm band in the presence of azide, 106
 - D. Titrations of the EPR signals in the presence of azide, 108
 - E. Carbon monoxide, 109
 - F. Cyanide, 109
- V. pH dependence of the midpoint potentials of the haems, 111
 - A. The involvement of protons, 111
 - B. Simulation, 111
 - C. Significance of the pK values found, 113
- VI. Conclusion, 115

Chapter 6. Kinetics and catalytic mechanism, 117

- I. Introduction, 117
- II. On the reduction of dioxygen to water, 117
- III. Kinetics of the reaction with O_2 , 119
- IV. Intermediate steps in the reduction of dioxygen, 121
 - A. The "oxy" species or Compound A, 121

- B. The “peroxy” intermediate and Compound C, 124
- C. The nature of Compound B, 129
- D. The intermediates beyond Compound B, 131
- V. Tentative catalytic mechanism of O_2 reduction, 133
- VI. Reduction of cytochrome oxidase by cytochrome *c*, 135
 - A. Electron transfer between cytochromes *c* and *a*, 135
 - B. Steady state kinetics and mechanism of the cytochrome *c* reaction, 137
 - C. “Resting” and “ O_2 -pulsed” states of the enzyme, 139
 - D. Electron transfer sequence, 140
- VII. Conclusion, 141

Chapter 7. Electron transfer and energy transduction, 142

- I. Molecular aspects of redox-linked proton translocation, 143
 - A. General principles, 143
 - B. Basic elements and properties of a redox-linked proton pump, 143
 - C. A general model of redox-linked proton translocation, 144
 - D. Shifts in E_m and pK and kinetic performance of the pump, 147
 - E. Proton-conducting channels, 148
 - F. Experimental predictions from the model, 148
- II. The proton pump of cytochrome oxidase, 149
 - A. Identification of the redox element, 149
 - B. The two states of cytochrome *a*, 150
 - C. Sidedness of the redox-linked acid/base groups, 152
 - D. A possible role of subunit III in proton translocation, 152
 - E. The role of the haem a_3/Cu_B centre in energy transduction, 154
- III. Reversed electron flow and other energy-dependent phenomena, 155
 - A. Partial reversal of the O_2 reaction, 155
 - B. The spectral shift in ferrocycytochrome *a*, 157
 - C. Energy-dependent changes in measured E_m values, 158
- IV. A reciprocating site mechanism, 159
 - A. Definition of the reciprocating site mechanism, 161
 - B. Evidence for and against dimeric functioning of cytochrome oxidase, 161
 - C. The nature of the monomer/monomer interactions, 163
 - D. “Resting” and “pulsed” states of the dimer, 166
 - E. On the role of Cu_A in cytochrome oxidase, 167
 - F. Structural implications and general significance, 168
- V. Conclusions, 170

Epilogue: Why a proton pump? 171

References, 174

Notes added in proof, 190

Index, 192

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, <i>der Atmungsferment</i> , ferric iron being reduced by foodstuffs and reoxidized by oxygen.
1925	Keilin rediscovered McMunn's pigments and identified them as three species, the <i>cytochromes a</i> , <i>b</i> and <i>c</i> .
1926–33	Warburg <i>et al.</i> 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 <i>der Atmungsferment</i> . 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 <i>c</i> as electron donor to the terminal oxidase, which they called cytochrome <i>c</i> oxidase.
1939	Keilin and Hartree showed using their microspectroscope and with the aid of several inhibitors that their previous "cytochrome <i>a</i> " was, in fact, composed of two different species, only one of which (called cytochrome <i>a</i> ₃) reacted with ligands. The remainder of the original "cytochrome <i>a</i> " retained this name.
1953	Chance <i>et al.</i> showed that the CO-ferrocycytochrome <i>a</i> ₃ is photo-dissociable with a dissociation spectrum identical to Warburg's "photochemical action spectrum". This was the final proof for the co-identity of <i>der Atmungsferment</i> and cytochrome <i>a</i> ₃ .
1954	Maley and Lardy and Lehninger showed that oxidation of cytochrome <i>c</i> by O ₂ is coupled to oxidative phosphorylation.
1958–61	Okunuki <i>et al.</i> , Hatefi <i>et al.</i> and Griffiths and Wharton developed the methods for isolation and purification of cytochrome oxidase (which by now was the name for the cytochrome <i>aa</i> 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 (1979a), Azzi (1980).

