The mechanism by which succinate dehydrogenase transfers electrons from succinate to ubiquinone and the cytochromes b and c, of the membranous \( b-c \) segment of the mitochondrial respiratory chain is not known. An interesting aspect of this electron transfer is that, whereas the oxidation-reduction of succinate and ubiquinone are 2-electron transfer processes, the iron-sulfur centers of SDH, which are intermediate oxidation-reduction components between succinate and ubiquinone, are 1-electron carriers. Thus, the oxidation of succinate and reduction of ubiquinone most likely proceed through the transient formation of half-reduced forms, such as ubisemiquinone in the instance of ubiquinone.

The mechanism of reduction of ubiquinone is of interest because of Mitchell's (2) postulate that transmembranous oxidation-reduction of ubiquinone may account for active transport of protons outward across the inner mitochondrial membrane by a direct chemiosmotic mechanism in which proton transport is stoichiometrically coupled to electron transfer (2-4). This possible function of ubiquinone has received added emphasis with the recently proposed proton-motive \( Q \) cycle mechanism of electron transfer, by which ubiquinone would transport protons for both the second and third sites of oxidative phosphorylation (5, 6). Consequently, it is important to identify the carriers which participate in the oxidoreductions of ubiquinone, ubisemiquinone, and ubiquinol and to establish their presumed transmembranous location.

In attempting to establish whether SDH is a direct donor for reduction of ubiquinone and, if so, to understand the mechanism of this reaction, various investigators have noted the anomaly that membranous SDH has succinate-ubiquinone oxidoreductase activity but that purified SDH does not catalyze reduction of ubiquinone by succinate (7-9). The recent isolation of a low molecular weight protein from the \( b-c \) segment (10), which confers succinate-ubiquinone reductase activity on soluble SDH, appears to represent an important step toward understanding how SDH reduces ubiquinone.

Another aspect of the succinate-ubiquinone reductase reaction which is not understood is whether the reduction of ubiquinone to ubiquinol involves two oxidation-reduction donors which sequentially reduce ubiquinone and ubisemiquinone or whether this reaction involves a single donor for catalyzed dismutation reaction as proposed by Kroger and Klingenberg (Kroger, A., and Klingenberg, M. (1973) Eur. J. Biochem. 34, 358-368).


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Bernard L. Trumpower‡ and Zachary Simmons

From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755

Diminished Inhibition of Mitochondrial Electron Transfer from Succinate to Cytochrome c by Thenoyltrifluoroacetone Induced by Antimycin*

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The thenoyltrifluoroacetone inhibits succinate-cytochrome c reductase activity of resolved succinate-cytochrome c reductase complex. Dixon plots of [succinate-cytochrome c reductase activity] \(^{-1} \) [succinate-ubiquinone reductase activity] \(^{-1} \) versus concentration of inhibitor are consistent with there being a single site of inhibition by thenoyltrifluoroacetone in resolved reductase complex. This agrees with results obtained with succinate-ubiquinone reductase complex by Mowery and co-workers (Mowery, P. C., Steenkamp, D. J., Ackrell, B. A. C., Singer, T. P., and White, G. A. (1977) Arch. Biochem. Biophys. 178, 495-506).

When electron transfer from succinate to cytochrome c is partially inhibited by antimycin, thenoyltrifluoroacet!...
Diminished Efficacy of Thenoyltrifluoroacetone

ubiquinone followed by a noncatalyzed dismutation (11). Backstrom and co-workers (12) showed that ubisemiquinone is formed in detectable quantities in mitochondria, and recent findings by low temperature EPR spectroscopy indicate that a spin-coupled ubisemiquinone pair is closely associated with a high potential iron-sulfur center (S-3) of succinate dehydrogenase (13, 14). By virtue of its interaction with the iron-sulfur center, ubisemiquinone may be stabilized during respiration. However, the existence of stable forms of ubisemiquinone does not preclude the involvement of a noncatalyzed dismutation reaction in mitochondrial respiration.

As an approach to understand more fully the mechanism of electron transfer from SDH to ubiquinone, we have examined the inhibition of electron transfer in resolved succinate-cytochrome c reductase complex by thenoyltrifluoroacetone, a metal chelator which inhibits succinate-ubiquinone reductase activity (15). Ackrell and co-workers (16) showed that TTFA inhibits oxidation of iron-sulfur center S-3 of SDH, and EPR studies indicate that TTFA disrupts the interaction between the ubisemiquinone pair and center S-3 (17, 18). However, these findings do not establish whether TTFA inhibits the reduction of ubiquinone to ubisemiquinone, the reduction of ubiquinone to ubiquinol, or both of these reactions.

