TRANSPORT AND BIOENERGETICS IN BIOMEMBRANES

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JAPAN SCIENTIFIC SOCIETIES PRESS *Tokyo*
PLENUM PRESS *New York London*

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Published jointly by
JAPAN SCIENTIFIC SOCIETIES PRESS, Tokyo
ISBN 4-7622-5332-4
and
PLENUM PRESS, New York London
ISBN 0-306-41282-9
Library of Congress Catalogue Card No. 82-051050

Distributed in all areas outside Japan and Asia between Pakistan and Korea by PLENUM PRESS, New York London.

Printed in Japan

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Preface

Biomembranes are not only principal constituents of the cell but also a major site of biological activity. Studies on biomembranes are, therefore, crucial for furthering our understanding of life processes. A special research project on biomembranes involving more than 100 investigators was conducted under a grant from the Ministry of Education, Science and Culture of Japan between 1978 and 1981. This has resulted in marked progress on basic biomedical studies on biomembranes in Japan. This book is a compilation of several major developments made during this project in the field of "Transport and Bioenergetics in Biomembranes." Developments in other fields constitute the contents of a companion volume, "Structure, Dynamics, and Biogenesis of Biomembranes," edited by Sato and Ohnishi. In these two volumes the authors review recent advances which have been primarily made in their own laboratories and include relevant work carried out by other investigators.

Nine topics are dealt with in this volume. Ozawa reviews purification and properties of four particulate complexes of the mitochondrial electron transfer chain and reports the crystallization of Complex III (ubiquinol-cytochrome *c* reductase) and Complex IV (cytochrome *c* oxidase). The structure and function of H^+-ATPase from a thermophilic bacterium and reconstitution of the F_0 portion of the enzyme from their subunits are reviewed by Kagawa. The reconstitution of some functionally competent hybrid enzymes from subunits obtained from thermophilic bac-

terium and *E. coli* is also reported. Futai and Kanazawa report cloning of the *E. coli* H^+-ATPase gene and complete sequencing of its DNA, leading to a clarification of the primary structures of all subunits of this enzyme.

Anraku summarizes the present knowledge on the molecular organization and physiological functions of bacterial amino acid transport systems, placing emphasis on the biochemical sensing reactions of active transport, whereas the mechanism of Na^+-coupled transport of organic solutes in animal cells is discussed by Hoshi and Himukai. Tada and coworkers review recent developments in the study of Ca^{2+}-ATPase of the sarcoplasmic reticulum with special reference to a newly found membrane protein, phospholamban, as a regulator of this enzyme. Detailed studies of the binding of Na^+ and K^+ to Na^+,K^+-ATPase are reported by Matsui.

Although the microsomal electron transfer system has nothing to do with transport and bioenergetics, a review of this membrane-bound enzyme system by Yamano is included in this volume, together with a chapter on mitochondrial cytochrome P-450 and its involvement in steroidogenesis by Katagiri.

Active transport of a number of ions and molecules across membranes requires secondary energy input, and in certain membrane-bound transport enzymes such as H^+-ATPase and Na^+, K^+-ATPase primary energy transformation also takes place. Thus transport and bioenergetics in biomembranes are closely related. Cytochrome P-450-containing systems, though less related to the subjects of the book, have resemblance to the energy-yielding electron transfer chain in organization. We hope that this book will provide the reader with an insight into electron transfer and energetic functions of biomembranes and, together with the companion volume, will stimulate future progress in membrane research.

July 1982

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Mitochondrial Electron Transport System

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At the beginning of this century, Michaelis (*1*) opened the door to a concept of respiration in which a mitochondrion in the living cell is the site of oxido-reduction on the principle of *supravital staining* of mitochondria with Janus green. Warburg (*2*) developed the concept that an iron-containing catalyst, the *Atmungsferment*, which is sensitive to cyanide and to carbon monoxide, catalyzes most of the utilization of oxygen by cells. In 1925, Keilin (*3*) rediscovered MacMunn's pigments in cells, "cytochromes," and showed them to undergo characteristic changes in the redox state in intact muscle. Using a hand spectroscope, Keilin (*4–6*) demonstrated that a chain of cytochromes (*a*, *b*, and *c*) could transfer electrons from hydrogen donors at one end to molecular oxygen at the other. He demonstrated that this electron transfer chain was present in a wide variety of aerobic organisms such as animals, insects, and bacteria, and that the complement of cytochromes was fairly constant from organism to organism. Although some components of the electron transfer chain such as coenzyme Q (CoQ) (*7*), iron sulfur (*8*), and copper (*9*), which were found by Green's group around the 1960's, were missed by the hand spectroscope, the skeleton of the electron transfer chain was established by Keilin at that time. Also, the particulate nature of the electron transfer

chain was first recognized by him from a Keilin-Hartree preparation of homogenized heart muscle particles (*10*).

In 1956, Green's group developed the tactics and methodologies (*11*) required for the isolation of inner mitochondrial membrane in large quantity that is identified as the seat of electron transfer and coupled ATP synthesis. Hatefi and others of the Wisconsin group (*12–14*) fragmented the electron transfer chain of the inner membrane into four complexes (Complexes I to IV) and reconstituted the electron transfer chain from these particulate segments.

