Biologically Active Principles of Natural Products

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PREFACE

In honour of Karl Folker's 75th birthday a symposium was held at Lehigh University, Bethlehem, Pennsylvania, USA, in the end of 1981. Due to the excellent survey articles presented by worldwide known specialists in different fields of natural products' chemistry the idea was born during the symposium to make the valuable information accessible to all colleagues having missed the chance to hear the lectures at Lehigh University. However, this relatively late upcoming idea took quite some time to become realized as the authors wanted updated manuscripts to be published.

The now available volume "Biologically Active Principles of Natural Products" reflects Professor Folker's lifelong research activities: The unifying theme of Dr. Folkers' research might be termed "Chemical Regulation of Life Processes". Research highlights include isolation and structural studies of antibiotics, including penicillin and streptomycin, structure determination and synthesis of vitamin B_6, synthesis of pantothenic acid and analogs, isolation, structure elucidation and synthesis of biotin, isolation and crystallization of vitamin B_{12}, discovery and identification of mevalonic acid, isolation, identification, synthesis and elucidation of the biosynthetic pathway for coenzyme Q, and identification of thyrotropin releasing hormone.

Currently, active research efforts at the Institute for Biomedical Research in Austin are directed toward isolation and synthesis of immuno and hormonally active peptides, elucidation of nutritional requirement for vitamin B_6 and identification of vitamin B_6 deficiency states and establishment of requirements for coenzyme Q, discovery of coenzyme Q deficiency states and development of replacement therapies.

Dr. Folkers' research accomplishments have been reported in more than 700 research publications and in frequent lectures throughout the world. He has received many honors including the American Chemical Society Award for Meritorious Work in Pure Chemistry, the Mead Johnson Award for research on vitamin B_6, the Harrison-Howe Award (ACS Rochester Section), the Spencer Award (ACS Kansas City Section), the Perkin Medal and the Nichols Medal (ACS New York Section), the Van Meter Prize of the American Thyroid Association, and an Alexander von Humboldt-Stiftung Award. He was the recipient of the first Robert A. Welch International Award and Medal for his research on life processes.

He is a member of the National Academy of Sciences and is one of two U.S. members of the Society Italiana de Scienze Farmaceutica. He has served as president of the American Chemical Society, received honorary doctorates from Philadelphia College of Pharmacy and Science, University of Uppsala, University of Wisconsin, and University of Illinois and has held many distinguished lectureships and organized and participated in many international congresses and symposia.

Several articles in the book discuss the recently discovered application of ubiquinone and its mechanism of action for treatment of heart diseases. Ubiquinone opens to the cardiologist a completely new principle of therapy. Also of medical importance is the now eluciated biosynthetic pathway of tetrahydro-L-biopterin and the treatment of patients suffering from atypical phenylketonuria with hydro-L-biopterins.

Survey articles about vitamin B_{12}, biotin, steroid hormones, hypothalamic hormones, substance P, gastrointestinal peptide hormones, nerve growth factors and peptide antibiotics inform the natural products' chemist, pharmacist and physician about the most recent results in different research laboratories.

Exciting new discoveries for the isolation and structure elucidation of natural products are covered by several chapters and also new synthetic approaches for this class of compound are surveyed.

A great deal of credit for the book goes to Dr. Heide Voelter who looked after every stage of the preparation of the manuscript. We would like to thank our publishing house Georg Thieme Verlag Stuttgart · New York for their encouragement, co-operation, and patience.

Tübingen, *Wolfgang Voelter*
Bethlehem, March 1984 *G. Doyle Daves, Jr.*

CONTENTS

STUDIES ON THE ROLE OF COENZYME Q_{10} IN THE INNER MITOCHONDRIAL MEMBRANE

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Introduction

Coenzyme Q_{10} has previously been found to be involved in electron transport. The chemiosmotic model proposes that coenzyme Q_{10} is involved in proton translocation across an asymmetric mitochondrial membrane. However, some data do not support this role for coenzyme Q_{10}. For example, short chain ubiquinols are oxidized by cytochrome c, even though their transmembrane diffusion rates are too slow to support enzymic activity. Also ubiquinol is oxidized preferentially at the matrix side of the inner membrane, rather than the predicted cytoplasmic side. Further, antimycin is found to inhibit the reduction of cytochrome C_1 as a single turnover step, although the chemiosmotic theory does not predict this. Finally, results utilizing dicyclohexylcarbodiimide suggest a possible uncoupling of electron transfer from proton translocation.

