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INHIBITORY EFFECT OF α-TOCOPHEROL AND ITS DERIVATIVES ON BOVINE HEART SUCCINATE-CYTOCHROME *c* REDUCTASE

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α-Tocopherol and its derivatives inhibit succinate-cytochrome *c* reductase activity at a concentration of 0.5 μmol/mg protein in 50 mM phosphate buffer, pH 7.4, containing 0.4 % sodium cholate when α-tocopherol is predispersed in sodium cholate solution. The inhibitory site is located at the cytochrome *b*-c_1 region. Succinate-ubiquinone reductase activity of succinate-cytochrome *c* reductase was not impaired by treatment with α-tocopherol. The α-tocopherol-inhibited succinate-cytochrome *c* reductase activity can be reversed by the addition of ubiquinone and its analogs. When ubiquinone- and phospholipid-depleted succinate-cytochrome *c* reductase was treated with α-tocopherol followed by reaction with a fixed amount of 2,3-dimethoxy-6-methyl-5-(10-bromodecyl)-1,4-benzoquinone and phospholipid, the amount of α-tocopherol needed to express the maximal inhibition was only 0.3 μmol/mg protein. When ubiquinone- and phospholipid-depleted enzyme was treated with a given amount of α-tocopherol and followed by titration with 2,3-dimethoxy-6-methyl-5-(10-bromodecyl)-1,4-benzoquinone, restoration of activity was enhanced at low concentrations of ubiquinone analog, indicating that α-tocopherol can serve as an effector for ubiquinone. The maximal binding capacity of α-[^{14}C]tocopherol, dispersed in 50 mM phosphate buffer containing 0.25% sodium cholate, pH 7.4, to succinate-cytochrome *c* reductase was shown to be 0.68 μmol/mg protein. A similar binding capacity, based on cytochrome *b* content, was observed in submitochondrial particles. Binding of α-tocopherol to succinate-cytochrome *c* reductase not only caused an inhibition of enzymatic activity but also caused a reduction of cytochrome c_1 in the absence of substrate, a phenomenon analogous to the removal of phospholipids from the enzyme preparation. Furthermore, binding of α-tocopherol to succinate-cytochrome *c* reductase decreased the rate of reduction of cytochrome *b* by succinate. Since electron transfer from succinate to ubiquinone was not affected by α-tocopherol treatment, the decrease in reduction rate of cytochrome *b* by succinate must be due to a change in environment around cytochrome *b*. These results as well as the fact that reactivation of α-tocopherol-inhibited enzyme requires only low concentrations of ubiquinone were used to explain the inhibitory effect as a result of a change in protein conformation and protein-phospholipid interaction rather than the direct displacement of ubiquinone by α-tocopherol. This deduction was further supported by the fact that no ubiquinone was released from succinate-cytochrome *c* reductase upon treatment with α-tocopherol.

Introduction

α-Tocopherol and its derivatives are generally known as Vitamin E. The biochemical function of

Abbreviations: Q_2, 2,3-dimethoxy-5-geranyl-6-methyl-1,4-benzoquinone; $Q_0(CH_2)_{10}Br$, 2,3-dimethoxy-6-methyl-5-(10-bromodecyl)-1,4-benzoquinone.

this vitamin is not yet clear. It is believed that one of the main functions of vitamin E is that of a biological antioxidant [1,2]. It may act by scavenging free radicals and singlet oxygen [3–6], thus assisting in maintenance of the structural integrity of the membranes [7,8] of cells or organelles. Effects of vitamin E on membrane stability and permeability have also been proposed [8].

Kitabchi et al. [9] have demonstrated the existence of a specific binding site (receptor) for α-tocopherol in the adrenocortical cell membranes, and the binding was shown to be sensitive to heat and trypsin treatment, indicating that a protein is, at least partly, involved in the α-tocopherol binding. The α-tocopherol-binding (transfer) protein has also been shown to exist in the cytosol of rat liver [10,11]. These results suggest that α-tocopherol plays an important role in the membrane through binding to a specific membrane protein.

The structural similarity between α-tocopherol, especially its quinone form, and ubiquinone, and the acceptance of the role of the latter in mitochondrial and photosynthetic electron-transfer chains led us to investigate the possible role of α-tocopherol in the electron-transfer reaction. In fact, before the identification of ubiquinone, α-tocopherol was believed to be directly involved in electron transfer [12], however, this idea has since been disproved. Recent study of ubiquinone has shown that in at least one step, the ubisemiquinone radical is the active species of Q in the reaction sequence of electron transfer [13]. Since α-tocopherol is capable of undergoing a redox change upon accepting and donating an electron, it could possibly serve as an electron mediator or as an effector of Q. On the other hand, the ability of α-tocopherol to act as a free radical scavenger may disrupts electron transfer by abstracting an electron from the ubisemiquinone radical, which would result in inhibition. Indeed, at moderate concentrations of α-tocopherol an inhibition of succinate-cytochrome *c* reductase is observed, and a specific saturation behavior of binding is observed in succinate-cytochrome *c* reductase or in submitochondrial particles. In this report, we wish to present a detailed study of the inhibition of succinate-cytochrome *c* reductase by α-tocopherol and its derivatives and the reactivation of the α-tocopherol-inhibited enzyme by addition of Q analogs. A preliminary report has been presented [14].