Recent advances in the field of chromatography, such as hydrophobic interaction and affinity chromatography, make it possible to handle these hydrophobic particulate complexes more easily than two decades before. Phospholipids bound to the complexes and impurities attached to the phospholipids could be depleted under control without loss of enzymic activity. Thus, phospholipid depletion from the complexes resulted in intensive purification and in the reduction of the hydrophobic nature of the complexes, even leading to their crystallization. Here, I will describe the characteristics and molecular profiles of these purified complexes of the electron transfer chain, and the crystallization of particulate cytochromes by tactics developed in our laboratory.

I. COMPLEX I (NADH-CoQ OXIDOREDUCTASE)

In 1962, Hatefi *et al.* (*14*) first described the resolution of NADH-CoQ reductase (Complex I) from NADH-cytochrome *c* reductase (Complexes I–III) in the mitochondrial electron transfer chain. It is constituted of flavoprotein, a series of iron sulfur protein and some mitochondrial structural proteins. Also, it contains about 20% phospholipids by weight (*15*). It is reported that removal of phospholipids by cholate treatment causes a loss of NADH-CoQ reductase activity; thus phospholipids are directly involved in this reaction (*16*).

In this section, intensive purification and phospholipids depletion of Complex I by hydrophobic interaction chromatography and some molecular profiles of the purified enzyme (*17*) will be described.

1. Depletion of Phospholipids

Complex I prepared from beef heart mitochondria by the method of Hatefi (*18*) was bound on a column (1×23 cm) of phenyl-Sepharose

CL-4B equilibrated with 20 mM Tris-HCl, pH 8.5, containing 0.5% deoxycholate and 0.2 M NaCl. To purify and to deplete the phospholipids, Complex I on the column was washed with 500 ml of the same buffer. Due to the washing, several peaks of impure proteins were eluted from the column (Fig. 1) together with phospholipids. Then, the proteins bound on the column were eluted with the buffer containing 1% octa-ethyleneglycol mono *n*-dodecylether ($C_{12}E_8$). A fast moving sharp peak (Peak I) showed high NADH-CoQ reductase activity, and a slowly moving peak (Peak II) showed no activity. Other impure proteins remained on the column. They were eluted from the column with the buffer containing 1% sodium dodecyl sulfate (SDS).

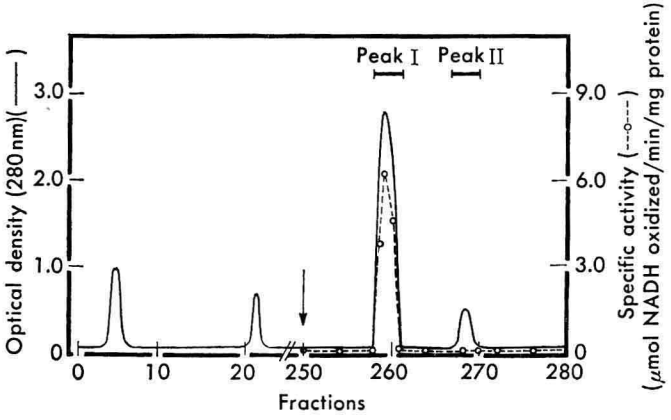

Fig. 1. Phenyl-Sepharose CL-4B column chromatography of Complex I. Complex I (80 mg protein) was applied to a column (1 × 23 cm) of phenyl-Sepharose CL-4B and was washed with 500 ml of 20 mM Tris-HCl, pH 8.5, containing 0.5% deoxycholate and 0.2 M NaCl, and was eluted with the buffer containing 1% $C_{12}E_8$ in place of deoxycholate. The arrow indicates the point where the elution was started. The flow rate was about 38 ml/hr and 2.0 ml was collected in each fraction. NADH-CoQ reductase activity of the samples was measured by the method of Hatefi (*18*).

2. *Subunit Composition*

The SDS-urea gel electrophoresis by the method of Downer *et al.* (*19*) (Fig. 2) showed that Peak I consisted of 9 subunits, Peak II, 12 subunits, and Complex I, 17 subunits. The FMN content (2.07 nmol/mg protein) of Peak I was 1.5 times higher than that of Complex I. Peak I was depleted of phospholipids from 20% (w/w) to 0.5% (Table I). It

