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Graham W. Pettigrew
Geoffrey R. Moore

Cytochromes c

Biological Aspects

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With 68 Figures

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Series Preface

During the past few decades we have witnessed an era of remarkable growth in the field of molecular biology. In 1950 very little was known of the chemical constitution of biological systems, the manner in which information was transmitted from one organism to another, or the extent to which the chemical basis of life is unified. The picture today is dramatically different. We have an almost bewildering variety of information detailing many different aspects of life at the molecular level. These great advances have brought with them some breath-taking insights into the molecular mechanisms used by nature for replicating, distributing and modifying biological information. We have learned a great deal about the chemical and physical nature of the macromolecular nucleic acids and proteins, and the manner in which carbohydrates, lipids and smaller molecules work together to provide the molecular setting of living systems. It might be said that these few decades have replaced a near vacuum of information with a very large surplus.

It is in the context of this flood of information that this series of monographs on molecular biology has been organized. The idea is to bring together in one place, between the covers of one book, a concise assessment of the state of the subject in a well-defined field. This will enable the reader to get a sense of historical perspective – what is known about the field today – and a description of the frontiers of research where our knowledge is increasing steadily. These monographs are designed to educate, perhaps to entertain, certainly to provide perspective on the growth and development of a field of science which has now come to occupy a central place in all biological studies.

The information in this series has value in several perspectives. It provides for a growth in our fundamental understanding of nature and the manner in which living processes utilize chemical materials to carry out a variety of activities. This information is also used in more applied areas. It promises to have a significant impact in the biomedical field where an understanding of disease processes at the molecular level may be the capstone which ultimately holds together the arch of clinical research and medical therapy. More recently in the field of biotechnology, there is

another type of growth in which this science can be used with many practical consequences and benefit in a variety of fields ranging from agriculture and chemical manufacture to the production of scarce biological compounds for a variety of applications.

This field of science is young in years, but it has already become a mature discipline. These monographs are meant to clarify segments of this field for the readers.

Cambridge, Massachusetts

ALEXANDER RICH
Series Editor

Foreword

It is no easy task to bring order into the inchoate mass of current data relating to structure and function of cytochromes c. A vast literature has accumulated in the last few decades consequent on the application of new methodologies based on improved X-ray diffraction procedures and ever more increasingly sophisticated versions of a great variety of spectroscopies (NMR, EPR, infrared, EXAFS, laser Raman, etc.). Understandably, this bewilders the novice and often proves baffling to the expert who ventures outside an area of specialisation. It is fortunate that there are some willing and competent to accept the challenge to produce a text which can serve as an adequate guide. None are better equipped, by virtue of familiarity with the older knowledge, and personal experience as contributors to the new, than the authors of the present text. They have produced an all-inclusive coverage of relevant data, and – more important – thoughtful summaries of current knowledge which provide a basis for not only a better understanding of cytochrome c function but also for reasonable assessment of results of future research. Thus the text is not merely a compilation of data but an integrated presentation of findings evaluated as to reliability and significance. There will be some effort involved in reading the text but it will be well rewarded as an increasing comprehension of the material is attained.

Investigations on cytochrome c structure and function through the years have reflected accurately the general history of protein research. The quarter century, which began in the 1920s with David Keilin's epochal findings, can be considered a classical era centered mainly on the nature of cytochrome c in eukaryotic mitochondrial systems. Transition to a comparative biochemistry of cytochromes c began in the 1950s with attention paid to non-mitochondrial systems. The explosive development of physical biochemistry and molecular biology produced a flood of new information bringing promise of better perceptions of structural bases for the function of cytochrome c and heightened appreciation of the many mechanisms made possible by these heme proteins in supporting their general function as mediators of coupled electron transfer in both prokarytic and eukaryotic bioenergetic

transduction. Thus, the appearance of this text is most timely. It meets the urgent need of researchers and students at all levels of sophistication for an adequate aid in assessing progress toward an eventual understanding of how cytochromes c function.