Experimental Procedures

Bovine heart submitochondrial particles [15], succinate-cytochrome *c* reductase [15] and its Q- and phospholipid-depleted preparation [16] were prepared and assayed according to previously reported methods. The concentration of essential components such as cytochrome *b* [17], cytochrome c_1 [18], phospholipid [19] and Q [20] were determined by the reported methods. Ubiquinone in the α-tocopherol-treated succinate-cytochrome *c* reductase was extracted by pentane according to the method of Redfearn [20] and the separation of ubiquinone from α-tocopherol was achieved by thin-layer chromatography. The samples were spotted on silica gel thin-layer plates containing fluorescence indicator and developed with 3% ethanol in benzene. The R_f values for Q_{10} and α-tocopherol in this solvent system were 0.9 and 0.76, respectively. Protein was estimated by the biuret method [21] in the routine assays and by the method of Lowry et al. [22] in the binding study. Spectrophotometric measurements were done at room temperature in a Cary spectrophotometer, model 219. Radioactivity was assayed in a Beckman SL-100 and SL-3150 liquid scintillation counter.

Horse cytochrome *c*, type III, sodium cholate, deoxycholate and Q_6 were purchased from Sigma. α-Tocopherol and α-tocopherol quinone were products of Eastman Kodak. The purity of α-tocopherol and α-tocopherol quinone was checked by thin-layer chromatography. α-[^{14}C]Tocopherol and α-tocopherol acetate were gifts from Hoffmann-La Roche (to Professor P. Marfey, Department of Biology, State Universtity of New York at Albany, NY). 2,3-Dimethoxy-5-geranyl-6-methyl-1,4-benzoquinone (Q_2) and 2,3-dimethoxy-6-methyl-5-(10-bromodecyl)-1,4-benzoquinone [$Q_0(CH_2)_{10}Br$] were synthesized in our laboratory according to the previously reported methods [23].

Studies on inhibition of succinate-cytochrome *c* reductase, ubiquinol-cytochrome *c* reductase and succinate-Q reductase activities by α-tocopherol were performed by addition of succinate-cytochrome *c* reductase to sodium cholate-dispersed

α-tocopherol. 50-μl aliquots of succinate-cytochrome *c* reductase (17.7 mg/ml) were incubated with 0.95 ml of 50 mM phosphate/0.4% sodium cholate buffer, pH 7.4, containing various concentrations of α-tocopherol at 0°C for 20 min. After incubation, each sample was diluted with 1 ml of 50 mM phosphate buffer, pH 7.4, and succinate-cytochrome *c* reductase, succinate-Q reductase and ubiquinol-cytochrome *c* reductase activities were assayed. The α-tocopherol solution was prepared by gently mixing 175 μl α-tocopherol (170 mM in 95% ethanol) with 1.0 ml of 20% sodium cholate at room temperature until clear. The solution was then diluted with 50 mM phosphate buffer, pH 7.4, and sonicated in the dark under an argon atmosphere at 15°C for 2 min at 8 mA. The sonicated solution was centrifuged at 45 000 rpm for 2 h in a Beckman L5-50B centrifuge, rotor 50.2 Ti. The insoluble, floating precipitate, if any, was removed to yield an α-tocopherol concentration of 0.58 mM which was diluted to the desired concentrations with 50 mM phosphate buffer, pH 7.4, containing 0.4% sodium cholate for the inhibitory studies. The concentration of α-tocopherol was determined spectrophotometrically, using $\epsilon_{1\%}^{292\ nm} = 76$.

Binding of α-tocopherol to succinate-cytochrome *c* reductase was determined by centrifugal sedimentation after incubation of the enzyme with α-[^{14}C]tocopherol. About 11 μmol α-[^{14}C]tocopherol (50 nCi/μmol) in 0.1 ml ethanol were mixed with 0.63 ml of 20% sodium cholate and diluted with 24.3 ml of 50 mM phosphate buffer, pH 7.4. The solution was then sonicated at an output of 8 mA in a Branson sonic oscillator, model 110, for a total of 2 min (4 × 30 s), under argon in the dark at about 15°C. The dispersed solution was then diluted with another 25 ml of 50 mM phosphate buffer, pH 7.4, and kept refrigerated. The final concentration of α-tocopherol is 230 μM in the presence of 0.25% sodium cholate. 0.4–2.4 mg succinate-cytochrome *c* reductase in 0.6 ml of 50 mM phosphate buffer, pH 7.4, containing 0.25 % sodium cholate were mixed with 9.4 ml of α-tocopherol solution and incubated at 0°C for 20 min with constant stirring. After incubation, 8 ml of the mixture were centrifuged at 45 000 rpm for 5 h in a Beckman centrifuge, L5-50B, rotor 50. The succinate-cytochrome *c* reductase and bound α-tocopherol were recovered in the precipitate. The supernatant solution, which contains unbound α-tocopherol, was removed completely. The precipitate was homogenized in 1 ml of 50 mM phosphate buffer, pH 7.4, containing 0.25 M sucrose and the protein concentration and succinate-cytochrome *c* reductase activity were determined. The α-tocopherol concentration in the precipitate was determined by ^{14}C radioactivity.

Results

Inhibitory effect of α-tocopherol in succinate-cytochrome c reductase and its Q- and phospholipid-depleted preparation

α-Tocopherol inhibited succinate-cytochrome *c* reductase activity about 95% at a concentration of 0.5 μmol/mg protein. Fig. 1 shows results of the titration of succinate-cytochrome *c* reductase with α-tocopherol in the presence of 0.4% sodium cholate. A 50% inhibition occurred at a concentration of 0.17 μmol α-tocopherol/mg protein. The inhibition was only observed when succinate-cytochrome *c* reductase was preincubated with α-tocopherol which was dispersed in sodium cholate or other detergents. Preincubation of succinate-cytochrome *c* reductase with α-tocopherol in ethanol or direct introduction of ethanolic α-tocopherol solution into the assay system produced very little inhibitory effect even when the concentration of α-tocopherol used was as high as that exhibiting maximal inhibition in the preincubated system.

