Mitochondrial Ubiquinone Homologues, Superoxide Radical Generation, and Longevity in Different Mammalian Species

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Rates of mitochondrial superoxide anion radical (O$_2^-$) generation are known to be inversely correlated with the maximum life span potential of different mammalian species. The objective of this study was to understand the possible mechanism(s) underlying such variations in the rate of O$_2^-$ generation. The hypothesis that the relative amounts of the ubiquinones or coenzyme Q (CoQ) homologues, CoQ$_6$ and CoQ$_{10}$, are related with the rate of O$_2^-$ generation was tested. A comparison of nine different mammalian species, namely mouse, rat, guinea pig, rabbit, pig, goat, sheep, cow, and horse, which vary from 3.5 to 46 years in their maximum longevity, indicated that the rate of O$_2^-$ generation in cardiac submitochondrial particles (SMPs) was directly related to the relative amount of CoQ$_6$ and inversely related to the amount of CoQ$_{10}$ extractable from their cardiac mitochondria. To directly test the relationship between CoQ homologues and the rate of O$_2^-$ generation, rat heart SMPs, naturally containing mainly CoQ$_6$ and cow heart SMPs, with high natural CoQ$_{10}$ content, were chosen for depletion/reconstitution experiments. Repeated extractions of rat heart SMPs with pentane exponentially depleted both CoQ homologues while the corresponding rates of O$_2^-$ generation and oxygen consumption were lowered linearly. Reconstitution of both rat and cow heart SMPs with different amounts of CoQ$_6$ or CoQ$_{10}$ caused an initial increase in the rates of O$_2^-$ generation, followed by a plateau at high concentrations. Within the physiological range of CoQ concentrations, there were no differences in the rates of O$_2^-$ generation between SMPs reconstituted with CoQ$_6$ or CoQ$_{10}$. Only at concentrations that were considerably higher than the physiological level, the SMPs reconstituted with CoQ$_6$ exhibited higher rates of O$_2^-$ generation than those obtained with CoQ$_{10}$. These in vitro findings do not support the hypothesis that differences in the distribution of CoQ homologues are responsible for the variations in the rates of mitochondrial O$_2^-$ generation in different mammalian species.

A current hypothesis of aging postulates that oxidative stress/damage is a major causal factor in the attrition of functional capacity occurring during the aging process (1–6). The basic tenet of this hypothesis is that there is an intrinsic imbalance between the reactive oxygen species (ROS), that are incessantly generated in the aerobic cells and the antioxidative defense against them, thereby resulting in the accrual of steady-state levels of oxidative molecular damage. The direct evidence in support of this hypothesis is that the augmentation of antioxidative defenses by simultaneous overexpression of Cu/Zn superoxide dismutase, which converts superoxide anion radicals (O$_2^-$) into H$_2$O$_2$, and catalase, which removes H$_2$O$_2$, retards the age-associated increase in the levels of molecular oxidative damage and extends the life span of Drosophila melanogaster by one-third (7, 8).

Although there are several intracellular loci for the generation of O$_2^-$ (the first molecule in the ROS series), it is widely accepted that the mitochondrial electron transport chain is the main source of O$_2^-$ (9, 10). Previous studies in this laboratory have indicated that the rate of mitochondrial O$_2^-$ generation varies greatly, even in the same type of tissue, among different mammalian species and is inversely related to the maximum life span potential (MLSP) of the species (11, 12). The inverse relationship between the rate of O$_2^-$ generation and MLSP was found to hold in a sample of mammalian species as well as a group of dipteran insect species (11–13).

The question that arose out of these studies and that is also the subject of this investigation is what is the underlying mechanism for the variations in the rates of mitochondrial O$_2^-$ generation in different species? Although opinions vary (14), a number of experimental studies in the literature suggest that ubiquinones modulate the rate of mitochondrial O$_2^-$ generation (10, 15–18). Ubiquinones (2,3-dimethoxy-5-methyl-6-multiprenyl-1,4-benzoquinone), or coenzyme Q (CoQ), is a quinone derivative with a chain of 1–12 isoprene units in the different homologue forms (CoQ$_n$) occurring in nature. Relatively short-lived mammalian species such as the mouse and the rat primarily contain CoQ$_6$, whereas the larger long-lived mammals such as man predominantly exhibit CoQ$_{10}$ (19). The present study tests the hypothesis that variations in the rate of O$_2^-$ by cardiac submitochondrial particles (SMPs) in different mammalian species are related to the relative CoQ$_6$ and/or CoQ$_{10}$ content. The hypothesis was prompted by the fact that longevity of non-primate mammalian species tends to be inversely correlated with the rate of mitochondrial O$_2^-$ generation and directly correlated with the body mass.