The purpose of this paper is to describe our finding that the efficacy of inhibition of electron transfer from succinate to cytochrome c by TTFA is diminished under conditions where electron transfer is partially inhibited by antimycin and to discuss the possible mechanism of electron transfer from succinate dehydrogenase to ubiquinone in light of these results.

EXPERIMENTAL PROCEDURES

Materials—Cytochrome c (type III), bovine superoxide dismutase, ubiquinone-10, antimycin, and dichlorophenolindophenol were obtained from Sigma. Thenoyltrifluoroacetone was obtained from Fisher. Soybean phosphatides were obtained from Applied Associates, Woodside, N. Y., and purified before use (19). DBH, an analogue of ubiquinone-2 having a decyl side chain, was synthesized as described by Wan and co-workers (20). The quinone was stored as a 72 mM solution (e = 16.0 mM cm−1) in ethanol at −10°C. Thenoyltrifluoroacetone was stored as a 500 mM solution (determined gravimetrically) in ethanol at −10°C and serial dilutions were made therefore so that concentrations of TTFA desired in the assays could be obtained by adding 5 μl of ethanol solution/1 ml of reaction mixture. Antimycin was stored in ethanol and its concentration was determined spectrophotometrically (21). Solutions of superoxide dismutase, 1 mM in 20 mM potassium phosphate, 100 mM NaCl, pH 7.0, were prepared fresh daily.

Preparation of Succinate-Cytochrome c Reductase Complex—Bovine heart mitochondria were prepared in 0.25 M sucrose, 10 mM potassium phosphate, 10 mM EDTA, pH 7.4, and stored at 70°C. The mitochondria were thawed and washed to remove peripheral proteins by homogenizing with 1 liter of 0.1 M sucrose, 100 mM NaCl, pH 7.0, were prepared fresh daily.

Preparation of Succinate-Cytochrome c Reductase Complex—Bovine heart mitochondria were prepared in 0.25 M sucrose, 10 mM potassium phosphate, 10 mM EDTA, pH 7.4, and stored at 70°C. The mitochondria were thawed and washed to remove peripheral proteins by homogenizing with 1 liter of 0.1 M sodium phosphate, pH 7.4/10 g of mitochondria in a 250-ml loose-fitting Teflon-glass homogenizer and then centrifuging for 60 min at 15,000 × g. The mitochondria were suspended two more times in the same manner and collected. Solid ammonium sulfate was added to obtain 25% saturation. The mixture was centrifuged 60 min at 4°C, after which the volume was measured and ammonium sulfate was added to obtain 35% saturation (61 g/liter). After centrifuging 20 min, the mixture was centrifuged 60 min at 37,500 × g and the supernatant was collected. Solid ammonium sulfate was added to obtain 50% saturation (94 g/liter) and the mixture was centrifuged 30 min at 37,500 × g.

The dark red pellet obtained from 50% ammonium sulfate was dispersed in 0.1 M sodium phosphate, 0.5% cholate, 0.5 mM EDTA, pH 7.4, by gentle homogenization with a loose-fitting homogenizer to obtain 2 ml of red sediments. The mixture was centrifuged 60 min at 12 to 18 h at 4°C, during which time it became turbid due to aggregation of residual cytochrome c oxidase, and then it was centrifuged 90 min at 78,500 × g and the reductase complex was recovered as a bright red supernatant. To remove cholate, the reductase complex was dialyzed 16 to 18 h against 30 volumes of 0.25 M sucrose, 10 mM sodium phosphate, pH 7.4, after which it was recovered by centrifugation for 60 min at 37,000 × g, suspended at 15 to 20 mg/ml in 0.0 M sucrose, 10 mM TES, pH 7.4, and stored at 70°C.

As discussed elsewhere (22), with the exception of the preparation described by Yu and co-workers (23), most preparations of succinate-cytochrome c reductase complex reported in the literature have low succinate-cytochrome c reductase activity (<1 unit/mg). This consideration is relevant to the findings reported here since we have observed that if higher amounts of cholate (0.5 to 0.8 mg/ml of protein) are used in the preparation, the resulting reductase has cytochrome c reductase activity of less than 1 unit/mg and this is increased 2- to 3-fold by addition of ubiquinone (22). Likewise, Yu and co-workers (23) noted a partial recovery of activity in the case of succinate-ubiquinone reductase activity of 8 to 11 units/mg. On freezing, the succinate-cytochrome c reductase activity drops to 2.5 to 3.5 units/mg; this activity is restored 50 to 100% by adding phospholipid plus ubiquinone nono-10 (22).