When the α-tocopherol-treated succinate-cytochrome *c* reductase was assayed for succinate-Q and ubiquinol-cytochrome *c* reductase activities, it was found that the succinate-Q reductase activity was not inhibited by the treatment of α-tocopherol, indicating that electron transfer from succinate to Q is not impaired. When reduced Q_2 was used as substrate, ubiquinol-cytochrome *c* reductase activity in the α-tocopherol-treated succinate-cytochrome *c* reductase decreased parallel to the succinate-cytochrome *c* reductase activity, although to a lesser extent (see Fig. 1). Maximal inhibition of ubiquinol-cytochrome *c* activity was about 78% when succinate-cytochrome *c* reductase was treated with α-tocopherol at a concentration of 0.6 μmol/mg protein. The lowered extent of inhibition of ubiquinol-cytochrome *c* reductase activity

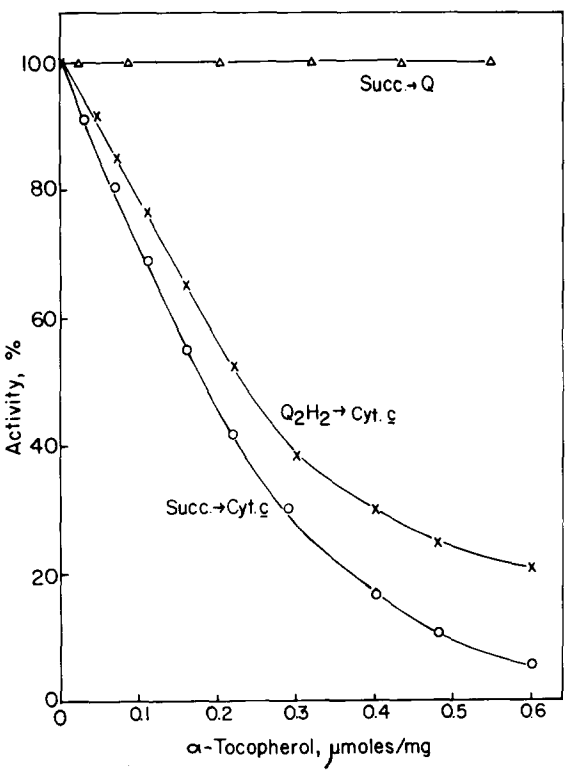

Fig. 1. Effect of α-tocopherol on the succinate-cytochrome *c*, succinate-Q and ubiquinol-cytochrome *c* reductase activities of succinate-cytochrome *c* reductase. 50-μl aliquots of succinate-cytochrome *c* reductase (17.7 mg/ml) were added to 0.95 ml of 50 mM phosphate/0.4% sodium cholate buffer, pH 7.4, containing various concentrations of α-tocopherol, and were incubated at 0°C for 20 min before the activities were assayed. The succinate-Q reductase activity was assayed by following the reduction of dichlorophenolindophenol by succinate after treatment of succinate-cytochrome *c* reductase with antimycin A (equimolar concentration to cytochrome c_1). The α-tocopherol solution was prepared by mixing 175 μl of α-tocopherol (170 mM in 95% ethanol) with 1.0 ml of 20% sodium cholate. When the mixture became completely clear, a 50 mM phosphate buffer, pH 7.4, was slowly added to a final volume of 50 ml. The diluted solution was then sonicated for 2 min at 8 mA under argon atmosphere and in the dark at 15°C. The sonicated solution was centrifuged at 45000 rpm for 2 h to remove any insoluble floating material. The final concentration of α-tocopherol, estimated spectrophotometrically on a Cary 219 spectrophotometer, using $\epsilon_{1\%}^{292\ nm} = 76$, was 0.58 mM.

observed in the α-tocopherol-treated reductase is at least partly due to the reversal of inhibition by the added substrate, Q_2H_2. The concentration of Q_2H_2 in the assay mixture is very high compared to the Q in the enzyme.

The inhibition of succinate-cytochrome *c* reductase by α-tocopherol was dependent on the presence of sodium cholate. The sodium cholate dependency of α-tocopherol inhibition is shown in Table I. Since α-tocopherol and its derivatives are insoluble in aqueous solution, dispersion in detergent is absolutely necessary to gain access to the enzyme and to express their inhibitory effect. Sodium cholate has been found to be the most suitable detergent to disperse α-tocopherol because succinate-cytochrome *c* reductase is known to be stable at fairly high concentration of cholate, and since succinate-cytochrome *c* reductase and its Q- and phospholipid-depleted preparation were prepared in sodium cholate solution. The maximal concentrations of α-tocopherol, α-tocopherol quinone and α-tocopherol acetate in 0.4% sodium cholate are 1.3, 0.3 and 0.1 mM, respectively.

