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The Relative Essentiality of the Antioxidative Function of Coenzyme Q—The Interactive Role of DT-Diaphorase

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Abstract—This paper will address two aspects regarding the antioxidative role of coenzyme Q (CoQ): (1) Is the antioxidant function of CoQ primary or secondary (coincidental), i.e. was this molecule selected during evolution to function primarily as an essential functional component of the mitochondrial electron transfer chain and oxidative phosphorylation processes, is its antioxidative capability merely a coincidence of its hydroquinone structure, or was its synthetic enzyme sequence selected on the basis of the advantage to the evolving organism of both functions of CoQ? (2) What is the mechanism whereby the hydroquinone (antioxidant) form of CoQ ($CoQH_2$) is maintained in high proportion in the various and many membranes in which it resides, and in which an obvious electron transfer mechanism to reduce it is not present? The essentiality of the antioxidative role of $CoQH_2$ will be explored and compared to other primary and secondary antioxidants. Recent evidence implicating the two-electron quinone reductase, DT-diaphorase, in the maintenance of the reduced, antioxidant state of CoQ during the oxidative stress of exhaustive exercise will be presented, and a hypothesis concerning the evolutionary significance of DT-diaphorase will be offered.

Introduction

Evolving in the presence of oxygen must have presented a paradox to potentially aerobic organisms. On the one hand, oxygen was in abundant supply and chemically well suited to act as the terminal electron acceptor for the major energy transferring and conserving system, the mitochondrial electron transfer chain. In addition, the end product of these reactions, water, was not toxic and was easily excreted. On the other hand, molecular oxygen also had the capacity to accept an unpaired electron leading to the formation of damaging oxygen free radicals. In order to survive the evolutionary process of natural

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selection and to establish themselves as species, it was necessary for aerobic organisms to develop and select successful means of preventing the formation of free radicals and/or mechanisms to convert them to nontoxic molecules (i.e. antioxidant defenses), and to repair free radical damage to the various cellular constituents.

Discussion

We synthesize a number of enzymes such as superoxide dismutases, catalases and peroxidases in order to convert oxidizing molecules, formed during metabolism, to innocuous chemical species. In addition, we obtain via nutritional means a number of antioxidants synthesized by other species for their own antioxidant protection, and utilize them as essential antioxidant defence devices. Among the latter category are vitamin E (α-tocopherol), β-carotene (a form of vitamin A) and vitamin C (ascorbic acid). Although β-carotene and ascorbic acid have important functions in addition to antioxidation, they, and especially vitamin E for which no other function is known (Tappel, 1965; Maguire *et al.*, 1989), may be assigned to the category of "Primary or Essential Antioxidants". A number of other compounds whose primary functions may be unrelated to antioxidation but which, due to some aspect of their molecular structure and reactivity relating to their oxidation–reduction potential or reducing capacity, are able to perform as antioxidants, have been referred to as "Secondary or Incidental Antioxidants" (Beyer, 1990, 1992). A number of such molecules are formed, either as a result of anabolism or catabolism, whose primary functions, for which they were selected during evolution, are not related to their secondary, or incidental, antioxidative capacity. The extent and diversity of such secondary antioxidants in biological systems may be appreciated by perusing Table 1 which contains a list of such compounds. It should be acknowledged that many of the compounds listed in Table 1 have been shown to have met the criteria regarding "How to characterize a biological antioxidant" offered by Halliwell (1990).

Table 1. A partial list of secondary or coincidental antioxidants functional in plant and animal systems*

N-Acetylcysteine	Dihydrolipoid acid	Lactoferrin	Proteolytic enzymes
Anserine	Diphenylamine	Lipoate	Protoporphyrin
Apomorphine	Ergothioneine	Melanins	Pyrroloquinolinequinones
Azelaic acid	Estrogens	Melatonin	Pyruvate
Bilirubin	P-ethanolamine	Monoamines	Riboflavin
Biliverdin	Flavonoids	Neopterin	Spermine
Ca^{2+} channel blockers	Glycated proteins	Nitric oxide	Tannic acid
Caffeine	Hemocyanin	Orotic acid	Taurine
Carnitine	Hemoglobin	Ovothiol	Thioctic acid
Carnosine	Histidine	Phytic acid	Thioproline
Ceroloplasmin	Homocarnosine	Polyamines	Urate
Chlorophylls	Hylauronic acid	Procyanidins	Vanillin
Cholesterol	Hypotaurine	Propinylcarnitine	Xanthophylls

*Due to space limitations, literature references to the table contents are not included. A complete list of references may be requested from the author.