The experiments reported here were performed with freshly prepared reductases and reductase which was stored frozen. In the latter instance, the reductase was reconstituted with 2 μl of phospholipid plus 57 nmol of ubiquinone-10/mg of protein by mixing, in sequence: 20 μl of 200 mM sodium phosphate, 200 mM sodium succinate, 10 mM EDTA, pH 7.5; 300 μg of reductase complex (20 to 30 μl); 20 μl of sonicated phospholipid/ubiquinone mixture (25); 36 μl of 20 μl of sodium cholate, pH 7.5; and sufficient water to yield 200 μl. This mixture was incubated 60 min at 4°C and then diluted to 2 ml by addition of 40 mM sodium phosphate, 20 mM sodium succinate, 0.5 mM EDTA, pH 7.5.

Assays—Succinate-cytochrome c reductase activity was measured in a mixture containing 20 μl of 250 mM sodium succinate, 0.5 mM ubiquinone, 0.5 mM KCN, 0.25 mM KCN, pH 7.5. The cyanide was added as a neutralized solution immediately before starting the reaction by adding 1.5 or 3.0 μg of reductase complex. The zero order rate of cytochrome c reduction was calculated from the initial absorbance increase at 550 nm using εredox = 18.5 mM−1 cm−1.

Succinate-ubiquinone reductase activity was measured with 48 μM DBH, a synthetic analogue of ubiquinone-2 (20), in a mixture containing 62 μM dichlorophenolindophenol, 40 mM sodium phosphate, 20 mM sodium succinate, 0.02% Tween 80, 0.5 mM EDTA, 0.25 mM KCN, pH 7.5. Cyanide was added immediately before starting the reaction with enzyme as above. Reduction of dichlorophenolindophenol followed spectrophotometrically at 600 nm on a Gillford 2400S spectrophotometer employing an absorbance offset (cf. Ref. 9). After an initial lag of 1 to 3 s, the rate was calculated using Δε = 19.1 mM−1 cm−1.

Rates of cytochrome b reduction by succinate were measured at 8°C in an Aminco DW-2A dual wavelength spectrophotometer using the wavelength pair 562 versus 577 nm and a 2-mm band-pass. The reaction mixture contained 650 μg of reductase complex in 1.4 ml of 90 mM sodium phosphate, 0.45 mM EDTA, 0.5 mM KCN, pH 7.5.

Reduction of cytochrome b was initiated by adding 1 μl of 12 μl of succinate. Amounts of TTFA and antimycin added are indicated in the figure legends. Using the ED101 chamber stirrer supplied by the manufacturer, it was possible to obtain a mixing time of approximately 1 s if the volume of the reaction mixture was 1.4 to 1.5 ml.

Rates of electron transfer for the cytochrome c reductase and ubiquinone reductase reactions, measured at 30°C, are expressed in units of 1 electron equivalent, a unit being defined as 1 microgram equivalent of electrons transferred/min. For convenience of represen-
tation, data in the figures are presented as percent of the control activity, measured in the absence of inhibitors. Control activities are indicated in the legends.

To construct Dixon plots (26) for inhibition of electron transfer activities by TTFA, it was necessary to correct for the rate not inhibitable by TTFA (see Fig. 1 and Ref. 27). This correction was determined by plotting activity versus [TTFA concentration]−1, which yielded a smooth curve. By extrapolation of these curves to their intercept on the vertical axis, it was calculated that 5 to 7% of the cytochrome c reductase and 10 to 13% of the ubiquinone reductase activity was not inhibitable by TTFA. After applying these percentages to the rate obtained in the absence of TTFA and subtracting the TTFA-insensitive rate thus calculated from the rates obtained at varying concentrations of TTFA, the resulting Dixon plots were linear.

RESULTS

Effect of TTFA on Electron Transfer Activities of Resolved Reductase Complex—For the experiments reported here, resolved succinate-cytochrome c reductase complex offers several advantages compared to less resolved preparations such as submitochondrial particles or the more highly fragmented succinate-ubiquinone reductase complex (Complex II), in which inhibition of electron transfer by TTFA has been most extensively examined (16, 27, 28). Nelson and co-workers (29) first proposed that TTFA is competitive with ubiquinone and recent evidence indicates that TTFA disrupts an interaction between ubisemiquinone and the high potential iron-sulfur center, S-3, of succinate dehydrogenase (17, 18). On the basis of this earlier work, we reasoned that, if there are separate donor sites for reduction of ubiquinone and ubisemiquinone and if inhibition by TTFA involves only a single site, then the efficacy of TTFA may depend on the oxidation-reduction poise of the two ubisemiquinone couples, Q/QH. and QH2./QH2.