As indicated in Table I the inhibition of α-tocopherol is not only dependent on the final concentrations of cholate but also dependent on the manner of dispersion of α-tocopherol in sodium cholate solution. The reason for this is that when α-tocopherol is mixed with a high concentration of cholate prior to dilution with buffer, a better dispersion of α-tocopherol can be obtained and a pronounced inhibitory effect is observed. It can also be seen in Table I that when the final concentration of cholate is higher than 0.5% a severe denaturation of the enzyme occurs even in the absence of α-tocopherol. This phenomenon became even more pronounced when a more dilute protein solution was used. The concentration of α-tocopherol required to inhibit succinate-cytochrome *c* reductase activity is rather high as compared to other potent inhibitors such as antimycin A. It is, therefore, of interest to see whether or not a better inhibition can be obtained if Q and phospholipid are removed from succinate-cytochrome *c* reductase before the addition of α-tocopherol and followed by replenishing of phospholipid and Q. Fig. 2 shows the inhibitory effect of α-tocopherol on the Q- and phospholipid-depleted succinate-cytochrome *c* reductase. The depleted enzyme was first incubated with various amounts of α-tocopherol for 2 min before addition of 100 nmol $Q_0(CH_2)_{10}Br$/mg protein. The $Q_0(CH_2)_{10}Br$ replenished enzyme was then mixed with phospholipid at 0.8 mg/mg protein. The enzymatic

TABLE I

EFFECT OF SODIUM CHOLATE CONCENTRATION ON THE INHIBITION OF SUCCINATE-CYTOCHROME *c* REDUCTASE BY α-TOCOPHEROL

[a] [(αT+SC)+Pi]+SCR indicates that aliquots of 7 μl of α-tocopherol (αT) (170 mM in ethanol) were added to various amounts (0.01–0.2 ml) of 20% sodium cholate (SC) before they were diluted to 2 ml with 50 mM phosphate buffer (P_i), pH 7.4. The α-tocopherol solutions were then sonicated for 2 min at 8 mA output in the dark under argon atmosphere. 0.95-ml aliquots of the sonicated α-tocopherol solution with the indicated cholate concentration were added to 50 μl of succinate-cytochrome *c* reductase (SCR) (17.7 mg/ml). The activity of succinate-cytochrome *c* reductase was assayed after incubation at 0°C for 30 min. [b] [αT+(SC+P_i)]+SCR indicates that aliquots of 7 μl of α-tocopherol, 170 mM in ethanol, were added to 2 ml of 50 mM phosphate buffer, pH 7.4, containing the indicated concentration of cholate. The rest of the procedure was the same as that described for a.

[Cholate] (%)	Relative activity (%)		
	[(αT+SC)+P_i]+SCR [a]	[αT+(SC+P_i)]+SCR [b]	[(SC+P_i)]+SCR
0	97	100	100
0.1	40	93	96
0.2	20	82	92
0.3	12	75	87
0.5	5	65	78
1.0	4	30	35

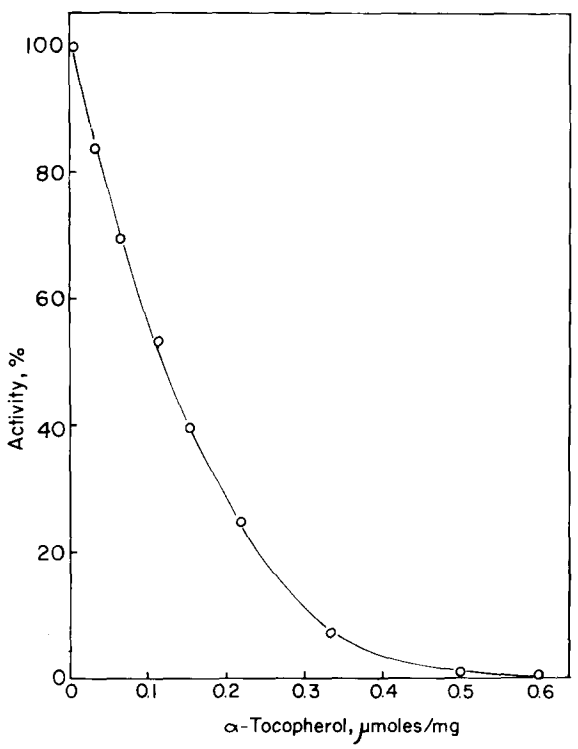

Fig. 2. Inhibitory effect of α-tocopherol on Q- and phospholipid-depleted succinate-cytochrome *c* reductase. 50 μl of the Q- and phospholipid-depleted succinate-cytochrome *c* reductase (15 mg/ml) were added to 0.95 ml of 50 mM phosphate buffer, pH 7.4, containing 0.4% sodium cholate and various concentrations of α-tocopherol. After incubation at 0°C for 2 min, 10 μl of $Q_0(CH_2)_{10}Br$ (7.6 mM in 95% ethanol) were added and the mixture was incubated for another 2 min before 30 μl of asolectin (20 mg/ml) were added. The succinate-cytochrome *c* reductase activity was assayed after 45 min incubation at 0°C. The 100% activity represents 8 μmol cytochrome *c* reduced/min per mg protein.

activity was determined after 30 min incubation at 0°C. As indicated in Fig. 2, the inhibitory behavior of α-tocopherol on depleted succinate-cytochrome *c* reductase is similar to that of intact succinate-cytochrome *c* reductase except that the amount of α-tocopherol required for complete inhibition as well as half inhibition is lower than that of intact reductase. The amount of α-tocopherol required for the complete and half inhibition was found to be 0.3 and 0.11 μmol α-tocopherol/per mg protein, respectively.