The question has been presented: "Why are secondary metabolites (natural products) biosynthesized? (Williams *et al.*, 1989). Several possibilities exist: (1) temporary mutation, (2) evolution in progress, (3) waste or detoxification products, (4) functional metabolic role and (5) repellent or attractant role. CoQ, which has received attention as an antioxidant in recent years, would appear to fit into category 4 as it was discovered as an essential member of the mitochondrial electron transfer chain by Crane *et al.* (1957) and as a functional component of the oxidative phosphorylation mechanism by Peter Mitchell in 1975. The antioxidative capacity of CoQ was first reported by Lea and Kwietney (1962) and the superior antioxidant capacity of the hydroquinone form of CoQ, equivalent to that of vitamin E, was reported in 1966 by Mellors and Tappel. This observation has been repeated many times in many laboratories since then, especially in Laura Landi's laboratory in Bologna (Landi *et al.*, 1984, 1987, 1990a,b, 1991, 1992; Cabrini *et al.*, 1991; Fiorentini *et al.*, 1991, 1993; Merati *et al.*, 1992; Solaini *et al.*, 1987), Lars Ernster's laboratory in Stockholm (Ernster, 1984; Beyer *et al.*, 1986, 1987; Glinn *et al.*, 1992; Ernster *et al.*, 1992; Ernster and Forsmark-Andrée, 1993), Lester Packer's (Lang *et al.*, 1987; Kagan *et al.*, 1990; Hiramatsu *et al.*, 1991; Packer *et al.*, 1991), Roland Stocker's (Stocker *et al.*, 1991; Mohr *et al.*, 1992; Suarna *et al.*, 1993; Ingold *et al.*, 1993), Jan Karlsson's (Karlsson, 1987; Karlsson *et al.*, 1993) and my laboratory in Ann Arbor (Beyer, 1988). From these and other outstanding studies and other laboratories, the antioxidant properties of $CoQH_2$ would appear to be well established. This subject has been reviewed by this author on several occasions (Beyer, 1989a, 1990a,b, 1992; Beyer *et al.*, 1986, 1987; Beyer and Ernster, 1990, Ernster and Beyer, 1991). Because of its primary role in such a vital and central function as mitochondrial electron transport and oxidative phosphroylation, it was assumed that its exclusive location was in the inner mitochondrial membrane and that its only function was in mitochondrial energy release and transformation.

Beginning with the work of Sasty *et al.* (1961), Jayaraman and Ramasarma (1963), Crane and Morré (1977), and then the explosion of information from Gustav Dallner's laboratory in Stockholm (Kalén *et al.*, 1987; Elmberger *et al.*, 1989; Appelqvist *et al.*, 1991; Thelin *et al.*, 1992; Åberg *et al.*, 1992; Swiezewaka *et al.*, 1993), it became

Table 2. Distribution of Coenzyme Q in subcellular fractions from rat liver. Values are means ± S.E. of seven experiments (modified from Kalèn *et al.*, 1987)

Subcellular fraction	Coenzyme Q_9 (μg/mg protein)
Homogenate	0.79 ± 0.08
Golgi vesicles	2.62 ± 0.15
Lysosomes	1.86 ± 0.18
Mitochondria	1.40 ± 0.16
Inner mitochondrial membrane	1.86 ± 0.13
Endoplasmic reticulum	0.15 ± 0.02
Peroxisomes	0.29 ± 0.04
Plasma membrane	0.74 ± 0.07
Cytosol	0.02 ± 0.004

apparent that CoQ was distributed widely in various membranes of the cell. Table 2, from Dallner's laboratory, shows the wide distribution of CoQ and its concentrations in those compartments. It is notable that the concentration of CoQ is higher in the Golgi membranes, and as high in the lysosomal membrane, than in the inner mitochondrial membrane. The question of the function of CoQ in these diverse membranes was difficult to resolve until it was shown that, depending on the tissue, for the most part, well over 50% of the resident CoQ in a variety of rat and human tissues is in the reduced, antioxidant form (Åberg *et al.*, 1992).

