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Electron Transport Between Cytochrome c and Alpha Tocopherol

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SUMMARY: Using liposomes we have demonstrated an electron transfer between tocopherol (vitamin E) and cytochrome c. Reduced cytochrome c protects vitamin E from oxidation induced either directly by ultraviolet light or indirectly by soybean lipoxygenase-catalyzed oxidation of arachidonic acid. Oxidized cytochrome c is reduced by tocopherol and tocopherol homologues (chromanols) resulting in accumulation of tocopheroxyl radicals which we detected by ESR. The peak height of the ESR spectrum of tocopheroxyl radicals (which is proportional to the amount of radical present) is proportional to the ratio of reduced to oxidized cytochrome c. In mitochondrial membranes succinate-cytochrome c reduction is inhibited by antimycin A. Addition of exogenous chromanols facilitates a by-pass of the antimycin A blocked electron pathway, and succinate-dependent cytochrome c reductase activity is restored. Cytochrome c may act as a water-soluble complement to the lipid-soluble ubiquinol in regenerating mitochondrial tocopherol from tocopheroxyl radical.

When alpha-tocopherol, which is considered the principal lipid-soluble chain breaking antioxidant in membranes (1), reacts with a peroxyl radical it is converted to its one electron oxidation product, the tocopheroxyl radical. It is possible to detect the tocopheroxyl radical by ESR in mitochondrial membranes isolated from rat livers enriched with vitamin E through dietary supplementation and exposed to an exogenous alpha-tocopherol oxidant. Concomitant measurement of alpha-tocopherol content demonstrates that alpha-tocopherol consumption accompanies tocopheroxyl radical appearance (2). The molecular mechanisms of the regeneration (recycling) of alpha-tocopherol from the tocopheroxyl radical are not yet fully understood. Ascorbate has been shown to reduce the tocopheroxyl radical back to tocopherol in vitro (3), but accumulation of ascorbate in the mitochondrial intermembrane space is not likley due to the presence of ascorbate-cytochrome oxidase, which readily oxidizes ascorbate (4).

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Abbreviations: MOPS, (3-[N-morpholino]propane sulfonic acid); PMC, 2,2,5,7,8, penta-methyl 6-hydroxy chromanol; SMP sub-mitochondrial preparation.

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The inner mitochondrial membrane contains both ubiquinol and alpha tocopherol, both of which have been reported to be effective scavangers of reactive free radicals (5-9). While some controversy exists as to which compound is the primary scavanger in mitochondrial membranes, ubiquinol is effective in preventing lipid peroxidation in liposomes in the absence of alpha tocopherol (3) and it may well have an antioxidant function in mitochondria (9-13). Studies using succinate-ubiquinone reductase incorporated into liposomes have shown that ubiquinol can recycle tocopheroxyl radicals in liposomes (14). While reduced succinate-ubiquinone reductase in the absence of quinone is ineffective in recycling alpha-tocopherol, reduced cytochrome c has been shown to to prevent alpha-tocopherol loss in mitochondria during oxidative stress in the presence of KCN (2).

In the present study we investigated the interaction of cytochrome c with alpha tocopherol and tocopheroxyl radical using both liposomes and mitochondrial membranes. The liposomes were used to determine if reduced cytochrome c can protect alpha-tocopherol from oxidative consumption independently of ubiquinol and to assess electron exchange between cytochrome c and alpha tocopherol/tocopheroxyl radical. Mitochondrial membranes were used to investigate potential roles of interactions of chromanols with cytochrome c .

MATERIALS AND METHODS

Liposome Preparation: Liposomes were prepared by dissolving phosphatidylcholine (Avanti Chemical Co.) (1.5mg), phosphatidyl serine (1.5mg), and various amounts of d,l-alpha-tocopherol, depending on the experiment (Sigma Chemical Co) in chloroform-hexane, then drying the mixture with a stream of nitrogen gas. 1ml of 50mM (3-[N-morpholino]propane-sulfonic acid) (MOPS), pH 7.5 was added to the dried mixture, which was then stirred and sonicated to a milky suspension in a bath sonicator. Subsequent sonication of the sample with a Branson sonicator using the micro-tip for 1-2 minutes resulted in a clarification of the suspension and liposome formation. Liposomes prepared in a similar manner have been used in previous studies (14). Alpha-C-1 chromanol,(2,2,5,7,8, penta-methyl 6-hydroxy chromane) and alpha-C-6-chromanol (2,5,7,8, tetramethyl-2 (4-methyl pentyl)-6-hydroxy chromane) are homologues of alpha tocopherol which have shorter hydrophobic tails than alpha tocopherol. Stock ethanol solutions of the homologues (100mM) were added to the liposomes to the final indicated concentrations. These reagents were a gift from Prof. E. Evstigneeva, Institute of Fine Chemical Technology, Moscow, Russia.