The nature of α-tocopherol inhibition on succinate-cytochrome c reductase

The structural similarity between Q and α-tocopherol led us to suspect that α-tocopherol might have some effector activity when low concentrations of Q are present in the succinate-cytochrome *c* reductase. This possibility was tested

using an α-tocopherol-treated, Q- and phospholipid-depleted succinate-cytochrome *c* reductase followed by titration with Q. Fig. 3 shows the effect of α-tocopherol on the reactivation of succinate-cytochrome *c* reductase activity from the Q- and phospholipid-depleted enzyme by various amounts of ubiquinone. In this case, $Q_0(CH_2)_{10}Br$ was used. The Q- and phospholipid-depleted succinate-cytochrome *c* reductase was treated with a given concentration of α-tocopherol before it was subjected to $Q_0(CH_2)_{10}Br$ titration. When Q- and phospholipid-depleted enzyme was treated with lower levels of α-tocopherol, $Q_0(CH_2)_{10}Br$ at lower concentrations restored more activity from depleted enzyme than in the sample without treatment of α-tocopherol. The enhancement of the restored activity was more apparent when lower concentrations of α-tocopherol was used. The lipophilic nature of both ubiquinone and α-tocopherol has somewhat complicated the kinetic analysis. At the present time no further pursuit of this matter has been undertaken. One possible explanation for the enhancement of Q restoration of activity by α-tocopherol when $Q_0(CH_2)_{10}Br$ concentrations are well below the saturation level comes from the fact that Q is bound to multiple sites in succinate-cytochrome *c* reductase, such as those bound to succinate-Q reductase and ubiquinol-cytochrome *c* reductase. Enhancement of activity by low concentrations of α-tocopherol also indicates that α-tocopherol is not bound to the same site as that of Q, because there is no activity restoration from depleted enzyme as is observed when a low concentration of α-tocopherol is used in the absence of Q. The possibility exists, although not likely, that low concentrations of α-tocopherol may have a stabilizing effect as a substitute for Q in the depleted enzyme complex and thereafter be displaced by the Q which is added back.

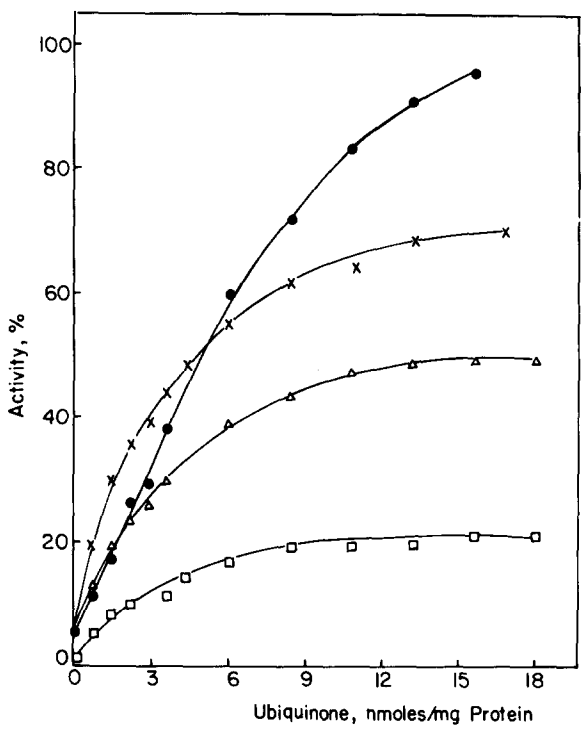

Fig. 3. Titration of the α-tocopherol-treated Q- and phospholipid-depleted succinate-cytochrome *c* reductase with $Q_0(CH_2)_{10}Br$. The Q- and phospholipid-depleted succinate-cytochrome *c* reductase was treated without (●———●) or with α-tocopherol at the final α-tocopherol concentrations of 72 nmol/mg (×——×); 145 nmol/mg, (△——△); and 217 nmol/mg (□——□) in the same manner as that described for Fig. 2. 0.5-ml aliquots were withdrawn and titrated with the indicated concentrations of $Q_0(CH_2)_{10}Br$. The mixture was incubated at 0°C for 2 min before addition of asolectin. The enzymatic activity was assayed after incubation at 0°C for 45 min. The 100% activity equals 8 μmol cytochrome *c* reduced/mg per min.

Reactivation of α-tocopherol inhibited succinate-cytochrome c reductase with Q derivatives

Fig. 4 shows the reactivation of α-tocopherol-inhibited succinate-cytochrome *c* reductase by Q_2, $Q_0(CH_2)_{10}Br$ and Q_6. Among the Q derivatives tested, Q_2 shows the greatest efficiency in reactivation. The maximal reactivation (0.4 μmol/mg protein) is about 75% of the maximal activity in the presence of saturated Q. As indicated in Fig. 4, the succinate-cytochrome *c* reductase as prepared is somewhat Q deficient. An approx. 18% increase in activity was observed when 50 nmol Q_2/mg protein was added to the prepared succinate-cytochrome *c* reductase. $Q_0(CH_2)_{10}Br$, which is more effective than Q_2 in restoration of succinate-cytochrome *c* reductase activity from its depleted preparation [24], showed only about 80% of the activity of Q_2 in the reactivation of α-tocopherol-inhibited enzyme. Q_6 has only about 30% activity in restoring the α-tocopherol-inhibited enzyme, although Q_6 is as active as Q_2 in restoring succinate-cytochrome *c* reductase activity to the depleted prep-