EXPERIMENTAL PROCEDURES

Materials—All solvents used were of HPLC grade (Fisher). Ubiquinone-9, ubiquinone-10, (±)-tocopherol and ferricytochrome c (Type VI), superoxide dismutase, retinoic acid, antimony A, and 4,4-trifluoro-1-(2-thienyl)-1,3-butanedione (TFDA) were purchased from Sigma.

1 The abbreviation used are: ROS, reactive oxygen species; CoQ$_n$ coenzyme Q$_n$; CoQ$_{10}$; CoQ$_{10}$; MLSP, maximum life span potential; O$_2^-$, superoxide anion radical; SMPs, submitochondrial particles; TFDA, 4,4-trifluoro-1-(2-thienyl)-1,3-butanedione; HPLC, high performance liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid.
Variations in the Distribution of CoQ Homologues in Mitochondria of Different Species—Comparisons of the concentrations of CoQ₉ and CoQ₁₀ extracted from the heart mitochondria were made in nine different mammalian species, namely mouse, rat, guinea pig, rabbit, pig, goat, sheep, cow, and horse. The data, presented in Table I and Fig. 1, indicate that both the total as well as the relative concentrations of CoQ₉ and CoQ₁₀ in heart mitochondria vary greatly in different species. The total concentration of mitochondrial CoQ₉, i.e. CoQ₉ + CoQ₁₀, varied about 2-fold in different species with the rank order:
Coenzyme Q and Radical Generation

CoQ and Radical Generation—To determine the relationship between mitochondrial CoQ content and the rate of O$_2$ generation in different species, the amounts of CoQ$_{9}$ and of CoQ$_{10}$ were plotted against the average rates of O$_2$ generation by SMPs, partially determined in the context of previous studies (12). As shown in Fig. 1A, the amount of CoQ$_{9}$ was directly correlated and that of CoQ$_{10}$ was inversely correlated (Fig. 1B) with the rate of O$_2$ generation in different species. There was no correlation between the total amount of CoQ content and the rate of O$_2$ generation (data not presented).

The results of the correlational study, presented in Fig. 1, led to the question of whether the relationship between mitochondrial CoQ homologues and the rate of O$_2$ generation was purely coincidental or causally related. To investigate the possible existence of a causal relationship, SMPs were experimentally depleted of native CoQ and reconstituted with either CoQ$_{9}$ or CoQ$_{10}$. These studies were conducted on cardiac SMPs of rat, a short-lived species containing relatively high amounts of CoQ$_{9}$, and of cow, a representative of the long-lived species containing relatively low CoQ$_{10}$ content.

Effect of Depletion of CoQ Homologues on the Rates of Oxygen Consumption and O$_2$ Generation in Rat Heart SMPs—Repeated extractions with pentane were found to exponentially deplete the amount of native CoQ from the rat heart SMPs (Fig. 2, inset); the amount remaining after six serial extractions was about 4.5% of the total amount extractable by hexane. In contrast, apparently due to the much lower natural content of CoQ$_{10}$, only three extractions with pentane were sufficient to deplete SMPs of CoQ$_{10}$ to a level below the detection threshold of 0.2 nmol (i.e. 0.015 nmol/mg of SMP protein).

To determine the effect of pentane extractions on the functional state of the SMPs, rates of oxygen consumption and O$_2$ generation were determined after each extraction procedure. The rate of succinate-supplemented oxygen consumption was highest in the unextracted SMPs, decreasing linearly following each extraction procedure, reaching 25% of the initial value after seven successive extraction procedures (Fig. 2). Addition of antimycin A and TTFA greatly reduced (to <2%) the rate of oxygen consumption by the depleted SMPs, whereas rotenone had no effect, indicating that O$_2$ consumption observed was specifically due to succinate oxidase activity. NADH did not, in most instances, stimulate the rate of oxygen consumption by the depleted SMPs.