M. D. KAMEN

Preface

Cytochromes c play a central role in biological electron transport systems and, because of their small size and their stability, have been a popular subject for study in the general areas of protein chemistry and redox reactions. Our knowledge of cytochrome c has advanced through the contributions of disciplines as different as, for example, physical chemistry and microbial physiology. A comprehensive review must therefore deal with this diversity of interest and be intelligible to the chemist and biologist alike. To achieve such a synthesis is a daunting task but we feel it is an important one in providing a basis for developing research.

This book is divided into two volumes which correspond roughly to a biologist's view of cytochrome c and a chemist's view. However, this division was largely dictated by considerations of size and the two volumes should be seen as a single review. The book is directed at the graduate student and the research worker. To this end, it is detailed in its treatment of the recent research literature and incorporates much methodological material. However, it is our hope that within the complexity of cytochrome c, we have found unifying themes which will be of interest to the more general reader.

We would like to thank all our colleagues who critically read sections of the manuscript and Maxine Pettigrew who helped in its preparation.

GRAHAM W. PETTIGREW
GEOFFREY R. MOORE

Contents

Chapter 3 The Function of Bacterial and Photosynthetic Cytochromes c

Chapter 4 The Biosynthesis of Cytochrome c

Chapter 1 Resolution, Characterisation and Classification of c-Type Cytochromes

Functional studies of cytochromes fall into one of two groups. In the first, the intact respiratory system is examined using methods which allow the resolution of individual components. In the second, components are isolated and characterised and their role studied in reconstituted partial systems. Relatively few studies incorporate both approaches and we suggest that this has led to problems in the interpretation of results.

A good example is the investigation of the role of cytochrome c_2 in bacterial photosynthesis (Chap. 3). Models of electron transport were proposed based on the light-induced absorbance changes in the intact photosynthetic system assuming that cytochrome c_2 was the single c-type cytochrome present. Only with the demonstration by Wood (1980) of the presence of a cytochrome c_1 could the kinetic results of the whole system be correctly re-interpreted. Conversely, studies on isolated components without reference to the intact system can be misleading. Thus the study of electron transfer between purified cytochrome c_3 and ferredoxin of the sulfidogenic bacteria has no physiological relevance because the two proteins are separated by the cell membrane and cannot interact in vivo (Chap. 3).

In the following we discuss methods for resolution of the whole system and methods for characterisation of individual purified components. In the final section we describe a classfication scheme for the cytochromes c.

1.1 Resolution

1.1.1 The Location of Cytochrome c

Gram-negative bacteria are surrounded not only by the cell membrane but also by a peptidoglycan cell wall and an outer membrane. Proteins which are secreted into the space between the cell membrane and the outer membrane are termed periplasmic. They can be selectively released by treatment with lysozyme and EDTA in a sucrose medium which provides osmotic support for the spheroplasts as they are formed. The fragile spheroplasts can then be osmotically shocked to release the cytoplasmic contents. Thus periplasmic, cytoplasmic and membrane fractions can be obtained (Fig. 1.1) and their composition investigated.

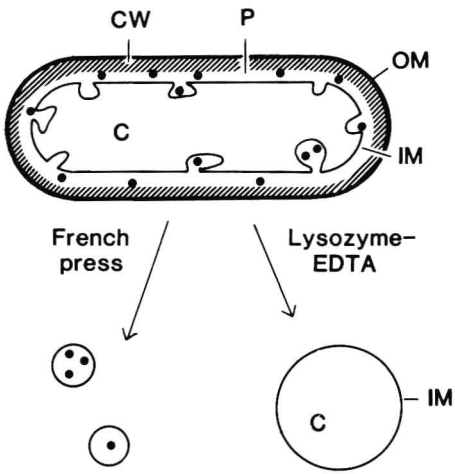

Fig. 1.1. Spheroplast formation in gram-negative bacteria. Gram-negative bacteria contain a periplasmic compartment (*P*) situated between the outer membrane (*OM*) and inner (plasma) membrane (*IM*). The cell wall (*CW*) is denoted by *hatching*. Passage of bacteria through a French pressure cell yields membrane vesicles which may contain periplasmic proteins (●) depending on the way the inner membrane fragments and reseals. In some bacteria, the inner membrane is highly invaginated, increasing the likelihood of trapping the periplasmic proteins in vesicles. Treatment with lysozyme and EDTA removes the outer membrane and cell wall and the spheroplasts that are formed contain an intact cytoplasmic compartment (*C*) if osmotically supported