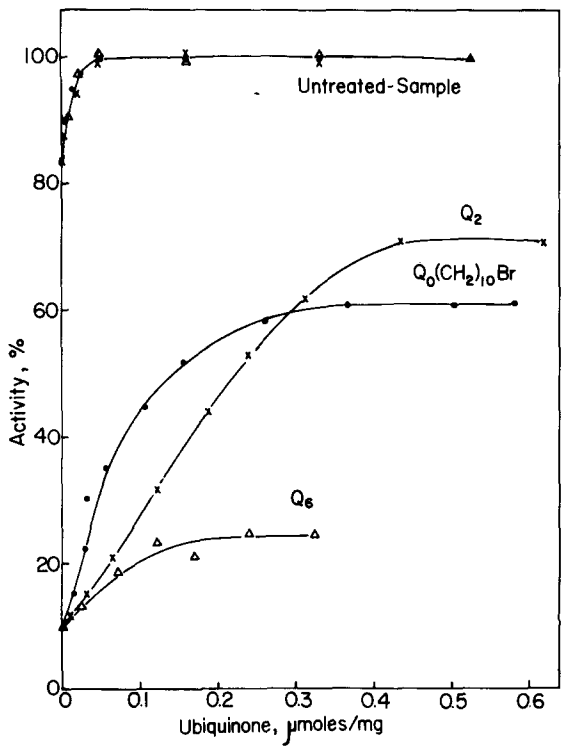

Fig. 4. Restoration of succinate-cytochrome *c* reductase activity from the α-tocopherol-treated enzyme by Q analogs. Succinate-cytochrome *c* reductase was treated with α-tocopherol (0.5 μmol/mg) by the procedure described in Fig. 2. Q analogs (in 95% ethanol) were added to both treated and untreated (control) samples. The maximal activity of untreated enzyme, which was used for 100% activity, was 8 μmol cytochrome *c* reduced/min per mg protein.

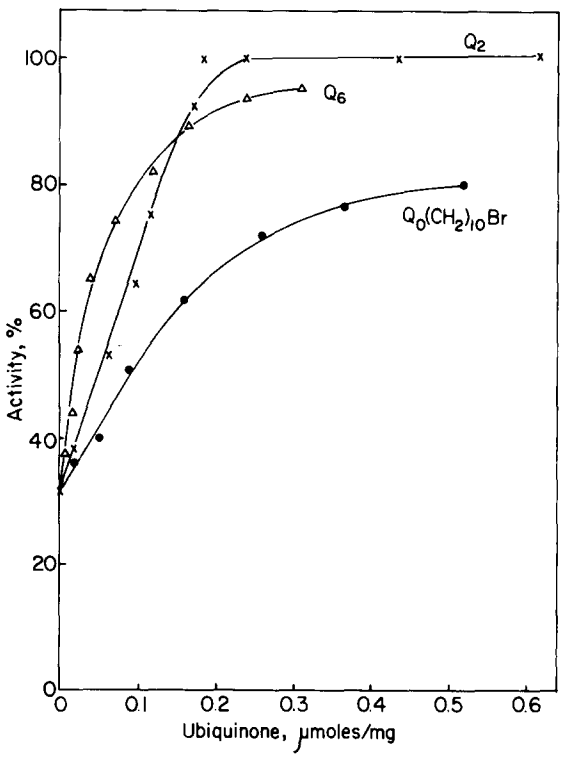

Fig. 5. Restoration of succinate-cytochrome *c* reductase activity from the α-tocopherol quinone-treated enzyme by Q analogs. The experimental conditions were the same as described in Fig. 4 except that α-tocopherol quinone was used (0.4 μmol/mg).

aration [16]. Although Q_2 has be greater reactivation capacity at higher concentration, at low concentration, $Q_0(CH_2)_{10}Br$ is much more effective. The Q_2 reactivation shows a sigmoidal titration curve whereas $Q_0(CH_2)_{10}Br$ and Q_6 exhibited hyperbolic behavior, for reasons not yet understood.

It should be mentioned that none of the ubiquinone analogs tested restored the full activity to the α-tocopherol-treated reductase. This was apparently not due to an insufficient addition of Q to the system, because saturation behavior was reached in the titration curves. A possible explanation would be the partially irreversible inactivation occurred during the treatment of reductase.

Fig. 5 shows the activation of α-tocopherol quinone-inhibited succinate-cytochrome *c* reductase by Q derivatives. The reactivation behavior is quite different from that of α-tocopherol-inhibited enzyme. The α-tocopherol quinone shows a 70% maximal inhibition whereas α-tocopherol inhibits succinate-cytochrome *c* reductase activity more than 90%. Q_2 reactivates the maximal succinate-cytochrome *c* reductase at a concentration of 0.2 μmol/mg protein, while $Q_0(CH_2)_{10}Br$ reactivates only 80% of the maximal activity at a concentration of 0.5 μmol/mg protein. Q_6 restores almost as much activity as does Q_2 in the α-tocopherol quinone-inhibited succinate-cytochrome *c* reductase. At low concentrations Q_6 is more effective than Q_2.

Reactivation of ubiquinol-cytochrome c reductase activity in α-tocopherol-treated succinate-cytochrome c reductase

The inhibition of succinate-cytochrome *c* re-

ductase by α-tocopherol has been located in the ubiquinol-cytochrome *c* reductase segment, as described in the previous section. When the α-tocopherol-treated succinate-cytochrome *c* reductase was reactivated by the addition of Q analogs, the succinate-cytochrome *c* reductase activity was restored but the ubiquinol-cytochrome *c* reductase activity was not. Since the oxidation of succinate by cytochrome *c* in this preparation is found to be antimycin A sensitive, the electron flow must go through the ubiquinol-cytochrome *c* reductase region. The inability to restore ubiquinol-cytochrome *c* reductase activity can only be attributed to the inaccessibility of substrate (reduced Q_2) to the enzyme system in the α-tocopherol-treated and Q-reactivated enzyme. In other words, the presence of α-tocopherol blocked the exchange between the bound Q, which is directly involved in electron transfer, and added substrate (Q_2H_2) [25].