A similar study was conducted on the effect of various pentane extractions on the rate of O$_2$ generation by the SMPs. Again, the rate of O$_2$ generation was highest in the unextracted SMPs and progressively declined with each sequential pentane extraction, reaching 45% of the control value after six extraction procedures, where less than 5% of the original CoQ was present (Fig. 3).

Overall the results of the depletion experiments indicated that even after six or seven serial extractions with pentane the SMPs exhibited succinate oxidase activity and were able to generate O$_2$ albeit at rates lower than the unprocessed SMPs.

Effects of Reconstitution of Rat Heart SMPs with CoQ Homologues—Rat heart SMPs that had been extracted with pentane six times, as described above, were reconstituted with different amounts of CoQ$_{9}$ and CoQ$_{10}$. Reconstitution with increasing amounts of CoQ$_{9}$ or CoQ$_{10}$ caused an initial steep increase in the succinate-supplemented rate of oxygen consumption, which was followed by a plateau (Fig. 4B, inset). No significant differences in the rates of oxygen consumption were observed between the SMPs reconstituted with equal amounts of CoQ$_{9}$ or CoQ$_{10}$.

Reconstitution of the depleted rat heart SMPs with increasing amounts of CoQ$_{10}$ resulted in an initial sharp rise in the rate of O$_2$ generation, followed by a more gradual increase (Fig. 4A, inset). At the highest concentration of repleted CoQ$_{9}$ used in
In this study, the rate of $O_2^-$ generation increased about 2-fold as compared with the depleted SMPs. Addition of increasing amounts of CoQ$_{10}$ to the depleted SMPs also caused an initial steep rise in the rate of $O_2^-$ generation, but unlike CoQ$_9$ further additions did not result in correspondingly increased rates of $O_2^-$ generation (Fig. 4A). For example, rat heart SMPs reconstituted with 50 nmol of CoQ$_9$ exhibited a rate of $O_2^-$ generation that was 40% higher than when an equal amount of CoQ$_{10}$ was used for reconstitution. The rates of succinate-supplemented oxygen consumption and $O_2^-$ generation by SMPs and CoQ concentrations, within the physiological range. Data are mean ± S.E. of three independent experiments.

**FIG. 4. Rates of oxygen consumption and $O_2^-$ generation in CoQ-depleted/reconstituted rat heart SMPs.** Freeze-dried SMPs were depleted of native CoQ homologues by six repeated pentane extractions and reconstituted with specific amounts of CoQ$_9$ or CoQ$_{10}$ in pentane. The reconstituted SMPs were dried and suspended in phosphate buffer, and rates of $O_2^-$ generation, shown in A, were measured as superoxide dismutase-inhibitable reduction of acetylated ferricytochrome c. Rates of oxygen consumption, shown in B, were determined polarographically with a Clark-type electrode using 7 mM succinate as a substrate. The insets depict the relationship between rates of $O_2^-$ generation and oxygen consumption by SMPs and CoQ concentrations, within the physiological range. Data are mean ± S.E. of three independent experiments.

**Effects of Reconstitution with CoQ$_9$ and CoQ$_{10}$ on Bovine Heart SMPs—**In contrast to the rat, bovine cardiac SMPs contain a relatively high amount of CoQ$_{10}$ and a small amount of CoQ$_9$ (see Table I). Depletion of bovine SMPs by six serial extractions with pentane achieved a 96% extraction of CoQ$_{10}$ and virtually the entire amounts of the detectable CoQ$_9$. Reconstitution of these depleted SMPs with varying concentrations of CoQ$_9$ or CoQ$_{10}$ indicated different patterns for the two homologues for the rate of oxygen consumption and $O_2^-$ generation. Augmentation of SMPs with relatively low amounts of CoQ$_9$ or CoQ$_{10}$ caused a sharp increase in both the rate of oxygen consumption (Fig. 5B, inset) and $O_2^-$ generation (Fig. 5A, inset), but at higher concentrations these rates leveled off. Within the physiological range of CoQ content (Table I), there were no differences in the rates of $O_2^-$ generation between SMPs reconstituted with CoQ$_9$ or CoQ$_{10}$ caused a sharp increase in both the rate of oxygen consumption (Fig. 5B, inset) and $O_2^-$ generation (Fig. 5A, inset), but at higher concentrations these rates leveled off. Within the physiological range of CoQ content (Table I), there were no differences in the rates of $O_2^-$ generation between SMPs reconstituted with CoQ$_9$ or CoQ$_{10}$ (Fig. 5A, inset). The differences in the maximal rates of $O_2^-$ generation were greater for CoQ$_9$ than for CoQ$_{10}$. For example, in the bovine SMPs, reconstituted with 50 nmol of CoQ$_9$, the rate of $O_2^-$ generation was 35% greater than in the SMPs reconstituted with an equal amount of CoQ$_{10}$ (Fig. 5A).