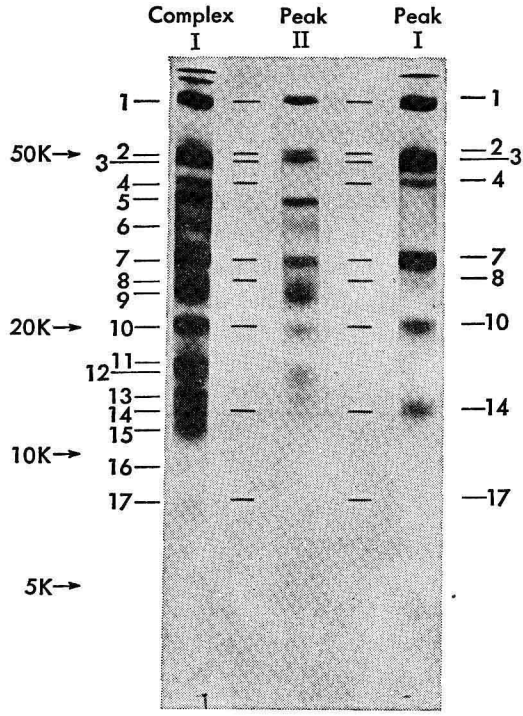

Fig. 2. Subunit composition of Complex I. Disc polyacrylamide gel electrophoresis was performed on 0.1% SDS/8 M urea gel, according to the method of Downer *et al.* (*19*), except that the total acrylamide concentration was 7.5% instead of 12.5%. Molecular weights in thousands are indicated by the postscript K, *i.e.*, 50K is 50,000.

TABLE I. Chemical composition of Peak I.

Component[a]	nmol/mg protein
FMN	2.07
Nonheme iron	54.7
CoQ	<0.3
Phospholipids	0.5%

[a] FMN was determined fluorometrically (*20*). Nonheme iron was estimated by the method of Brumby and Massey (*21*), CoQ by the method of Kröger (*22*), and phospholipids by the method of Chen *et al.* (*23*).

contained 54.7 natom of nonheme iron/mg protein and less than 0.3 nmol of CoQ/mg protein.

In order to examine whether Peak I possesses the same catalytic activity as Complex I, NADH-cytochrome *c* reductase activity was reconstituted from Peak I and Complex III. Table II shows the results that Complexes I–III were reconstituted in the complete system, and when CoQ_1 was eliminated from the assay system, the rate of cytochrome *c* reduction decreased to one-tenth of that in the complete system. When antimycin A, which is the specific inhibitor of Complex III, was added to the system, the cytochrome *c* reduction was almost abolished. These data show that Peak I can oxidize NADH and transfer the electron to cytochrome *c* *via* Complex III.

TABLE II. Reconstitution of NADH-cytochrome *c* reductase with Peak I and Complex III.

System	Cytochrome *c* reduced[a] (mol/min/mol FMN)
Complete[b]	2,960
$-CoQ_1$	339
$-CoQ_1+100$ μg antimycin	27

[a] NADH-cytochrome *c* reductase activity was measured according to the method of Hatefi and Stiggall (*24*). [b] The complete system contained Peak I (39 pmol FMN), Complex III (40 pmol cytochrome c_1), CoQ_1 (100 nmol), and cytochrome *c* (700 nmol). Activities were assayed in 1 ml at 30°C.

Table III shows the molecular weights and stoichiometry of subunits in Complex I and Peak I, which are calculated from the profiles of SDS-urea gel electrophoresis (Fig. 2). The molecular weight of Peak I was calculated to be 477,300, and this figure was almost the same as that based on the specific FMN content of Peak I.

The oxidized and dithionite-reduced spectra of Peak I are shown in Fig. 3, and also the difference spectrum in the inserted figure. By virtue of the removal of excess iron sulfur protein, a remarkable three-banded structure was clearly observed in the 450 nm region in the difference spectrum.

3. *Molecular Profile of Complex I*

According to Hatefi *et al.* (*25*), Complex I contains 14–17 polypeptides with molecular weights of 641,500–716,500. However, the stoichiometry and roles of subunits in the electron transfer reaction still remain obscure. In this study, we adopted a hydrophobic interaction chromato-

TABLE III. Molecular weights and stoichiometry of subunits in Complex I and Peak I.

Polypeptide	Complex I	Peak I	Stoichiometry
1	66,000	66,000	2
2	50,000	50,000	2
3	48,000	48,000	1
4	43,000	43,000	1
5	38,000		2
6	34,000		2
7	28,000	28,000	2
8	25,500	25,500	1
9	23,000		2
10	20,000	20,000	2
11	16,300		1
12	15,500		1
13	13,600		1
14	12,500	12,500	2
15	11,300		1
16	9,400		1
17	7,800	7,800	1
Total MW[a]	733,400	477,300	

[a] Molecular weights and stoichiometry were estimated from gels stained with Coomassie Blue.

graphy to purify Complex I and to deplete its phospholipids. As shown in Fig. 1, after removal of some impure proteins and phospholipids, a sharp peak of purified Complex I (Peak I) with high specific activity was eluted from the column prior to Peak II by using a nonionic detergent, $C_{12}E_8$. SDS-urea gel electrophoresis shows that this purified enzyme contains 9 subunits (Fig. 2), whereas Complex I before the purification contains 17 subunits.

In parallel with the decrease in the number of polypeptides, the specific FMN content is increased up to 2.07 nmol/mg protein (Table I). Twenty-six atoms of nonheme iron were present per mol of FMN. Both CoQ and phospholipids were depleted to less than 0.3 nmol/mg protein and 0.5% (w/w), respectively. Not only the NADH-CoQ reductase activity of the purified enzyme, but reconstituted NADH-cytochrome *c* reductase activity with Peak I and Complex III (Table II) indicated that the purified enzyme is really NADH-CoQ reductase itself. From the polypeptide composition in the purified enzyme shown in Table III, its molecular