Binding of α-tocopherol to succinate-cytochrome c reductase and submitochondrial particles

Since α-tocopherol and its derivatives are very insoluble in aqueous solution, the presence of dilute detergent is necessary to achieve the solubility needed for the binding studies. Fig. 6 shows the protein concentration-dependent binding of α-tocopherol to succinate-cytochrome *c* reductase. The maximum uptake of α-tocopherol by succinate-cytochrome *c* reductase is about 0.68 μmol/mg protein in a medium containing 0.25% sodium cholate, 50 mM phosphate buffer, pH 7.4. When the submitochondrial particle was used instead of succinate-cytochrome *c* reductase, a similar result was obtained based upon the content of cytochrome *b*. Submitochondrial particles bind 0.1 μmol α-tocopherol/mg protein. The cytochrome *b* content in the submitochondrial particles is about 0.6 nmol/mg protein whereas that in succinate-cytochrome *c* reductase is about 4 nmol/mg. Thus, the binding of α-tocopherol in submitochondrial particles and isolated succinate-cytochrome *c* reductase is about 0.17 μmol/nmol cytochrome *b* under the same conditions tested. It seems unlikely that any given protein in the cytochrome *b*-c_1 region is responsible for all the binding of α-tocopherol. More detail work is required before a definite statement regarding the protein and α-tocopherol interaction can be made.

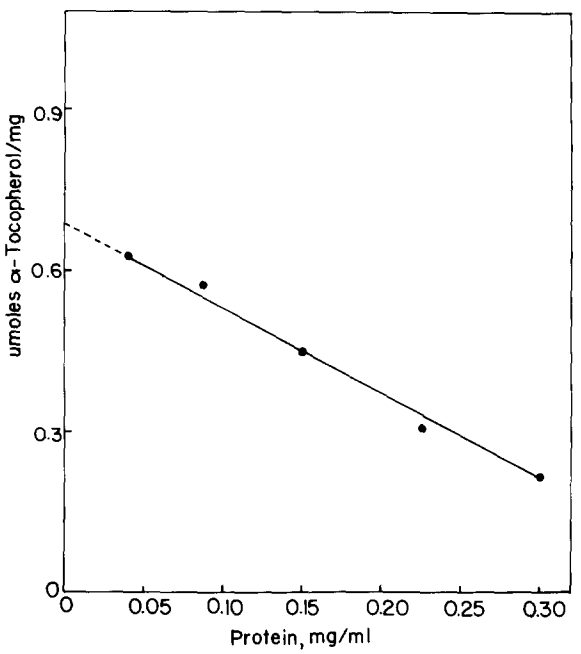

Fig. 6. Binding of α-tocopherol to succinate-cytochrome *c* reductase. The experimental conditions are detailed in Experimental Procedures.

Effect of α-tocopherol on the reduction of cytochromes b and c_1

Table II shows the effect of α-tocopherol on the reduction of cytochromes *b* and c_1 in succinate-cytochrome *c* reductase. In the isolated succinate-cytochrome *c* reductase preparation, succinate reduced about 80% of the cytochrome *b* which could be reduced by sodium dithionite, and 100% of the cytochrome c_1. When succinate-cytochrome *c* reductase was treated with α-tocopherol at the concentration of 0.5 μmol/mg protein, all the cytochrome c_1 became reduced in the absence of substrate (succinate), a phenomenon similar to that observed when phospholipids are removed from the intact enzyme [27]. The total amount of cytochrome *b* reducible by succinate in the intact and α-tocopherol-treated succinate-cytochrome *c* reductase is nearly the same but the rate of cytochrome *b* reduction by succinate in the latter was greatly decreased. Addition of exogenous Q analogs to the α-tocopherol-treated succinate-cytochrome *c* reductase reoxidized cytochrome c_1 to the original oxidized state and restored the reduc-

TABLE II

EFFECT OF α-TOCOPHEROL ON THE REDOX STATE OF CYTOCHROMES *b* AND c_1

SCR, succinate cytochrome *c* reductase.

Preparations	Succinate-reducible cytochrome *b* (%) [a]	Cytochrome c_1 reduced without substrate (%) [b]
Intact SCR	80	< 2
α-Tocopherol-treated SCR	75	100
α-Tocopherol-treated SCR + $Q_0(CH_2)_{10}Br$	80	< 2

[a] Based on the total amount of cytochrome *b* reducible by $Na_2S_2O_4$.

[b] Based on the total amount of cytochrome c_1 reducible by ascorbate.

tion rate of cytochrome *b* by succinate to a rate similar to that of the intact preparation. The reduction of cytochrome c_1 by treatment with α-tocopherol indicates the presence of some component in succinate-cytochrome *c* reductase, originally in the reduced state, which became oxidized perhaps by the change of E_m upon treatment with α-tocopherol. The reversible behavior upon reactivation by Q indicates that the component involved is directly related to Q, and the reoxidation of cytochrome c_1 is caused by the redox change of that particular component and thus received electrons from cytochrome c_1 rather than that Q serves as an electron acceptor.

Discussion

Since the solubility of α-tocopherol and its derivatives in aqueous solution is very low, study of the effect of these compounds on the electron-transfer reaction requires special techniques in the preparation of reagent solutions. Under the conditions described in the previous section, in 50 mM sodium/potassium phosphate buffer, pH 7.4, containing 0.4% sodium cholate, suitable concentrations of α-tocopherol, α-tocopherol quinone and α-tocopherol acetate can be obtained and the detergent solution itself is not harmful to the enzyme. Higher concentrations of vitamin E can be obtained if the concentration of detergent is increased. It should be stressed that mixing the ethanolic solution of α-tocopherol with a high concentration of detergent (20%) prior to dilution is absolutely necessary in oder to make the described solution. The solution was routinely kept at 0.4% sodium cholate as the enzyme is stable at this detergent concentrations. Direct addition of α-tocopherol or its derivatives in ethanolic solution to the enzyme assay mixture did not result in inhibition.