Preparation of cytochrome c, measurement of alpha-tocopherol, and determination of protein: A 20mM solution of horse heart cytochrome c (Sigma type III) was prepared in deionized water. Half of the cytochrome c solution was reduced with dithionite (0.2M) buffered with 50mM MOPS, pH 7.5 without adding a substantial excess of reductant. Cytochrome c reduction was monitored by measuring the absorbance of sample at 550nm. Both the reduced and oxidized samples were dialyzed for 24 hours against anaerobic 5mM MOPS, pH 7.5, 4^{o}C, using 1ml of sample/500ml of dialysis buffer. The dialyzing buffer was changed three times. After dialysis the cytochrome c samples were concentrated in the dialysis bag by removal of buffer with dry Sephadex. The reduced sample was assayed to be sure there was no detectable remaining dithionite by measuring oxygen consumption polarographically in aerobic buffer. The reduced cytochrome c solution was >96% reduced and contained no residual dithionite. The oxidized cytochrome c was checked to see if it contained any appreciable reduced component by monitoring oxygen consumption polarographically in the presence of submitochondrial particle (SMP) membranes. No detectable reduced cytochrome c was found in the oxidized sample. Alpha-tocopherol concentration was measured using HPLC with an amperometric detector as previously described (15).Protein was determined by the method of Lowry et al. (16).

Oxidation of membrane-bound alpha-tocopherol: For direct oxidation of alpha-tocopherol by cytochrome c, liposomes with 0.08 mg/ml alpha-tocopherol were diluted 1:10 in MOPS buffer, 50 mM, pH 7.5,and oxidized cytochrome c was added to a final concentration of 2 mM.

Ultraviolet irradiation was used to directly induce tocopheroxyl radical formation from tocopherol. Liposomes, containing 0.08 mg/ml alpha-tocopherol were diluted 1:5 in MOPS buffer, 50 mM, pH 7.5. For irradiation in which cytochrome c was present its concentration was 2 mM. The sample (0.2 ml) was placed in a 0.2mm path length quartz cell 13cm from the output of a Xenon lamp (Solar Light Company, Philadelphia, PA, Model 14S). The sample was mixed every 10 minutes, as the beam did not irradiate the entire sample. As alpha-tocopherol has an absorption peak at 291nm (17) and cytochrome c has an absorption minimum at around 290nm (18) a band width filter was used to select ultraviolet irradiation near these wavelengths. An HCC-3 band-width filter (from Mashpriborintorg Co. U.S.S.R.) was placed two cm in front of the sample. The absorption of this filter at 310nm was 0.44, while at 338nm and 282nm the absorption was >2.5. Samples were kept aerobic during incubations.

Soybean lipoxygenase-catalyzed oxidation of arachidonic acid was used for enzymatic formation of peroxyl radicals, resulting in accumulation of tocopheroxyl radical and loss of alpha-tocopherol, as in previous studies. (2,14). Stock liposomes as described above, containing 0.08 mg/ml alpha-tocopherol were diluted 1:10 in MOPS buffer, 50mM pH 7.5. The arachidonic acid was added to 0.15mM from a 30 mM stock solution. The cytochrome c concentration was 3 mM and the reaction was started by addition of lipoxygenase to a final concentration of 0.19 mg/ml.

Reduction of cytochrome c by liposomal alpha-tocopherol: Liposomes were prepared with differing concentrations of alpha tocopherol. Oxidized cytochrome c (133uM) was addded to these liposomes and the initial rate of cytochrome c reduction was measured at 37°C by following its absorbance increase at 550 nm.

ESR Measurements: ESR measurement of tocopheroxyl radical was made with a Varian E109E X-band spectrometer. Liposomes with alpha tocopherol (1.5mg lipid/ml, 0.04mg/ml alpha tocopherol), in 50mm MOPS, pH 7.5 were oxidized by either oxidized cytochrome c or lipoxygenase (0.06mg, 15units/ml) and arachidonic acid (2mM), or a mixture of reduced and oxidized cytochrome c (2.8 mM total concentration). For the (lipoxygenase + arachidonic acid) system, samples were run in the presence of reduced or oxidized cytochrome c (2mM). ESR spectra were recorded at 100mW microwave power, modulation was 2.5 gauss at 100 KHz and the scan speed was 12.5 gauss/minute. Samples were measured in gas permeable tubing over which passed a stream of oxygen or nitrogen as has been previously described (2).