To further determine whether CoQ$_9$ and CoQ$_{10}$ content above the in vivo level had a different effect on the rate of $O_2^-$ generation, freeze-dried unextracted bovine SMPs were augmented with CoQ$_9$ or CoQ$_{10}$. As shown in Fig. 6, the rates of $O_2^-$ generation were stimulated to a greater extent by the addition of CoQ$_9$ than CoQ$_{10}$. The differences in the rate of $O_2^-$

**FIG. 5. Rates of oxygen consumption and $O_2^-$ generation in CoQ-depleted/reconstituted bovine heart SMPs.** Freeze-dried SMPs were depleted of native CoQ by six repeated extractions with pentane and reconstituted with specific amounts of CoQ homologues as described in Fig. 4, and the rates of $O_2^-$ generation, shown in A, were measured. Plot B shows the rates of succinate-supplemented oxygen consumption of the SMPs from the same set of experiments. The insets depict the relationship between rates of $O_2^-$ generation and oxygen consumption by SMPs and CoQ concentrations, within the physiological range. Data are mean ± S.E. of three independent experiments; S.E. is not shown in the inset.
generation tended to increase with the augmented amounts of CoQ₉ or CoQ₁₀ in the SMPs.

**DISCUSSION**

Results of this study indicated that rates of $O_2^\cdot$ generation in different mammalian species are directly correlated with the amounts of mitochondrial CoQ₉ and are inversely related to the CoQ₁₀ content. The *in vitro* depletion/reconstitution studies do not, however, support the hypothesis that variations in the relative concentrations of CoQ₉ and CoQ₁₀ are directly involved in the modulation of rates of $O_2^\cdot$ generation in mitochondria. Although it is widely believed that the components of the mitochondrial respiratory chain, located within the domains of NADH- and succinate-cytochrome c reductase, are the main sites of $O_2^\cdot$ generation (9, 10), there are at least two different schools of thought about whether or not autoxidation of ubisemiquinone is indeed the actual source of $O_2^\cdot$ generation. The view, implicating ubisemiquinone (15–18), is based on the following evidence: (i) rates of $O_2^\cdot$/H₂O₂ generation by SMPs and/or mitochondria are highest in the presence of rotenone and antimycin A with succinate as the substrate (9); (ii) bovine heart SMPs, depleted of endogenous CoQ and reconstituted with variable amounts of exogenous CoQ, exhibit a linear relationship with the amount of quinone added (15, 16); (iii) rates of $O_2^\cdot$ generation by isolated NADH-ubiquinone reductase particles, supplemented with different CoQ homologues, were found to be linearly dependent on the amount of exogenous CoQ (17); and (iv) in reconstituted SMPs, activities of succinate dehydrogenase and succinate-cytochrome c reductase reached a plateau at relative low concentrations of reducible CoQ, whereas the rate of H₂O₂ generation was linearly related to a higher range of CoQ concentration (16). In the opposing view (14), such observations do not necessarily establish a direct association between autoxidation of ubisemiquinone and $O_2^\cdot$ generation. It is argued that such findings are also compatible with the interpretation that the iron-sulfur centers in mitochondrial respiratory complex I (NADH-ubiquinone reductase), II (succinate-ubiquinone reductase), or III (ubiquinolcytochrome c reductase) may be the sources of $O_2^\cdot$ generation. While the results of the present study are insufficient to resolve this controversy, they however support the view that CoQ is directly or indirectly associated with the modulation of the rates of $O_2^\cdot$ generation, since experimental variations in its content have a clear effect on the rate of $O_2^\cdot$ generation.