To test this possibility, it was desirable to manipulate the oxidation-reduction poise of ubiquinone in a system in which the oxidation-reduction reactions of ubiquinone are apparently rate-limiting. The endogenous activity of SDH in succinate-cytochrome c reductase complex, measured with phenazine methosulfate plus dichlorophenolindophenol, is 1.5 to 2.0 times greater (22), and the ubiquinol-cytochrome c reductase activity is approximately 5 times greater (see "Experimental Procedures") than the succinate-cytochrome c reductase activity. Succinate-cytochrome c reductase activity of resolved reductase complex thus appears to satisfy the kinetic requirements anticipated, in that electron transfer through ubiquinone appears to be rate-limiting.

Resolved reductase complex is also desirable from the standpoint that interpretation of results is not complicated by considerations related to changes in membrane potential or ATPase activity, or both (30). Because the apparent antagonism between TTFA and ubiquinone was observed as an alteration in the oxidation-reduction poise of cytochrome b (29), it seemed that succinate-cytochrome c reductase complex may be preferable to Complex II, in which at least a portion of the b cytochrome is damaged in preparation (22, 28). In the reductase complex used here, cytochromes b-562 and b-566 are both present and demonstrable as high and low potential components, respectively, and less than 10% of the total b cytochrome is reactive to CO (22).

The inhibition of succinate-cytochrome c reductase complex by TTFA is shown in Fig. 1. The pattern of inhibition is notable in two respects. First, with both cytochrome c reductase (Fig. 1a) and ubiquinone reductase (Fig. 1b) activities, a portion of the activity is not inhibited by TTFA. Using reciprocal plots to calculate the amount of activity not inhibitable by TTFA, we found a consistent difference between the succinate-ubiquinone reductase and succinate-cytochrome c reductase activities, in that 10 to 13% (0.80 to 1.40 units/mg) of the ubiquinone reductase but only 5 to 7% (0.35 to 0.50 units/mg) of the cytochrome c reductase activity was not inhibitable by TTFA. A possible explanation for the incomplete inhibition of electron transfer by TTFA is discussed below.

Second, if the rates of electron transfer in the presence of TTFA are corrected for the uninhibitable activity, the resulting Dixon plots for inhibition of ubiquinone reductase and cytochrome c reductase activities are linear (Fig. 1, insets). This agrees with the results of Mowery and co-workers (27), who obtained linear Dixon plots for inhibition of succinate-ubiquinone reductase activity in Complex II and succinate oxidase activity of submitochondrial particles. Thus, our results appear to confirm that, in the span of the respiratory chain between succinate and cytochrome c, there is only a single site of TTFA inhibition or, if there are multiple sites, their affinities for TTFA are indistinguishable.

For noncompetitive inhibition as manifested by TTFA (cf. Ref. 27) one can calculate apparent K values from Dixon plots (25). Inhibition of both cytochrome c reductase (Fig. 1a) and...
ubiquinone reductase (Fig. 1b) activities occurs with apparent 
$K_i = 14 \pm 2 \mu M$, which further indicates a common single site 
for inhibition of these two activities. These $K_i$ values are 
significantly higher than that reported for the succinate-ubiquinone 
reductase activity of Complex II (1.6 $\mu M$; Ref. 27), and this 
difference may reflect a fundamental aspect of the manner 
in which TTFA inhibits electron transfer as discussed below.

Effect of TTFA on Electron Transfer Activities of Resolved 
Reductase Complex in the Presence of Antimycin—Addition 
of antimycin to respiring mitochondria or submitochondrial 
particles causes a coincident decrease of the apparent potential 
of the $n = 2$ ubiquinone couple, $Q/QH_2$, as the oxidation-
reduction poise of the two ubisemiquinone couples is shifted 
in the direction of the reduced forms, $QH_2/QH_2$ (31, 32). In 
addition, inhibition of electron transfer by antimycin is essen-
tially irreversible ($K_a = 10^{-11} M$) such that this inhibitor does 
not dissociate on dilution of the particles (33). Thus, by 
preincubating reductase complex with controlled amounts of 
antimycin ($<1 \text{ mol/mol of cytochrome } c_1$), it is possible to 
limit the rate of electron transfer through the complex from 
succinate to cytochrome $c$, thereby causing reducing equivalents 
to accumulate at low potential on the substrate side of the 
antimycin block and shifting the poise of the ubisemiquin-
one couples in the direction of the reduced forms.

