Complete inhibition of dihydro-orotate oxidation and superoxide production by 1,1,1-trifluoro-3-thenoylacetone in rat liver mitochondria

Kottarappat N. DILEEPAN and James KENNEDY

Enzymology Research Laboratory, Veterans Administration Medical Center, 4801 Linwood Boulevard,
Kansas City, MO 64128, U.S.A.

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1,1,1-Trifluoro-3-thenoylacetone was shown to cause complete inhibition of dihydro-orotate oxidation in rat liver mitochondria as measured by orotate formation and the rate of dihydro-orotate-dependent reduction of 2,6-dichlorophenol-indophenol or cytochrome *c*. The inhibition by trifluorothenoyleacetone was dose-dependent, and a concentration of 1 mM completely inhibited dihydro-orotate dehydrogenase activity. 1,10-Phenanthroline, another iron-chelating agent, also caused total inhibition of the liver enzyme. Whereas the iron chelators inhibited 100% of dihydro-orotate dehydrogenase activity in liver mitochondria, they inhibited only a maximum of 72% in the case of the brain enzyme. The inhibition by trifluorothenoyleacetone was not prevented by addition of phenazine methosulphate or ubiquinone. Dihydro-orotate dehydrogenase-mediated generation of superoxide was abolished when the enzyme was fully inhibited by trifluorothenoyleacetone or when the electron-transport system was blocked by antimycin A. These results suggest that the iron component(s) of dihydro-orotate dehydrogenase is of strategic importance for catalytic activity and transfer of reducing equivalents from the primary enzyme to the electron-transport chain. Furthermore, the study indicates that production of superoxide radicals during dihydro-orotate dehydrogenase-catalysed oxidation of dihydro-orotate may be at the cytochrome *b-c*₁ segment of the electron-transport chain (as a consequence of auto-oxidation of ubisemiquinone) rather than at a site on the primary enzyme.

The enzymic oxidation of dihydro-orotic acid to orotic acid has been studied in several microbial sources and animal tissues with a view towards understanding its possible role in the regulatory mechanisms of pyrimidine biosynthesis (for a review see Jones, 1980). This obligatory step is catalysed by dihydro-orotate dehydrogenase (DHO-DHase, EC 1.3.3.1), the fourth enzyme in the pathway for the synthesis of uridylic acid *de novo*. In eukaryotic cells, DHO-DHase is located in the mitochondrion (Kennedy, 1973; Chen & Jones, 1976) and is linked to the electron-transport chain (Miller *et al.*, 1968; Forman & Kennedy, 1975).

Abbreviations used: DHO-DHase, dihydro-orotate dehydrogenase (EC 1.3.3.1); DCIP, 2,6-dichlorophenol-indophenol; TTFA, 1,1,1-trifluoro-3-thenoylacetone; QH₂, ubiquinol; QH•, ubisemiquinone.

In earlier reports a mechanism was presented in which the primary dehydrogenase reduced ubiquinone and cytochrome *b* and produced O₂•⁻ through an unidentified cofactor (Forman & Kennedy, 1975, 1976). Furthermore, TTFA, an iron-chelating agent, was found to cause partial inhibition of DHO-DHase activity without affecting O₂•⁻ production. Studies with a purified preparation of DHO-DHase revealed iron to be the only potentially reducible cofactor present (Forman & Kennedy, 1978). However, differences in catalytic properties between the isolated and membrane-bound forms of the enzyme made assignment of O₂•⁻ production to iron less certain. The present studies were carried out to re-evaluate the effect of iron chelators in order to gain further insight into the role of iron in dihydro-orotate oxidation and O₂•⁻ production in isolated mitochondria.

Materials and methods

Materials

Bovine serum albumin, L-dihydro-orotate, DCIP, cytochrome *c*, TTFA, 1,10-phenanthroline, phenazine methosulphate, ubiquinone-10, sodium succinate and sucrose (grade 1) were obtained from Sigma Chemical Co. All reagents used were of analytical grade. Superoxide dismutase was generously given by Dr. Amelia Cudd, Department of Biochemistry, Duke University Medical Center, Durham, NC, U.S.A.