Results of this study should be interpreted in light of the fact that the preparatory procedures involving freeze-drying and pentane extractions, although widely employed (15–17, 26, 27), have an irreversible effect on the functional state of SMPs. For example, freeze-drying of SMPs followed by depletion and reconstitution with the original (natural) amount of CoQ decreased the rate of $O_2^\cdot$ generation by about 30% and of oxygen consumption by about 60%. However, the rates of $O_2^\cdot$ generation and oxygen consumption of pentane-extracted SMPs, reconstituted with relatively high concentrations of CoQ₉ or CoQ₁₀, reached the level comparable to the unextracted freeze-dried preparations. In both the rat and the bovine heart SMPs there were no differences in the rates of $O_2^\cdot$ generation between SMPs reconstituted with the *in vivo* amounts of CoQ₉ or CoQ₁₀. Only at concentrations higher than those present under physiological conditions relatively higher rates of $O_2^\cdot$ generation were obtained with CoQ₉ than with CoQ₁₀. The same tendency was observed when unextracted bovine SMPs were augmented with CoQ₉ or CoQ₁₀. Altogether results of depletion/reconstitution and/or augmentation studies suggest that CoQ₉ and CoQ₁₀ differ in their *in vitro* effect on the rate of $O_2^\cdot$ generation only at concentrations that exceed the *in vivo* amounts.

A question arising from this study and also having some bearing on the interpretation of the present results concerns the nature of the structural differences between CoQ₉ and CoQ₁₀ molecules that apparently exert an effect on the rate of $O_2^\cdot$ generation, albeit at high concentrations. Indeed, the molecular structural differences between CoQ₉ (C₅₆H₈₄O₄; $M_r$ 794; melting point 44–45 °C) and CoQ₁₀ (C₆₅H₉₀O₄; $M_r$ 862; melting point 49 °C) (28) are relatively minor. Nevertheless, the relative length of the polyprenoid chain and the resultant effects on the hydrophobicity of the molecule have been shown to have an effect on the location of the molecule within the phospholipid bilayer of the cell membrane. Although the relative position of CoQ₉ and CoQ₁₀ in the phospholipid bilayer has not been precisely determined, the CoQ homologues with relatively short polyprenoid chains are believed to lie closer to the surface of the bilayer, whereas the long chained ones are thought to be nearer to the center of the bilayer (29–32). For example, studies by Kagan et al. (33) have shown that short chain ubiquinols are relatively more efficient in inhibiting Fe²⁺-ascorbate-induced lipid peroxidation, suggesting that the polyprenoid chain length has an effect on the interaction between the quinols and the ROS present in the aqueous phase.

Studies by Matsura et al. (34) on rat and guinea pig hepatocytes also implicate major differences in antioxidant efficiency between the reduced CoQ₉ and the reduced CoQ₁₀. In response to the hydrophilic radical initiator, 2,2-azobis(2-aminopropane) dihydrochloride, CoQ₉ was found to be preferentially oxidized as compared with CoQ₁₀ homologue and thus may be more accessible to ROS present in the surrounding phase. This mechanism, albeit hypothetical, may underlie the relatively higher rates of $O_2^\cdot$ generation in highly CoQ₉-rich SMPs observed in this study.

Functional differences between CoQ₉ and CoQ₁₀ have also been reported by Edlund et al. (35), who found that treatment of mumps virus-infected cultured neurons with CoQ₁₀ protected the cells from degeneration, whereas no effects were observed in response to CoQ₉ treatment. Results of the present *in vitro* studies demonstrate that CoQ₉ and CoQ₁₀ homologues can differentially affect the rate of $O_2^\cdot$ generation at high concentrations; however, the *in vivo* variations in rates of mitochondrial $O_2^\cdot$ generation among different mammalian species

![Image](73x531 to 283x729)

**FIG. 6. Rates of $O_2^\cdot$ generation in CoQ-augmented bovine heart SMPs.** Freeze-dried unextracted SMPs were supplemented with known amounts of CoQ₉ or CoQ₁₀ in pentane, dried, and suspended in phosphate buffer. Rates of $O_2^\cdot$ generation were measured as superoxide dismutase-inhibitable reduction of acetylated ferricytochrome c. Data are mean ± S.E. of three independent experiments.
cannot be explained on the basis of relative CoQ$_9$ or CoQ$_{10}$ content.

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