If reductase complex is treated with an amount of antimycin 
which partially inhibits succinate-cytochrome $c$ reductase 
activity, the remaining activity is less sensitive to inhibition by 
TTFA than is the activity in the absence of antimycin, as 
shown in Fig. 2. This decreased efficacy of TTFA is apparent 
at concentrations as high as 500 $pM$ but is most obvious at low 
concentrations of TTFA (Fig. 2, inset). In the presence of 
antimycin, 10 $\mu M$ TTFA inhibits only 5% of the succinate-

cytochrome $c$ reductase activity, while in the absence of 
antimycin more than 60% of the activity is inhibited by this 
same concentration of TTFA. Also, there is a slight stimula-
tion of cytochrome $c$ reductase activity at low concentrations 
of TTFA in the presence of antimycin, while no such effect is 
seen in its absence. This stimulation is examined in more 
detail below.

Control experiments, previously reported (1), indicate that 
the diminished efficacy of TTFA is not attributable to heter-
ogeneity of the reductase complex population in terms of its 
sensitivity to antimycin, since the cytochrome $c$ reductase 
activity can be fully inhibited by amounts of antimycin stoi-
chiometric with the $c_1$ content of the complex and this sensi-
tivity to antimycin is not changed in the presence of TTFA.

Dixon plots of [succinate-cytochrome $c$ reductase activ-
ity]$^{-1}$ versus TTFA concentration in the presence of antimycin 
were nonlinear (not shown), having an upward deflection at 
low concentrations of TTFA, in contrast to the linear plots 
observed in the absence of antimycin (Fig. 2a, b). If the linear 
portion of the Dixon plots was extrapolated to the horizontal 
axis, the resulting intercept was at $-29 \mu M$ TTFA, which 
would correspond to a 2-fold increase in $K_i$ for TTFA in the 
antimycin-treated reductase. However, the validity of this 
analysis depends on TTFA inhibition being noncompetitive 
in the presence of antimycin and such may not be the case.

Antimycin does not change the pattern of inhibition of 
succinate-ubiquinone reductase activity by TTFA, as shown 
in Fig. 3. In contrast to the effect seen on the cytochrome $c$ 
reductase activity of the same sample of reductase complex 
(Fig. 2), the TTFA titration curves of ubiquinone reductase 
activity in the presence and absence of antimycin are super-
imposable. We also examined the inhibition of succinate-di-
chlorophenolindophenol reductase activity by TTFA, using 
the same assay conditions as in Fig. 3, except omitting DBH 
from the reaction mixture (9), and found that antimycin did 
not lower the efficacy of TTFA in this reaction either (results 
not shown).

The diminished efficacy of TTFA was observed with freshly
prepared reductase complex (see Figs. 5 to 7 below) or reductase complex which was stored frozen and subsequently reconstituted with ubiquinone-10 plus phospholipid (Fig. 2). If reductase complex is repeatedly frozen and thawed, the succinate-cytochrome c reductase activity drops to less than 1 unit/mg. This loss of activity is probably due in part to dislocation of the endogenous ubiquinone, since addition of ubiquinone-10 plus phospholipid, but not phospholipid alone, increases the activity of preparations thus damaged 2- to 3-fold (22). Thus, it was of interest to examine the effect of TTFA in the presence and absence of antimycin on reductase complex which was extensively damaged by freezing. As shown in Fig. 4, antimycin elicited a marked change in the effect of TTFA on succinate-cytochrome c reductase activity. In addition to the diminished efficacy noted previously, low concentrations of TTFA caused a pronounced stimulation of cytochrome c reductase activity in the presence of antimycin (Fig. 4, inset).

From the results shown in Figs. 2 and 4, it is not clear whether the magnitude of the stimulation of cytochrome c reductase activity observed at low concentrations of TTFA is unique to preparations which have low activity due to freezing and thawing, or whether the stimulation by TTFA is dependent on the extent to which activity is inhibited by antimycin. Fig. 5 illustrates the effect of 10 μM TTFA on succinate-cytochrome c reductase activity of reductase complex treated with different amounts of antimycin. When no antimycin was added to the reductase complex, 10 μM TTFA inhibited succinate-cytochrome c reductase activity 61% (Fig. 5a). If sufficient antimycin was added to inhibit activity 58%, addition of TTFA inhibited only 34% of the remaining activity (Fig. 5b). And if succinate-cytochrome c reductase activity was inhibited 98% by antimycin, addition of TTFA produced a 223% increase in the rate of cytochrome c reduction (Fig. 5c).

Using this type of approach, it was possible to show that the effect of TTFA on succinate-cytochrome c reductase activity was dependent on the extent to which electron flow from succinate to cytochrome c was limited by antimycin as shown in Fig. 6. As increasing amounts of antimycin were added to the reductase complex, at approximately the point where cytochrome c reductase activity became inhibited 50 to 60%, the effect of addition of TTFA switched from one in which the remaining activity was inhibited by TTFA to one in which addition of TTFA stimulated the rate of cytochrome c reduction. The same stimulation was observed with 10 μM (Fig. 6a) and 100 μM (Fig. 6b) TTFA, both concentrations promoting a stimulation of cytochrome c reduction greater than 200% at low rates of respiration.