Under the described conditions, succinate-cytochrome *c* reductase binds 0.68 μmol α-tocopherol/mg protein, or 0.17 μmol/nmol cytochrome *b*. A similar binding capacity, 0.1 μmol/mg protein, was observed in the submitochondrial particles. The specific binding of α-tocopherol in adrenocortical membrane [9] and red blood cell membrane [26] has been reported. The binding capacity in red blood cell membrane was reported to be 12 and 70 pmol/mg protein [26] for high- and low-affinity sites, respectively. Since the detailed conditions for the reported values are not available, direct comparison of binding capacity of α-tocopherol between the submitochondrial particles and red blood cell membrane is not possible. The low solubility of α-tocopherol and its derivatives in aqueous solution made the idea of the existence of a specific binding or transporting protein for α-tocopherol plausible. The high binding capacity of succinate-cytochrome *c* reductase or submitochondrial particles has made the idea that α-tocopherol binds to a given protein in the complex or particle less attractive; more investigation is needed before a detail interaction between protein and α-tocopherol can be elucidated. Whether or not α-tocopherol exerts a regulatory function in the electron-transfer reaction of succinate-cytochrome *c* reductase remains to be determined. Although the concentration of α-tocopherol required to show an inhibitory effect on the isolated succinate-cytochrome *c* reductase is rather high, in the in vivo system the concentration required may be less.

Although the concentration of α-tocopherol required to inhibit succinate-cytochrome *c* reductase is rather high, the concentration of ubiquinone required to reactivate the α-tocopherol-inhibited enzyme is less than that of the α-tocopherol used.

A possible explanation would be that the binding of α-tocopherol caused a conformational change at the Q-binding site, thus decreasing the binding affinity for Q. When more Q was available in the system, the electron transfer proceeded without interruption. This can also explain why the activity of succinate-cytochrome *c* reductase can be restored but not the ubiquinol-cytochrome *c* reductase activity: α-tocopherol may occupy the site otherwise occupied by Q_2H_2 during its donation of an electron to bound Q. In other words, the presence of α-tocopherol prevents ubiquinol from gaining access to the protein or to bound Q and thus prevents the electron transfer between ubiquinol and bound Q taking place.

The finding that the activity of succinate-cytochrome *c* reductase but not that of ubiquinol-cytochrome *c* reductase can be restored from the α-tocopherol-inhibited enzyme by addition of Q and the fact that maximal enzymatic activity of isolated succinate-cytochrome *c* reductase is expressed [28] when the preparation contains only bound Q, i.e., 1 mol Q/mol cytochrome c_1, indicates that the major, if not the only, contribution of electron transfer from succinate to cytochrome *c* occurs via bound Q. Thus, the interference caused by α-tocopherol with the exchange between the bound and free forms of Q will not affect the electron transfer when succinate is used as substrate. A change in protein conformation induced by binding of α-tocopherol is indirectly supported by the observation that cytochrome c_1 became reduced when no substrate was added, a phenomenon which is similar to that observed when phospholipid is removed from succinate-cytochrome *c* reductase [16]. The removal of phospholipid from succinate-cytochrome *c* reductase is know to be associated with a conformational change [27]. The change in conformation seems to be very specific as the reactivation effectiveness of Q derivatives varied greatly, particularly when different α-tocopherol derivatives were used. Q_6 is very poor in reactivation of α-tocopherol-inhibited succinate-cytochrome *c* reductase activity but is very effective in restoration of the α-tocopherol quinone-inhibited enzyme.

Since α-tocopherol has no inhibitory effect on succinate-Q reductase, the lowered rate of reduction of cytochrome *b* by succinate can be better attributed to a change in cytochrome *b* itself than in the electron-donating system. Although the redox potential of cytochrome *b* is known to be highly sensitive to the phospholipid environment [29], the change in the reduction rate of cytochrome *b*, however, cannot be explained by the change of redox potentials resulting from a modification of membrane structure or environment in the α-tocopherol-treated enzyme complex, because the final extent of cytochrome *b* reduction by succinate is the same as that in the intact system.

The idea that the inhibitory effect of α-tocopherol on succinate-cytochrome *c* reductase is the result of protein conformational and membrane structural changes also gains support from the fact that less α-tocopherol is required to inhibit the Q- and phospholipid-depleted succinate-cytochrome *c* reductase than the intact preparation. In the depleted enzyme, the protein structure is in a less stable state, thus it is much easier to alter. Furthermore, in the absence of phospholipid and Q, as in the phospholipid- and Q-depleted enzyme, the binding of α-tocopherol may partly occur at the vacant Q-binding sites and such binding is not easily reversed by the addition of Q. This is different from the inhibitory effect of α-tocopherol in the intact system which is through the partial delocalization of ubiquinone, resulting from a change in protein conformation or from a change in protein-lipid interaction. The statement that addition of α-tocopherol to intact succinate-cytochrome *c* reductase causes only delocalization of Q but not the displacement of Q is supported by the fact that the same amount of Q is found in the α-tocopherol-treated and intact succinate-cytochrome *c* reductase after centrifugal sedimentation. No Q is released to the supernatant solution in both systems even though dilute cholate (0.4%) is present in both systems during the incubation and centrifugation procedures.

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