I. Metal centres

A. Nomenclature and chemistry

Cytochrome oxidase (ferrocytochrome *c* : O₂ 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

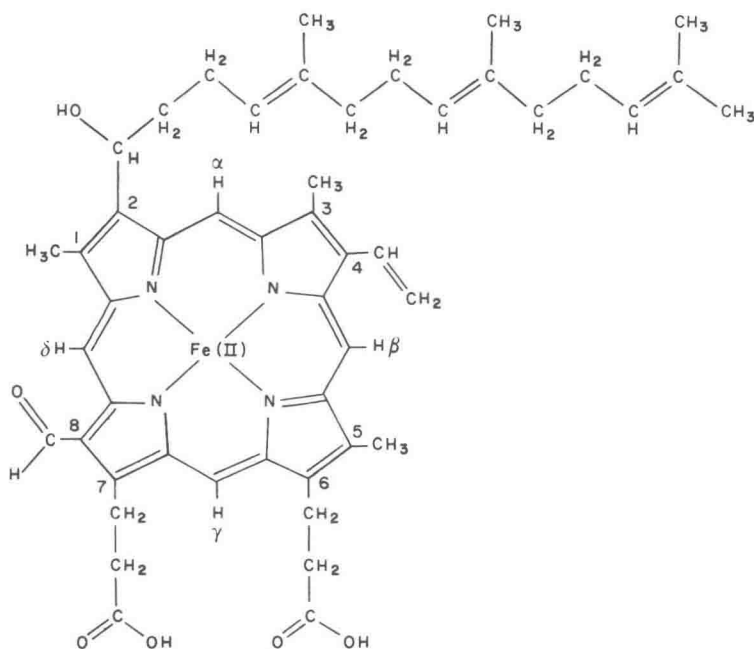


Fig. 2.1 The structure of haem A.

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 O₂, CO, etc., in the ferrous state, and HCN, HN₃, H₂S, 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-

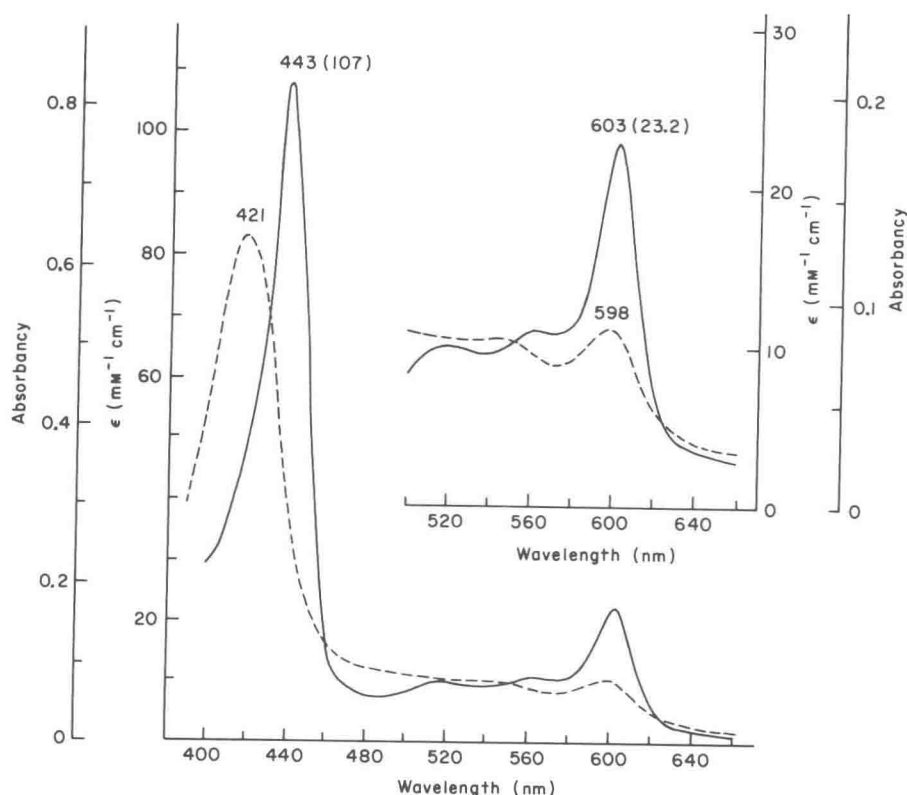


Fig. 2.2 Absolute spectra of fully reduced (—) and fully oxidized ("resting") (----) cytochrome oxidase. Extinction coefficients are on a haem A basis (should be multiplied by two to get the extinction on an aa_3 basis). From Vanneste (1966) with permission.

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