Once it was known that $CoQH_2$ functions as an effective intramembrane antioxidant and that it is present in the reduced state in essentially all cellular membrane compartments, the question arose as to the mechanism whereby CoQ in these membranes is maintained largely in the hydroquinone state. As early as 1986, my colleagues Kerstin Nordenbrand, Lars Ernster and I stated: "It is conceivable that mitochondrial DT-diaphorase maintains CoQ in the hydroquinone state thereby promoting its antioxidant function" (Beyer *et al.*, 1986). DT-diaphorase, otherwise known as NAD(P)H:Quinone Acceptor Reductase and menadione reductase, is an inducible 2-electron quinone reductase which has been shown by Charles Huggins to prevent carcinogenesis from several quinone carcinogens capable of quinone cycling and consequent free radical production (Huggins and Fukunishi, 1964; Huggins *et al.*, 1964, 1978; Huggins, 1979). About twenty years ago, Lars Ernster, a co-discoverer of DT-diaphorase, convinced Bruce Ames to demonstrate the antimutagenic properties of the liver S-9 fraction from rats in which this enzyme had been induced to high activity (Chesis *et al.*, 1984). Juan Seguar-Aguilar, Lars Ernster and I also showed that the activity of DT-diaphorase is induced to high levels in rapidly proliferating cells such as HepG2 hepatoblastoma and the Zajdela ascites hepatoma, and labeled it an anticancer enzyme (Beyer *et al.*, 1988).

Although considerable information is known about the structure, prosthetic group, mechanism, gene and other aspects of DT-diaphorase (see e.g. Ernster *et al.*, 1987), its normal function, i.e. the role for which it was selected during evolution, remains unresolved. In order to test the hypothesis that it may function to maintain membrane-bound CoQ in a reduced steady-state, we have assayed the extent of lipid peroxidation, the oxidation–reduction state of CoQ, and the DT-diaphorase activity of gastrocnemius muscles of control rats and rats injected with a potent inhibitor of DT-diaphorase, dicoumarol, and subjected them to severe oxidative stress by running them to exhaustion. All non-run control and dicoumarol-treated animals were killed and the gastrocnemius muscles removed and processed approximately 15 min following dicoumarol injection. The results of this experiment appear in Table 3. The extent of lipid peroxidation products, as thiobarbituric acid reacting substances (TBARS), were slightly higher in the dicoumarol-injected controls than in the non-treated controls (not significant). Running to exhaustion in a rodent treadmill increased TBARS about 3-fold in the non-dicoumarol injected rats and about 4-fold in the dicoumarol-injected, run-to-exhaustion group in which the DT-diaphorase activity was inhibited to approximately 1/3 of the non-inhibited level. These data are consistent with a role for DT-diaphorase in the maintenance of the reduced, antioxidant form of CoQ.

In order to test the DT-diaphorase-CoQ interaction further, we utilized an *in situ* technique, used previously in my laboratory (Starnes *et al.*, 1987), which overcomes

Table 3. Effect of dicoumarol administration on lipid peroxidation, oxidation–reduction state of coenzyme Q and DT-diaphorase activity of gastrocnemius muscle of rats at rest and following oxidative stress induced by treadmill running to exhaustion

Experimental condition	Lipid peroxidation	Coenzyme Q*(%) Oxidized	Reduced	DT-diaphorase activity
Control	22.6 ± 6.4	56	44	2.97 ± 0.64
Control + Dicoumarol	29.4 ± 3.7	62	38	1.04 ± 0.09
Run to exhaustion	62.7 ± 4.2	66	34	3.02 ± 0.72
Run to exhaustion + Dicoumarol	88.4 ± 7.2	82	18	0.92 ± 0.10

Lipid peroxidation as μmols MDA/g tissue wet weight. Dicoumarol administered i.p. at 3 mg/100 g body weight 15 min prior to exercise commencement. Control animals + dicoumarol were killed following a treatment period equivalent to time required for run to exhaustion. Oxidized and reduced states of CoQ were determined according to a modification of the method of Beyer (1989b). DT-diaphorase as nmols cytochrome c reduced/min/mg protein. Values reported as mean ± S.E.M. Each group consisted of eight animals.
*The concentration of total coenzyme Q in terms of pmoles/mg protein (224.6 ± 18.4) did not differ statistically between groups.

some of the disadvantages of both *in vivo* and *in vitro* techniques. Briefly, adult male Sprague–Dawley rats (*c.* 450 g) were anesthetized with Na-Pentobarbital, and the gastrocnemius–plantarus–soleus muscles of the left leg were exposed. The tendons were severed at the ankle, the knee joint stabilized, and the muscles were stretched 15–20% beyond resting length by attaching a weight of 100 g. For muscles which were to receive electrical stimulation, needle electrodes were inserted directly into the muscles and stimulated with two supramaximal pulses/sec for 90 min. The isotonic contractions were monitored to assure maximal contractions and the muscles were bathed with Ringer's solution at 37°C. Control values represent the contralateral limb which was treated similarly, but not stimulated. As in the previous experiment, dicoumarol was administered about 15 min prior to initiation of electrical stimulation.