Effect of TTFA on Reduction of Cytochrome b by Succinate in the Presence of Antimycin—Because the antimycin-dependent change in efficacy of TTFA is manifested in the succinate-cytochrome c reductase but not in the succinate-ubiquinone reductase reaction, it was of interest to examine the effects of TTFA and antimycin on reduction of cytochrome b in resolved reductase complex by succinate. In the absence of inhibitors, cytochrome b is reduced by succinate with a half-time less than 1 s at 8°C (Fig. 7a). The true half-time of this reaction is probably less than is apparent here, since the mixing time approaches 1 s. Addition of TTFA inhibits the reduction of cytochrome b.
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(a) aI
(b)

FIG. 6. Effect of TTFA on succinate-cytochrome c reductase activity of reductase complex treated with varying amounts of antimycin. The reductase complex was treated with the indicated amounts of antimycin prior to the start of the reaction. The results of succinate-cytochrome c reductase activity, expressed as per cent of the activity in the absence of antimycin, is shown by --- in both a and b. TTFA then was added to the ongoing cytochrome c reductase reaction as illustrated in Fig. 5. ---, extent to which 10 μM TTFA (a) or 100 μM TTFA (b) inhibited the cytochrome c reductase activity remaining in the presence of antimycin. In the absence of antimycin or TTFA, the cytochrome c reductase activity was 6.05 (a) and 6.47 (b) units/mg.

FIG. 7. Effect of TTFA on the reduction of cytochrome b in resolved reductase complex by succinate in the absence and presence of antimycin. The reduction of cytochrome b was monitored at 562 versus 577 nm in the dual wavelength spectrophotometer. As indicated, TTFA or 1 μg of antimycin/mg of reductase complex was added prior to initiating the reaction with succinate. The temperature of the mixture was 8°C. The numbers in parentheses indicate half-times for reduction of cytochrome b.

by succinate and the extent of inhibition increases with increasing amounts of TTFA (Fig. 7, b and d). This agrees with Baginsky and Hatefi's finding that TTFA inhibits reduction of cytochrome b in Complex II (28). Treatment of reductase complex with antimycin lowers the half-time for cytochrome b reduction in the presence of 50 μM TTFA from 7 to 1.8 s (Fig. 7, b and c) and in the presence of 500 μM TTFA from 25 to 4.6 s (Fig. 7, d and e). This antimycin-dependent decrease in inhibition was observed at concentrations of TTFA ranging from 2.5 μM to 1.0 mM (results not shown).

When the rate of electron transfer from substrate to cytochrome b is slow relative to the rate of electron transfer from cytochrome b to the high potential acceptors on the O₂ side of the antimycin block, the time required for reduction of cytochrome b may be determined by the time required to first reduce the high potential acceptors. Thus, for these experiments, we included controls to test whether the increased rate of cytochrome b reduction in the presence of TTFA caused by antimycin was attributable to antimycin preventing electron transfer through cytochrome b to the high potential acceptors, cytochrome c₁ and the iron-sulfur protein of the b-c₁ segment. We found that 2 mM ascorbate was added to the reaction mixture, which reduced cytochrome c₁ and presumably the iron-sulfur protein, the resulting inhibition of cytochrome b reduction by various concentrations of TTFA was not diminished, in contrast to the effect of antimycin on inhibition by TTFA. Thus, the effect of antimycin on inhibition of TTFA must be due to some effect other than preventing electron transfer to the high potential acceptors. The increased rate of cytochrome b reduction caused by antimycin is also not due to antimycin preventing reoxidation of cytochrome b, as might be expected with submitochondrial particles, since there is no flowthrough of reducing equivalents to O₂ in the resolved reductase complex.

Effect of Superoxide Dismutase on the Increased Rate of Cytochrome c Reduction Caused by TTFA in the Presence of Antimycin—The increased rate of cytochrome c reduction which results from addition of TTFA to an ongoing succinate-cytochrome c reductase reaction catalyzed by antimycin-treated reductase complex is eliminated by subsequent addition of superoxide dismutase as shown in Fig. 8a. If TTFA is added to the reaction mixture prior to starting the reaction with antimycin-treated reductase complex, the initial rate of cytochrome c reduction is greater than that obtained with antimycin-treated reductase complex in the absence of TTFA (compare Fig. 8, b and a). If superoxide dismutase is also included in
Inhibition by TTFA. For this explanation to apply, one b-cytochrome c reductase activity would be less sensitive to is 3- to 4-fold greater than these, it might be expected that if the ubiquinol-cytochrome c reductase activity is limited by the succinate-ubiquinone reductase complex in the absence of inhibitors. These results are evidence that the increased rate of cytochrome c reduction observed in the presence of TTFA and antimycin is due to formation of superoxide anion which reduces cytochrome c.