Mitochondrial membrane preparation, enzyme activities and rat liver submitochondrial particle (SMP) membranes were prepared by sonicating isolated liver mitochondrial membranes as previously described (19). Assays of succinate cytochrome c reductase were carried out at 37°C in 50mM MOPS buffer, pH 7.5. Succinate (10mM) was incubated with the SMP (at 37°C) in the presence of KCN to activate the succinate dehydrogenase fully and to deplete the enzyme of any bound oxaloacetate (20). Reduction of cytochrome c was measured by following its absorbance increase at 550 nm.

RESULTS

Protection of liposomal alpha tocopherol by addition of reduced cytochrome c. Oxidized cytochrome c added to liposomes containing alpha-tocopherol without any other oxidants resulted in a steady loss of alpha tocopherol (Fig. 1A). Reduced cytochrome c had no effect. Liposomes containing alpha tocopherol and cytochrome c were also exposed to oxidation either indirectly from lipoxygenase-catalyzed oxidation of arachidonic acid or directly from ultraviolet irradiation. Alpha tocopherol was consumed far more rapidly in the presence of oxidized cytochrome c than in the presence of reduced cytochrome c (Fig. 1 B, C). The rate of loss of alpha tocopherol with only added oxidized cytochrome c was less than when liposomes were oxidized by lipoxygenase or ultraviolet irradiation, in the presence of oxidized cytochrome c. Ultraviolet irradiation of liposomes with no cytochrome c added caused a slightly increased rate of alpha-tocopherol consumption compared to samples with oxidized cytochrome c This was probably due to increased ultraviolet absorption of alpha tocopherol in the absence of cytochrome c (data not shown).

Reduction of cytochrome c by alpha tocopherol and other chromanols. Liposomes containing differing alpha tocopherol concentrations reduced oxidized cytochrome c with an initial

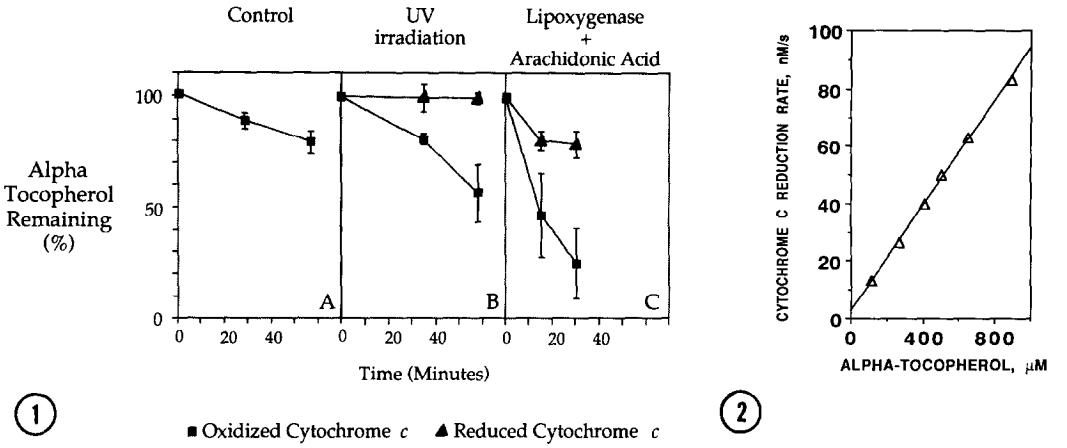

Figure 1. Protection of alpha-tocopherol by reduced cytochrome c in liposomes exposed to oxidants. Panel A,loss of alpha-tocopherol in the presence of oxidized cytochrome c (2mM); Panel B, loss of alpha-tocopherol induced by ultraviolet light in the presence of oxidzed or reduced cytochrome c; Panel C,Loss of alpha-tocopherol induced by arachidonic acid plus soybean lipoxygenase in the presence of oxidized cytochrome c. Error bars represent the standard deviation for each point with the initial measurement for each sample being defined as 100% tocopherol (each point represents 3 samples, except for panel C, in which each point represents 4 samples for reduced cytochrome c and 7 samples for oxidized cytochrome c).

Figure 2. Rates of reduction of cytochrome c by liposomal alpha tocopherol. Liposomes were prepared with different alpha tocopherol concentrations and the initial rate of succinate driven cytochrome c reduction was plotted against the alpha tocopherol concentration.

rate that was linear with respect to the alpha tocopherol concentrationn (Fig. 2). Reduction of cytochrome c also occured with liposomes containing 2,2,5,7,8, penta-methyl 6-hydroxy chromanol (PMC), which is a less lipophilic homologue of alpha tocopherol (data not shown).