Liver and brain mitochondria were prepared as described previously (Dileepan & Kennedy, 1983). Tissues from 150–200 g male Sprague–Dawley rats were homogenized in 9 vol. of 0.25M-sucrose, and mitochondria were isolated by centrifugation of the 600g supernatant at 8000g for 15 min. The mitochondrial pellet was washed twice in 0.15M-KCl and suspended in 0.15M-Tris/HCl buffer, pH 7.5, to one-third of the volume of the original homogenate. The mitochondrial preparation was used immediately for experiments with intact mitochondria or frozen at -20°C . For disrupted mitochondrial particles, the thawed preparations were washed with 0.15M-Tris/HCl buffer, pH 7.5, collected by centrifugation at 10000g for 15 min and resuspended in 0.15M-Tris/HCl buffer, pH 7.5, to one-third of the volume of the original homogenate.

Determination of dihydro-orotate oxidation

Oxidation of dihydro-orotate was measured by assaying the activity of DHO-DHase in three different ways: (1) by the direct determination of orotate formation; (2) by measuring the rate of dihydro-orotate-dependent reduction of DCIP; (3) by measuring the rate of reduction of cytochrome *c*.

Direct measurement of orotate formation was assayed as described previously (Dileepan & Kennedy, 1983). The reaction mixture contained 100mM-Tris/HCl buffer, pH 8.0, 1mM-L-dihydro-orotate, mitochondrial preparation equivalent to 200–500 μg of protein and, when present, the indicated amount of TTFA, all in a final volume of 2ml. The reaction was carried out at 37°C for 30 min (unless otherwise specified) with shaking and terminated by the addition of 2ml of 2M- HClO_4 . The concentration of orotate in the supernatant was measured by its absorbance at 280 nm (Kennedy, 1973). Blanks without substrate were run concurrently with each set of experiments.

The assays of DHO-DHase and succinate dehydrogenase activity with the use of DCIP (Miller *et al.*, 1968) or cytochrome *c* reduction were carried out as described previously (Forman & Kennedy, 1974, 1976) with minor modifications.

The reaction mixture contained 60mM-Tris/HCl buffer, pH 8.0, 0.66mM-L-dihydro-orotate, 1mM-KCN (unless otherwise specified), 43 μM -DCIP or 20 μM -cytochrome *c*, mitochondrial preparation equivalent to 200–500 μg of protein and, when present, the indicated amount of TTFA and other ingredients, all in a final volume of 3ml. The assay mixture for succinate dehydrogenase was the same except that 10mM-sodium succinate was used instead of dihydro-orotate. The reaction was initiated by addition of substrate after a 3 min pre-incubation. The changes in absorbance due to reduction of DCIP and cytochrome *c* were recorded spectrophotometrically at 600nm and 550nm respectively.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

One unit of enzyme activity is defined as 1 nmol of orotate formed or 1 nmol of DCIP or cytochrome *c* reduced in 1 min.

Results

TTFA caused virtually complete inhibition of dihydro-orotate oxidation in liver mitochondrial particles as measured by orotate formation and dihydro-orotate-dependent reduction of DCIP or cytochrome *c* (Table 1). The inhibition by TTFA was concentration-dependent, and total inhibition could be achieved at an inhibitor concentration of 1mM (Table 1 and Fig. 1). As shown in Fig. 1(a) (insert), there is rapid inhibition of dihydro-orotate-dependent reduction of cytochrome *c* at lower concentrations of TTFA, resulting in the loss of more than 50% of the activity by 10 μM -TTFA. A Dixon (1953) plot corresponding to the inhibition titration curve is linear and gives an apparent K_i of 23 μM (Fig. 1b). The inhibitory profiles were identical for intact mitochondria and disrupted mitochondrial particles (results not shown).

Ubiquinone and the artificial electron-acceptor phenazine methosulphate have been known to circumvent the blockade of the electron-transport chain by various inhibitors. However, in the present study neither phenazine methosulphate (0.1mM) nor ubiquinone (1mM) reversed TTFA-mediated inhibition of DHO-DHase activity as measured by reduction of DCIP (results not shown). Furthermore, addition of phenazine methosulphate or ubiquinone alone did not stimulate the rate of dihydro-orotate oxidation (results not shown), suggesting that the mitochondrial preparations contained sufficient amounts of endogenous electron acceptors for transporting reducing equivalents from the primary enzyme to the terminal acceptor.