Table 1.1. The cellular location of cytochrome c in *Pseudomonas stutzeri*

	Cyt. c (%)	ICDH (%)
Periplasmic	52	2
Cytoplasmic	8	98
Membrane	40	0

The amounts of total cytochrome c and isocitrate dehydrogenase (ICDH) present in each fraction were expressed as a percentage of the total present in the three fractions. (G. W. Pettigrew, unpublished results)

Wood (1983) has proposed that all c-type cytochromes are either periplasmic or are bound to the periplasmic side of the cell membrane. This model, which receives increasing experimental support, has important implications for the energy conserving mechanisms of bacterial oxidative phosphorylation (Chap. 3). The criterion for periplasmic location is the release of a protein without release of cytoplasmic contents and this is shown in Table 1.1 for *Pseudomonas stutzeri* where the cytochrome c is measured spectrophotometrically and isocitrate dehydrogenase is used as a cytoplasmic marker.

Less is known of gram-positive bacteria which lack the outer membrane. It may be that, in these organisms, cytochromes c are more tightly bound to the surface of the cell membrane (Jacobs et al. 1979) so that they are not lost through the relatively porous cell wall.

1.1.2 SDS Gel Electrophoresis and Heme Detection

The simple spectrophotometric method used in Table 1.1 cannot distinguish the individual cytochromes c that are present. This however is possible using SDS gel electrophoresis followed by heme detection.

One method of heme detection is based on the peroxidase activity of heme using 3 3′ 5 5′ tetramethylbenzidine as an oxidisable substrate (Thomas et al. 1976). The method was developed, and has been widely used, for proteins such as cytochrome P450 and cytochrome b which contain protoheme IX but, because this heme is not covalently bound, only a small fraction is retained after denaturation and this varies depending on the precise conditions employed.

On the other hand, c-type cytochromes contain covalently bound heme and are therefore ideally suited to the application of the peroxidase activity method after SDS gel electrophoresis (Goodhew et al. 1986). In Fig. 1.2, the method is applied to the fractions obtained during spheroplast preparation of aerobic and denitrifying *Pseudomonas stutzeri*. This experiment allows several important conclusions to be made.

First, individual cytochromes can be identified using purified markers. Second, the study of the general location of cytochrome c summarised in Table 1.1 is extended to define the location of the individual cytochromes. The figure of 8% total cytochrome appearing in the cytoplasmic fraction of Table 1.1 can be seen from Fig. 1.2 to be due mainly to membrane material which is difficult to fully sediment from the viscous spheroplast lysate. Third, membrane-bound c-type cytochromes are resolved and shown to be distinct from the soluble cytochromes. With few exceptions such cytochromes are rarely purified and are poorly characterised. Fourth, the effect of growth conditions on the complement of c-type cytochromes can be defined; cytochrome cd_1 (nitrite reductase) and a band of M_r 30 K are induced in denitrifying conditions, while a membrane-bound cytochrome c of M_r 32 K is characteristic of aerobic growth.

Sample preparation for electrophoresis usually involves the addition of a sulfhydryl reducing agent but this must be avoided if heme staining is to be performed because the ferrous iron is readily lost from the heme to give the porphyrin. However, although porphyrins have no peroxidase activity, they are fluorescent and this property is used in an alternative and equally sensitive method for the detection of c-type cytochromes in the presence of reducing agents (Wood 1981).

The heme-staining method is also usefully applied during purification of c-type cytochromes. Chromatographic peaks may contain single cytochromes