ESR measurements of tocopheroxyl radical. Adding either oxidized cytochrome c or lipoxygenase and arachidonic acid to liposomes containing tocopherol results in a tocopheroxyl radical ESR spectrum (2). When no oxidant or reduced cytochrome c is added there is no ESR signal (Fig. 3 A,B), whereas if oxidized cytochrome c is added the tocopheroxyl radical signal appears (Fig. 3 C). A similar signal appears if (lipoxygenase + arachidonate), rather than oxidized cytochrome c, is added (Fig. 3 D); this signal is completely abolished by the further addition of reduced cytochrome c (Fig. 3 E). If alpha-C-6-chromanol, added in an ethanol solution (2mM) is substituted for alpha tocopherol a chromanoxyl radical signal is also observed (data not shown). Changing the ratio of reduced/oxidized cytochrome c in the solution added to alpha tocopherol-containing liposomes resulted in the formation of tocopheroxyl radical ESR signals whose peak height was linearly dependent on the log of reduced cytochrome c/oxidized cytochrome c (Fig. 3 F). The formation of tocopheroxyl radical from tocopherol-containing liposomes induced by cytochrome c can also be measured anaerobically.

Redox cycling of chromanols in antimycin A-treated mitochondrial membranes. Succinate oxidation in mitochondria is inhibited by antimycin A, which specifically inhibits the cytochrome $b\text{-}c_1$ area of the mitochondrial respiratory chain (21). In KCN-treated SMP incubated with

Table 1
Cytochrome *c* Reduction By Succinate and Vitamin E Homologues In Rat Liver Submitochondrial Particles

Additions	Succinate	Antimycin A	Cytochrome *c* Reduction (nmoles/mg protein/minute)
	+	-	370.0
	+	+	23.9
+ α-C-1-Chromanol	+	+	225.8
+ α-C-1-Chromanol	-	+	57.2
+ α-C-6-Chromanol	+	+	78.8
+ α-C-6-Chromanol	-	+	27.9

Conditions: SMP (27μg protein/ml), Cytochrome *c* (100μM), KCN (2.0mM) in 50mM MOPS (pH 7.4 at 37°C). Succinate (50mM), antimycin A (1μg/ml) and chromanols (2mM).

combinations of antimycin A and added exogenous chromanols, the initial rate of cytochrome c reduction used to assess the electron flow through the cytochrome b-c_1 area) was much greater with than without succinate (Table 1). The more water-soluble homologue of tocopherol, alpha-C-1 chromanol, provided better electron bypass than alpha-C-6 chromanol. The sites of inhibition of antimycin and KCN and the hypothesized bypass of these sites by chromonols are shown in Fig 4.

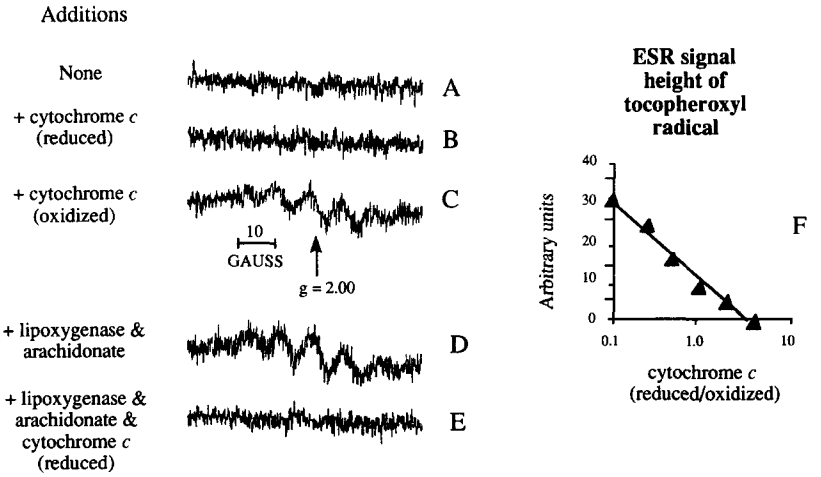

Figure 3. ESR spectra of tocopheroxyl radicals in liposomes containing alpha tocopherol. A) alpha-tocopherol containing liposomes. B) Same as A) plus reduced cytochrome c. C) Same as A) plus oxidized cytochrome c. D) Sames as A) plus lipoxygenase + arachidonate. E) Same as D) plus reduced cytochrome c. F) Tocopheroxyl radical signal peak height (peak indicated by an arrow), in the presence of 2.8 mM cytochrome c, reduced and oxidized as indicated.