Whereas DHO-DHase activity in liver mito-

Table 1. *Effect of TTFA on dihydro-orotate oxidation by rat liver mitochondrial particles measured by three different methods*

The assay system for dihydro-orotate-dependent reduction of DCIP or cytochrome *c* contained 60 mM-Tris/HCl buffer, pH 8.0, 0.66 mM-L-dihydro-orotate, 1 mM-KCN, 43 μ M-DCIP or 20 μ M-cytochrome *c*, mitochondrial particles equivalent to 200–500 μ g of protein and the indicated amount of TTFA in a final volume of 3 ml. The reaction was initiated by the addition of substrate after a 3 min pre-incubation. The reaction was carried out at 30°C and the change in absorbance due to reduction of DCIP or cytochrome *c* was recorded at 600 nm or 550 nm respectively. Orotate formation was assayed by the direct observation of the appearance of orotate. The reaction mixture contained 100 mM-Tris/HCl buffer pH 8.0, 1 mM-L-dihydro-orotate, mitochondrial particles equivalent to 200–500 μ g of protein and the indicated amount of TTFA in a final volume of 2 ml. The reaction was initiated by the addition of substrate after a 5 min pre-incubation and continued for 30 min. The reaction was terminated by addition of 2 M-HClO₄, and the concentration of orotate in the supernatant was measured by its absorbance at 280 nm. Units of activity are as defined in the Materials and methods section. Each value given is mean of the number of observations given in parenthesis.

Concn. of TTFA (mM)	Dihydro-orotate oxidation (units/mg of protein)		
	DCIP reduction	Cytochrome <i>c</i> reduction	Orotate formation
0 (control)	4.78 (5)	5.73 (4)	4.58 (2)
0.1	2.17 (2)	2.80 (2)	2.79 (2)
0.5	1.22 (3)	1.68 (2)	1.33 (2)
1.0	0.12 (4)	0.56 (2)	0.29 (2)

Table 2. *Effect of TTFA on DHO-DHase activity in brain mitochondrial particles*

Details of the assay procedures are given in the legend to Table 1 and in the Materials and methods section. Each value given is the mean of duplicate determinations.

Concn. of TTFA (mM)	DHO-DHase activity (units/mg of protein)	
	DCIP reduction	Orotate formation
0 (control)	2.63	2.57
0.5	0.76	1.53
1.0	0.76	1.37

chondria was totally inhibited by TTFA, the enzyme activity in brain mitochondria was inhibited only to a maximum of 70% (Table 2). 1,10-Phenanthroline (3 mM), another potent iron chelator, also caused complete inhibition of the liver

Table 3. *Effect of superoxide dismutase on DHO-DHase activity in liver and brain mitochondrial particles*

DHO-DHase activity was assayed by measuring the reduction of DCIP as described in the legend to Table 1 and in the Materials and methods section. The specified amount of superoxide dismutase was present during the pre-incubation time and throughout the reaction. Reaction was initiated by the addition of dihydro-orotate. Each value given is the mean of duplicate observations. Abbreviation: N.D., not determined.

Superoxide dismutase (μ g/assay)	DHO-DHase activity (units/mg of protein)	
	Liver	Brain
0	5.09	1.39
50	3.39	0.70
100	3.39	N.D.
200	3.39	N.D.

DHO-DHase activity, whereas it inhibited only 70% of the enzyme activity in the brain (results not shown).

It has been demonstrated earlier that DHO-DHase is capable of producing O₂^{•-} (Forman & Kennedy, 1975). Since superoxide dismutase scavenges O₂^{•-} in tissues, the difference in the rates of dihydro-orotate-dependent reduction of DCIP in the presence and in the absence of superoxide dismutase has been considered to represent the rate of O₂^{•-} formation. As shown in Table 3, addition of superoxide dismutase inhibited dihydro-orotate-dependent reduction of DCIP in liver and brain mitochondria by 33% and 50% respectively. Whereas superoxide dismutase caused inhibition of DHO-DHase-catalysed reduction of DCIP and cytochrome *c*, it did not alter the rate of orotate formation (results not shown).