Since its discovery in 1957 as a constituent of the mitochondrial respiratory chain (1), the role of Coenzyme Q_{10} (ubiquinone-10, Q_{10}) has been a highly controversial matter. Our studies, elicited by Professor Karl Folkers as early as in 1966, have shown that a Coenzyme Q (CoQ) having at least 6 isoprenoid units is required to support full oxidation of NADH in Coenzyme

Q-depleted mammalian mitochondria (2). Subsequent studies have shown that short-chain ubiquinones competitively inhibit mitochondrial NADH oxidation (3). A minimal requirement of the isoprenoid chain has also been found in bacterial chromatophores (4). The studies of the organic structural specificity of CoQ have been the basis for the therapeutical use of CoQ_{10} in a great deal of clinical studies (cf. 5).

The function of CoQ as an electron carrier between complexes I and II and complex III of the mitochondrial respiratory chain is still obscure. The chemiosmotic hypothesis postulates that respiration-driven proton translocation is affected through the asymmetric disposition of hydrogen and electron carriers in the respiratory chain. The Q-cycle proposed by Mitchell (6) (Fig 1) involves Coenzyme Q as a hydrogen carrier at the level of complex III. According to this hypothesis, CoQ is primarily involved in the mechanism of energy conservation, since the proton gradient (acid outside) would be the driving force for ATP synthesis (7).

There are several requirements for the Q-cycle that can be experimentally tested. We have provided experimental evidence against several of the postulations of the Q-cycle and other similar schemes of electron transfer in the cytochrome bc_1 region of the respiratory chain.

Turnover of the ubiquinol-cytochrome c reductase with short-chain ubiquinols

In spite of the requirement for long chain CoQ-homologs for restoration of NADH oxidation, short-chain ubiquinols are excellent substrates for oxidation by oxygen or cytochrome c. We have shown (8) that the maximal turnover of ubiquinol-1 at 20°C for cytochrome c reduction by complex III (ubiquinol-cyt.c reductase) approaches 200-250 s^{-1} either in mitochondria or in the isolated complex. No endogenous CoQ_{10} is required for such maximal activities. If a step involving diffusion of ubiquinol

ubiquinone across the lipid bilayer is required for ubiquinol-cyt.c reductase as postulated by the Q-cycle, then the transmembrane diffusion rate of Q_1 (either oxidized or reduced) should be less than 4-5 ms. We have shown (T. Fahmy, M. Degli Esposti and G. Lenaz, unpublished) in partial accordance with Futami et al. (9), that ferricyanide trapped inside phospholipid vesicles is reduced by ubiquinol-1 at low rates, with turnovers of CoQ across the membrane in the range of 1 s. In general, the transmembrane diffusion rate of Q_1 appears at least two orders of magnitude too slow to support enzymic activity (Table 1), if a transmembrane diffusion step is required for such activity.

Sidedness of the ubiquinol oxidation site

The Q-cycle postulates that ubiquinol is oxidized at the cytoplasmic side of the membrane in order to support proton extrusion out of the mitochondrion. We have investigated the rate of ubiquinol oxidation in mitochondria and in sonicated submitochondrial particles (SMP) which are inside-out with respect to membrane polarity. The rate constants and the initial rates of ubiquinol-cyt.c reductase are always higher and extrapolate to higher V_{max} in SMP than in uncoupled cyt.c depleted mitochondria (BHM-c) (M. Degli Esposti and G. Lenaz, unpublished). Disruption of the permeability barrier with deoxycholate enhances 4-fold the rate of ubiquinol oxidation in BHM-c (Table 2). The above data support the idea of a better accessibility of ubiquinol to the oxidation site at the matrix side of the membrane, which is exposed to the exterior in SMP.