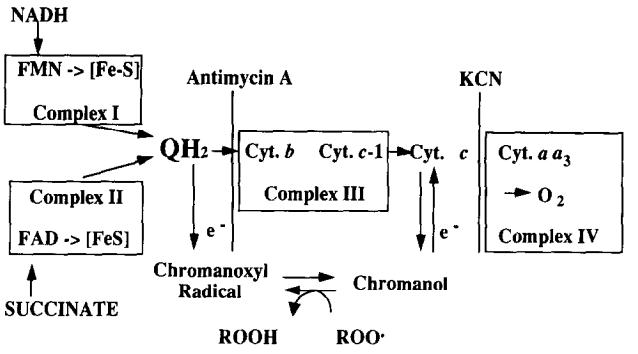

Figure 4. Schematic diagram of the electron carriers of the mitochondrial respiratory chain. The site of inhibition of the respiratory inhibitors antimycin A and KCN are indicated. Electron flow which bypasses Complex III is indicated.

DISCUSSION

We have demonstrated that reduced cytochrome c can prevent oxidative loss of alpha tocopherol in liposomes and oxidized cytochrome c is an oxidant for both alpha tocopherol and its less lipophilic homologues alpha-C-1-chromanol and alpha-C-6-chromanol. The interaction of tocopherol and tocopheroxyl radical with cytochrome c is the first demonstration of a reaction between alpha tocopherol and a redox protein. These results suggest a physiological role for cytochrome c as a reductant of mitochondrial alpha-tocopherol.

Electron transfer from cytochrome c to tocopheroxyl radical in mitochondria may serve an important function under physiological conditions by helping recycle vitamin E in the intermembrane space. The mitochondrial intermembrane space is a location where toxic free radicals, derived from the electron transport chain of the inner membrane, are found and these free radicals will initiate lipid peroxidation. Ascorbate, a water soluble reductant which is known to regenerate tocopherol from tocopheroxyl radicals (3) would likely not accumulate in this space as it is the site of ascorbate-cytochrome c oxidase, which would rapidly oxidize ascorbate. While the concentration of cytochrome c in the mitochondrial inter-membrane space is not known, as this space changes its volume appreciably depending on the respiratory state of the mitochondria (22), reduced cytochrome c as a polar and mobile reductant of tocopheroxyl radical is certainly present and may have a physiological role in alpha tocopherol recycling, serving as a complement to the lipid-soluble ubiquinol.

Furthermore, the reduction of tocopheroxyl radicals by reduced cytochrome c is more favorable to the energy balance of the cell than reduction by ubiquinol, as the redox midpoint potential of ubiquinol (+45mV) is significantly lower that that of cytochrome c (+254mV) (23). An electron passing from ubiquinol to cytochrome c via the respiratory chain passes through an ATP coupling site.

While ubiquinols are important reductants of mitochondrial tocopheroxyl radical, their function may be restricted to the mitochondrial inner membrane. Reduced cytochrome c may recycle a population of inner membrane tocopheroxyl radical that is not readily accessible to

ubiquinol and it could protect the inner side of the outer mitochondrial membrane. Since cytochrome c is impermeant to the membrane it would likely only react on the intermembrane space-accessible membrane. The protection provided by the reduced cytochrome c may be enhanced by the stable nature of tocopheroxyl radical (24). Cytochrome c is present in the intermembrane space and is normally 25% reduced in respiring isolated mitochondria (25) and 10-20% in perfused whole liver (26). Both ubiquinol and cytochrome c are mobile reductants in lipid and aqueous phases, respectively. Their mobility may have a role in their effectiveness in reduction of tocopheroxyl radical which presumably can be formed at multiple sites within the membrane.

The electron bypass of ubiquinol-cytochrome c reductase through chromanols (Table I & Fig. 4) represents a newly defined role for these compounds. Is there a physiological role for an electron shuttle around ubiquinol-cytochrome c reductase? Demonstration of an electron bypass around ubiquinol-cytochrome c reductase inhibited by antimycin A suggests two potentially new roles for chromanols. As added alpha-C-6 and alpha-C-1 chromanol stimulates electron flow around ubiquinol-cytochrome c reductase, dietary supplementation with chromanols or other phenolic drugs might be expected to increase basal metabolic rate by partial uncoupling of mitochondrial electron flow. Also, chromanols might be useful to bypass electron flow in patients with mitochondrial myopathies in which electron flow is blocked, or partially blocked in the ubiquinol-cytochrome c reductase region (27).

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