As an attempt to determine the site of O₂^{•-} generation during dihydro-orotate oxidation in mitochondria, the effects of antimycin A, superoxide dismutase and TTFA on dihydro-orotate-dependent reduction of added cytochrome *c* were examined (Table 4). The concentration of antimycin A used in this experiment was sufficient to block electron transfer at the cytochrome *b-c*₁ segment of the respiratory chain, whereas the TTFA concentration used was only sufficient to exert partial inhibition. Addition of superoxide dismutase inhibited cytochrome *c* reduction by 31%, the maximum inhibition expected, as the amounts of superoxide dismutase present in the system was sufficient to scavenge total O₂^{•-} produced. Antimycin A inhibited 77% of cytochrome *c* reduction in the absence of superoxide dismutase. Antimycin A apparently also blocked O₂^{•-} generation, since addition of superoxide

Fig. 1. Effect of TTFA on the DHO-DHase-catalysed reduction of cytochrome *c* in liver mitochondrial particles. DHO-DHase activity was assayed by measuring the rate of reduction of cytochrome *c* as described in the Materials and methods section. (a) Dependence of the enzyme inhibition on TTFA concentration. The inset shows the inhibitory profile at the lower concentrations of TTFA. (b) Dixon plot corresponding to the inhibitor titration curve (a). In this plot the enzyme activity (v) is the fraction of that in the absence of TTFA. Enzyme activity in the absence of TTFA was 5.90 units/mg of protein.

Table 4. Effects of superoxide dismutase (SOD), TTFA and antimycin A on dihydro-ornate-dependent reduction of cytochrome *c* in liver mitochondrial particles

Dihydro-ornate-dependent reduction of cytochrome *c* was assayed as described in the legend to Table 1 and in the Materials and methods section. In addition to the normal ingredients the assay system contained, when present, 50 μ g of superoxide dismutase, 30 μ g of antimycin A, 10 μ M-TTFA and liver mitochondria equivalent to 477 μ g of protein in a final volume of 3 ml. Each value given is the mean of duplicate observations.

Addition	Cytochrome <i>c</i> reduction (units/mg of protein)			Inhibition by SOD (%)
	-SOD	+SOD	O ₂ ^{•-} -dependent reduction	
None (control)	5.24	3.61	1.63	31
Antimycin A	1.20	1.22	0	0
TTFA	2.96	1.87	1.09	37
Antimycin A + TTFA	0.99	0.99	0	0

dismutase did not cause further inhibition of cytochrome *c* reduction. When DHO-DHase activity was only partially inhibited by TTFA, O₂^{•-} generation was inhibited to the same extent as inhibition of overall dihydro-ornate oxidation by chelator.

In this type of mitochondrial preparation, succinate dehydrogenase appears to be more sensitive to the effects of TTFA than does DHO-DHase, in the sense that less chelator is required to inhibit the former completely. Succinate dehydrogenase was completely inhibited by 0.5 mM-TTFA (Table 5).

Discussion

The results presented here indicate that TTFA and 1,10-phenanthroline cause complete inhibition of hepatic mitochondrial dihydro-ornate oxidation as determined by ornate formation and dihydro-ornate-dependent reduction of DCIP or cytochrome *c*. Although the chelating agents inhibited total dihydro-ornate oxidation in liver mitochondria, they exerted only partial inhibition in brain mitochondria. The latter finding is in agreement with earlier reports from this laboratory (Forman & Kennedy, 1975, 1976) demonstrating

Table 5. Comparison of the effects of TTFA on the activities of DHO-DHase and succinate dehydrogenase in liver mitochondrial particles in the presence and in the absence of KCN

The activities of DHO-DHase and succinate dehydrogenase were assayed by measuring the reduction of DCIP as described in the Materials and methods section. When present in the assay medium KCN was 1 mM. Values presented for DHO-DHase assayed in the presence of KCN are adopted from Table 1. Other results presented are the means of duplicate observations.