Antimycin sensitivity of cytochrome c_1 reduction in one single turnover

The Q-cycle proposes that the iron-sulfur protein of complex III is the first oxidant for ubiquinol, lying between the

Table 1

Kinetic constants of transmembrane reduction by ubiquinol-1 of ferricyanide trapped inside lecithin vesicles and of ubiquinol-1 cytochrome c reductase in mitochondria.

Kinetic constant	Lipid vesicles	Enzyme
Pseudofirst order k (s^{-1})(a)	0.04(b)	60
Half-times (s)(a)	14.3	0.01
Turnover number (s^{-1})	1	223

(a) Calculated with an amount of membrane containing 2 mg of phospholipids.

(b) The k is over 20 s^{-1} for ubiquinol-1 ferricyanide reaction in water under the same conditions.

Table 2

Ubiquinol cytochrome c reductase in BHM-c and SMP(a)

Exp.	Mitochondrial preparation	Activity
1	BHM-c	1.76
	SMP	4.56
	BHM-c + deoxycholate	7.49
2	BHM-c	0.46
	SMP	1.78
3	BHM-c	75
	SMP	148

(a) Exp. 1: v (mol $min.^{-1}mg^{-1}$) with 2 μM ubiquinol-2 and 14.5 μM cyt.c; exp. 2: V_{max} (mol. $min.^{-1}mg^{-1}$) with 2.4 μM cyt.c and varying ubiquinol-2; exp. 3: maximal turnovers with ubiquinol-1, calculated from double reciprocal plots of turnover numbers of fixed 15 μM cyt.c (mol c reduced/mol $c_1 \cdot s$).

electron donor and cyt.c_1 in an antimycin-insensitive pathway; according to such a view, antimycin inhibits steady-state electron transport in the cytochrome bc_1 region by preventing reoxidation of the b-cytochromes; antimycin should not, however, inhibit the rapid reduction of cyt.c_1 in a single turnover when a reductant is added to the fully oxidized complex (10). We have investigated the effect of antimycin on the rapid reduction of cytochromes b and c_1 by either succinate or ubiquinol-1 by a stopped-flow technique in a mitochondrial preparation consisting largely of succinate cytochrome c reductase (M.Degli Esposti and G.Lenaz, unpublished). Table 3 shows that the reduction of cyt.c_1 is inhibited by antimycin, while the biphasic reduction of cytochrome(s) b is accelerated. This result does also show that the first turnover of the reductase is antimycin-sensitive, indicating that the antimycin block is in a direct pathway leading to cyt.c_1.

DCCD inhibits proton translocation across the cytochrome bc_1 complex

Dicyclohexylcarbodiimide (DCCD) potently inhibits H^+-ATPases (11) preventing H^+ translocation by covalent bonding to a hydrophobic subunit. We have investigated the transmembrane pH difference induced by aerobic succinate oxidation in submitochondrial particles by evaluating the quenching of the fluorescence of 9-aminoacridine induced by proton release in the inner space of the particles (12); the quenching is inhibited by DCCD with half-inhibition at 40 nmol/mg protein, while electron transfer in the cytochrome bc_1 region is inhibited at much higher DCCD concentrations (Table 4). Under our experimental conditions the quenching associated with cytochrome oxidase is only marginally inhibited by DCCD, concentrations inducing full inhibition of the quenching driven by succinate: this finding indicates that the effects observed occur at the level of the cytochrome bc_1 complex. The decrease of ΔpH is

Table 3

Effect of antimycin on the rapid reduction of cytochromes b and c_1 by ubiquinol-1 (13 µM)

	control	antimycin[a]
	V_i (µM · s^{-1})	
cyt. b (562-570)	8	9
cyt. c_1 (552-540)	5.7	0.8

(a) Antimycin was added at a 3.5:1 ratio with cyt. c_1 (0.57 µM c_1 in the assay)

Table 4

Effect of DCCD on respiratory activities in SMP

Activity	DCCD for half-inhibition (nmol/mg protein)
Succinate oxidase	3000
Succinate driven quenching[a]	40
Succinate cytochrome c reductase	1100
Ubiquinol-1 cytochrome c reductase	900

(a) The basal quenching in absence of DCCD corresponds to a ΔpH of 2.8-3.1.