It can be seen in Fig. 8a that superoxide dismutase lowers the rate of cytochrome c reduction to a value somewhat less than that observed in the presence of antimycin (70 versus 123 units/mg). The likely explanation for this effect is that a portion of the cytochrome c reductase rate observed in the presence of antimycin is due to antimycin-induced production of superoxide anion as described by Boveris and co-workers (34). The increments in rates of cytochrome c reduction shown in Fig. 8 indicate that TTFA enhances production of superoxide anion approximately 3-fold above that caused by antimycin alone.

**DISCUSSION**

Our results with resolved succinate-cytochrome c reductase complex confirm previous findings (27) that inhibition by TTFA of electron transfer in the segment of the respiratory chain between succinate and cytochrome c involves only a single site of inhibitor action. For both succinate-ubiquinone reductase and succinate-cytochrome c reductase activities, the Dixon plots for inhibition by TTFA are linear, indicating that inhibition shows simple saturation kinetics in the resolved reductase complex as was found with Complex II (27). To the previous evidence that TTFA inhibits at a single site, we have added the finding that in the resolved reductase complex, the apparent K_i for inhibition of succinate-ubiquinone reductase and succinate-cytochrome c reductase activities is identical.

We have found that the efficacy of TTFA is lowered if electron transfer from succinate to cytochrome c is partially inhibited by antimycin. One possible explanation for the diminished efficacy is that resolved reductase complex may manifest pool function type kinetics (11, 32). If, in the absence of inhibitors, the rate of electron transfer from succinate to cytochrome c is limited by the succinate-ubiquinone reductase activity and if the ubiquinol-cytochrome c reductase activity is in 3 to 4 fold greater than these, it might be expected that if more than 75 to 80% of the ubiquinol-cytochrome c reductase activity was inhibited by antimycin, the remaining succinate-cytochrome c reductase activity would be less sensitive to inhibition by TTFA. For this explanation to apply, one b-c complex must be able to accept electrons from multiple dehydrogenase complexes, as if there were a homogenous and highly mobile pool of ubiquinone (11, 32).

Although we cannot exclude the possibility that pool function behavior may contribute partially to the decreased efficacy of TTFA, the increased rate of cytochrome c reduction seen in titration experiments, such as those in Figs. 2 and 4, and the decreased inhibition by TTFA of cytochrome b reduction in the presence of antimycin (Fig. 7) are not accounted for by pool function kinetics.

The diminished efficacy of TTFA in the presence of antimycin is partly due to production of superoxide anion which reduces cytochrome c nonenzymatically. This is most obvious under conditions where production of superoxide anion causes a net increase in rate of cytochrome c reduction (Figs. 5 and 8). However, the superoxide anion-dependent increment in rate of cytochrome c reduction is not sufficient to account for the extent to which the efficacy of TTFA is diminished by antimycin. The results in Figs. 5 and 8 indicate that superoxide anion accounts for 150 to 200 units/g of cytochrome c reduction. Thus, the increased rate of cytochrome c reduction caused by TTFA in the experiment of Fig. 4, which amounts to an increment in rates of 205 units/g, is quantitatively accounted for by superoxide anion. However, the experiment of Fig. 2 shows that 10 μM TTFA inhibits 1500 units of succinate-cytochrome c reductase activity in the absence of antimycin but inhibits only 300 units of activity in the presence of antimycin. This 1200 units/g difference in efficacy of TTFA is not accounted for by the measured rates of superoxide anion formation. Thus, as discussed below, a second factor which may contribute to the decreased efficacy is that ubisemiquinone, generated at the site of TTFA inhibition, may antagonize binding of the inhibitor.

In the segment of the electron transport chain between succinate and cytochrome c, the oxidation-reduction components which might generate superoxide anion by reacting with O_2 are the semiquinone form of FAD on SDH, the iron-sulfur centers of SDII, and ubisemiquinone. It seems likely that reaction of QH_2 with O_2 is responsible for production of superoxide anion in our experiments. Boveris and co-workers have shown that formation of superoxide anion in mitochondria treated with antimycin is dependent on the endogenous ubiquinone content (34) and autooxidation of ubiquinol in oxygenated buffers produces superoxide anion (35). Although we cannot unequivocally exclude the possibility that the flavin or one of the iron-sulfur centers of SDH, or both, might react with O_2 to form superoxide anion as a consequence of adding...
TTFA, the finding that solubilized SDH forms H₂O₂ at a very low rate in comparison to the parent submitochondrial particles (34) argues against these components as catalysts of superoxide anion production in this instance. In addition, if superoxide anion production catalyzed by flavin were a significant factor in our experiments, we would expect to see a diminished efficacy of TTFA in the succinate-ubiquinone reductase reaction. Such is not the case (see Fig. 3).