The results of the *in situ* stimulation experiments (Table 4) also demonstrate that inhibition of quinone reductase activity results in an increase in free radical damage, measured as TBARS via lipid peroxidation. Although such data are somewhat indirect and other explanations are plausible, they are consistent with a CoQ reductase function for DT-diaphorase under physiological conditions, and support the concept of an important function for DT-diaphorase in maintaining a significant level of the hydroquinone form of CoQ in the various membranes of the cell, thus allowing this hydroquinone to act as an effective antioxidant in such hydrophobic cellular compartments. Although the majority of DT-diaphorase activity resides in the cytosolic compartment, its assay *in vitro* requires the presence of non-ionic detergent for maximal activity. This suggests that the cytosolic DT-diaphorase may interact with membrane components located near the membrane–cytosol interface similar to the interaction of cytosolic ascorbate and membrane-bound α-tocopheroxyl radical to regenerate α-tocopherol, the reduced, antioxidant state of vitamin E (Beyer, 1994). The long (45 carbons in rats, 50 in humans) polyisoprenoid side chain of CoQ may allow

Table 4. Effect of dicoumarol administration on lipid peroxidation, coenzyme Q oxidation–reduction state and cytosolic DT-diaphorase activity of *in situ* electrically stimulated gastrocnemius muscle of rats

Treatment	Lipid peroxidation	CoQ (% reduced)	DT-diaphorase
Control*	27.7 ± 7.4	51	3.01 ± 0.41
Stimulated control	36.2 ± 5.4	36	2.87 ± 0.62
Dicoumarol*	44.4 ± 6.2	33	0.92 ± 0.21
Dicoumarol stimulated	96.4 ± 8.7	14	0.77 ± 0.17

In situ stimulation method as reported previously (Starnes *et al.*, 1987). Lipid peroxidation as μmols MDA/g tissue. 3 mg dicoumarol/100 g body weight administered 15 min prior to start of electrical stimulation.
*Control and dicoumarol-treated were the right contralateral gastrocnemius of left-limb-stimulated rats. Reduced state of CoQ was determined using a modification of Beyer (1989b). Cytosolic DT-diaphorase as nmols cytochrome c reduced/min/mg protein (Beyer *et al.*, 1988). Values as mean ± S.E.M.; n=8.

the quinone moiety of CoQ to move to the membrane surfaces (Siedo and Stidman, 1986; Lenaz *et al.*, 1992; Samori *et al.*, 1992; Fato *et al.*, 1986; Ondarroa and Quinn, 1986; Cornell *et al.*, 1987) in order to interact with, and receive reducing equivalents from DT-diaphorase. Very recent evidence (Salgado *et al.*, 1993) utilizing the magic angle spinning ^{13}C-NMR spin-lattice relaxation technique indicates that $CoQH_2$ can flip-flop across membranes, carrying reducing equivalents for energy conservation and/or antioxidation purposes. In addition, the report of three distinct CoQ pools in heart mitochondria (Jörgensen *et al.*, 1985) may be consistent with the notion of multiple function for CoQ.

Additional evidence supporting the concept of chemical interaction between DT-diaphorase and long polyisoprenoid side chain CoQ has appeared in recent reports of CoQ-binding proteins with DT-diaphorase activity in liver soluble fractions which may function to transport CoQ from its site of synthesis in the endoplasmic reticulum to various other sites (Kishi *et al.*, 1991), and the characterization of dicoumarol-sensitive NAD(P)H-dependent CoQ reductase activities in rat liver microsomes (Shigsemura *et al.*, 1993). It is also of interest that $CoQH_2$ has been reported to be a more efficient antioxidant with respect to lipid peroxidation of human low density lipoprotein when compared to α-tocopherol (Stocker *et al.*, 1991; Ingold *et al.*, 1993).

Conclusion

The question of whether CoQ should be considered an essential (primary) or a secondary (coincidental) antioxidant must be addressed. The voluminous litrature supporting its antioxidant role, in addition to beneficial effects of CoQ therapy, lacking detectable toxic side effects (Langsjoen *et al.*, 1991), effective in a large number of clinical pathologies reported in previous symposia on CoQ (Folkers and Yamamura, 1977, 1981, 1984, 1986; Folkers *et al.*, 1991; Lenaz, 1985; Lenaz *et al.*, 1990; Conference on Coenzyme Q, 1993; Yamamura *et al.*, 1980), would now appear

to support the concept of a primary, essential function for CoQ in acting as a chain-breaking antioxidant in the various membranes of the cell, in addition to its role in regenerating the active form of vitamin E.

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