not the result of an enhanced permeability of the membrane to protons; in fact the reversal of the quenching upon attaining anaerobiosis is faster in the controls than in DCCD-treated particles (Table 5), indicating that DCCD lowers H^+ permeability, even when the H^+ channel of ATPase is blocked by oligomycin (E.Meyer and G.Lenaz, unpublished).
These results indicate that electrons can flow in the cytochrome bc_1 complex under conditions where no proton translocation is occurring; the possibility of an "uncoupling" of electron transfer from proton translocation across the membrane is inconsistent with the theoretical postulation of the Q-cycle, where electron transfer is linked to proton translocation (2 protons per electron transversing complex III).
The known effects of DCCD on ATPase and other postulated H^+ pumps are consistent with the proposal that oxidation of ubiquinol by complex III is associated with H^+ translocation involving a proton conducting channel in the enzyme; DCCD is postulated to primarily block proton conduction through the channel, and only secondarily to inhibit electron flow.

Conclusions on the role of coenzyme Q

The involvement of CoQ in electron transfer is a rather established fact; the results of our investigation do not support, however, its role as a proton translocator across the membrane. Several lines of evidence have confimed the initial proposal of Green (13) that CoQ constitutes a mobile pool, shuttling electrons between the dehydrogenases and complex III (cf. Fig 1A). For such a role, a hydrophobic quinone is necessary in order to move back and forth in the lipid bilayer. Only long chain quinones appear to be sufficiently mobile in the bilayer to fulfill this requirement, thus explaining our previous results on the specificity of long chain CoQ homologs in NADH oxidation. Short chain CoQ homologs can be used as exogenous electron donor or acceptor , since they can readily

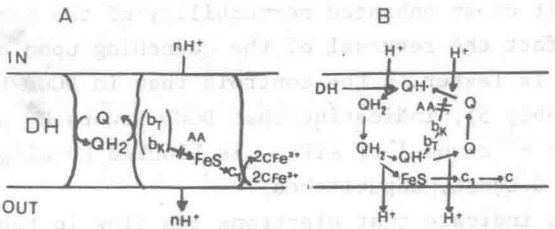

Fig.1 A: A classical scheme of electron transfer and proton translocation across complex III.
B: The protonmotive Q-cycle. AA, antimycin A; DH, dehydrogenase; FeS, iron-sulfur protein.

Table 5

Proton permeability of SMP(a)

Addition	k (s^{-1})	t/2 (s)
None	0.067	10
Oligomycin (2.5 µg/mg)	0.016	42
" + FCCP (4 nM)	0.138	5
" + DCCD (50 nmol/mg)	0.008	84

(a) Measured by the anaerobiosis-induced reversal of the quenching of 9-aminoacridine

Table 6

Effect of Q_{10} extraction on the viscosity of the inner mitochondrial membrane

Mitochondria	Polarization of DPH	Fluorescence lifetime (ns)	Viscocity (P)
Control	0.136	7.7	0.94
Extracted	0.144	7.1	1.40
Reconstituted	0.126	7.7	0.82

reach their sites of interaction in the redox complexes; they cannot however support full respiration by coordinated interaction among the complexes due to their insufficient rate of efficient diffusion in the lipid phase. We have shown by spectroscopic means that long chain quinones are largely contained in the hydrophobic membrane interior, although the quinone ring can freely diffuse to the membrane surface, particularly in its reduced form; on the other hand, short ubiquinones, mainly Q_1, are largely located at the membrane water interface (14). The length of Q_3 is almost equal to the width of a half bilayer, indicating that ubiquinones having a number of isoprenoid units lower than 4 are suited to fit between the lipid molecules of the bilayer, whereas the long-chain quinones can be easily accommodated in the hydrophobic interior. It is worth mentioning that CoQ_{10} is a bulky molecule, and its insertion in the membrane hydrophobic core is expected to induce a perturbation of its physical state. We have shown by fluorescence polarization of the probe diphenylhexatriene (DPH) that the viscosity of the membrane is reversibly increased by extraction of CoQ_{10} (R.Fato, E.Ferri and G.Lenaz, unpublished), (Table 6).

It is suggestive to postulate that one role of CoQ_{10} in the mitochondrial membrane is to ensure an optimal degree of fluidity in the lipid bilayer (15).

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