If QH₂ is the component which reacts with O₂ to form superoxide anion, a consequence of these results is that TTFA does not inhibit the reaction Q → QH₂, since addition of TTFA to antimycin-treated reductase complex would inhibit production of superoxide anion rather than stimulate it as we have observed. This finding, together with previous evidence that TTFA inhibits the reaction Q → QH₂ (30) and also inhibits reoxidation of iron-sulfur center S-3 (16), constitutes strong circumstantial evidence that center S-3 of SDH catalyzes the reaction QH₂ → QH₂ and that TTFA inhibits this reaction. This contrasts with the proposal (11, 37) that the reaction QH₂ → QH₂ might occur by a noncatalyzed dismutation reaction.

If TTFA inhibits the catalyzed reduction of QH₂ to QH₂, and if oxidation-reduction of cytochrome b is not required for succinate-ubiquinone reductase activity (28), then exogenously added quinones, such as DBH, and similar acceptors whose reduction is inhibited by TTFA must accept electrons from endogenous QH₂ or a currently unrecognized oxidation-reduction component on the O₂ side of QH₂. Thus, the slight lag observed in the succinate-ubiquinone reductase reaction (see "Experimental Procedures"), which is not observed in the succinate-cytochrome c reductase reaction (see Fig. 5), may reflect the time required for formation of a sufficient endogenous pool of QH₂ accessible to exogenous quinone acceptor, to yield zero order kinetics.

In addition, if TTFA inhibits reduction of QH₂ to QH₂, it is possible that the portion of the electron transfer activities not inhibitable by TTFA is due to the noncatalyzed dismutation reaction, 2 QH₂ → Q + QH₂, which could occur at a slow rate in the presence of TTFA. The explanation for why a greater rate of succinate-ubiquinone reductase activity (0.80 to 1.40 units/mg) than succinate-cytochrome c reductase activity (0.25 to 0.50 units/mg) is not inhibitable by TTFA is probably that the exogenous acceptor (DBH) oxidizes endogenous QH₂ faster than does the endogenous reductase acceptor. This explanation is supported by the fact that the succinate-ubiquinone reductase activity is significantly greater than the succinate-cytochrome c reductase activity (see Figs. 2 and 3).

In addition, the concentration of DBH (48 μM) is far in excess of that of reductase complex (3 to 6 nm) in these assays.

As a hypothesis regarding the site at which TTFA might bind in a manner which would inhibit the reaction QH₂ → QH₂, we suggest that TTFA intercalates between iron-sulfur center S-3 and the spin-coupled ubisemiquinone pair associated with this center (13, 14). In particular, we propose that the amount of TTFA required to inhibit respiration is dependent on the concentrations both of center S-3 and the associated ubisemiquinone(s). This is consistent with the findings that TTFA disrupts the interaction between center S-3 and the ubisemiquinone pair observed by EPR spectroscopy (17, 18) and that the amount of TTFA required to inhibit electron transfer is proportional to the amount of SDH added back to depleted particles (28). This also agrees with the suggestion by Nelson and co-workers (29) that TTFA is competitive with ubiquinone, except that we are suggesting a specific oxidation-reduction form, QH₂, as being antagonistic to the inhibitor.

We found that the apparent Kᵢ for inhibition by TTFA is 10-fold higher in resolved reductase complex than reported for Complex II. Since Complex II is highly enriched in SDH, our results might appear to contradict Baginsky and Hatefi's finding that the amount of TTFA required for inhibition is proportional to SDH content (28). A possible explanation for the higher Kᵢ obtained here is that the concentrations of ubisemiquinone associated with center S-3 may be higher in the resolved reductase complex and, thus, higher concentrations of TTFA would be required for inhibition. Likewise, the decreased efficacy of TTFA observed with antimycin-treated reductase complex and which resulted in parabolic Dixon plots, as mentioned above, can be attributed to increased amounts of ubisemiquinone associated with S-3 when electron transfer is partially inhibited by antimycin.

REFERENCES
Diminished Efficacy of Thenoyltrifluoroacetone

Diminished inhibition of mitochondrial electron transfer from succinate to cytochrome c by thenoyltrifluoroacetone induced by antimycin.

B L Trumpower and Z Simmons