Concn. of TTFA (mM)	Enzyme activity (units/mg of protein)	
	+ KCN	-KCN
DHO-DHase		
0	4.78	4.66
0.1	2.17	2.67
0.5	1.22	1.33
1.0	0.12	0
Succinate dehydrogenase		
0	9.33	6.0
0.1	2.67	1.33
0.5	0	0

partial inhibition of DHO-DHase activity by TTFA in brain mitochondria. However, earlier work showed only partial inhibition of the DHO-DHase activity in liver mitochondria as well. The discrepancy between the earlier and present results may be attributed, at least in part, to the quality of the mitochondrial preparations. In this regard several points need emphasis. First of all, DHO-DHase activity reported earlier (Forman & Kennedy, 1975, 1976) is only about 20% of what has been observed in the present experiments. This would indicate the mitochondrial preparations used previously either had contaminating particles or were only partially active. Secondly, since DHO-DHase is linked to the electron-transport chain (Forman & Kennedy, 1976), the optimum activity of the enzyme is dependent on the presence of surplus quantities of electron-transport components such as ubiquinone and cytochromes in the mitochondrial preparations. In the present study addition of ubiquinone did not enhance the overall DHO-DHase activity, whereas addition of ubiquinone stimulated dihydro-orotate-dependent reduction of DCIP in the earlier experiments (Forman & Kennedy, 1975). This indicates that the mitochondrial preparations used earlier possibly did not contain sufficient amounts of ubiquinone to carry out electron transfer efficiently, as the endogenous ubiquinone content is considered to be crucial for the electron transport (Boveris *et al.*, 1976). Thirdly, the accessibility of TTFA and 1,10-

phenanthroline to the iron centres also may have been a factor for discrepancy between these two studies. The inadequate accessibility of the chelating agents to the iron centres may have been the cause for the failure of complete inhibition of DHO-DHase activity in brain mitochondria, as these preparations are likely to trap small amounts of myelin. This reasoning is consistent with the finding that DHO-DHase purified from liver is completely inhibitable by TTFA, with a K_i of 20 μ M (Forman & Kennedy, 1978), which closely agrees with the present data obtained for liver mitochondrial particles ($K_i = 23 \mu$ M; Fig. 1b). Furthermore, the presence of 8 mol of iron/mol of enzyme and the stoichiometry of 1,10-phenanthroline binding (1 mol/mol of subunit) (Forman & Kennedy, 1978) support the notion that all the iron centres are saturable with iron chelators if they are freely accessible.

Inhibition of DHO-DHase activity by TTFA is not reversed by phenazine methosulphate or added ubiquinone, indicating that the iron component(s) of the enzyme is an absolute requirement for the transfer of reducing equivalents to the electron-transport chain and that phenazine methosulphate and ubiquinone do not compete for electrons at the non-haem iron centre. Using resolved succinate-cytochrome *c* reductase complex, Trumpower & Simmons (1979) have shown that inhibition by TTFA in the segment of the respiratory chain between succinate and cytochrome *c* involves only a single site of action. Therefore, if TTFA inhibited the reduction of ubisemiquinone (QH $\cdot$) to ubiquinol (QH $_2$), as suggested by Trumpower & Simmons (1979), a blockade in the quinone cycle should be expected. In the case of succinate dehydrogenase TTFA has been shown to interact with the iron-sulphur centre of the enzyme complex (Mowery *et al.*, 1977). In this regard, DHO-DHase seems to resemble succinate dehydrogenase.

There is substantial evidence suggesting that O $_2^{\cdot-}$ generation is at the cytochrome *b-c* $_1$ segment of the respiratory chain, resulting from auto-oxidation of QH $_2$ or QH $\cdot$ (Boveris & Chance, 1973; Boveris *et al.*, 1976; Cadenas *et al.*, 1977; Trumpower & Simmons, 1979). By using inhibitors that block specific sites of the cytochrome *b-c* $_1$ complex Ksenzenko *et al.* (1983) demonstrated that most of the O $_2^{\cdot-}$ produced during succinate oxidation is a consequence of auto-oxidation of an unstable QH $\cdot$. It seems reasonable to postulate that such unstable QH $\cdot$ could be produced during DHO-DHase-catalysed oxidation of dihydro-orotate, because interaction of ubiquinone with the iron centre of the 'primary enzyme' is likely to involve only a single electron transfer. According to the proposed model for O $_2^{\cdot-}$ generation (Ksen-

zenko *et al.*, 1983), in the absence of antimycin A or other site 2 inhibitors, $\text{QH}^{\bullet}$ formed on oxidation of QH_2 by $\text{FeS}_{\text{Rieske}}$ (Trumpower, 1981) or by a special $\text{FeS}_{\text{Rieske}}$ -associated ubiquinone molecule (Konstantinov *et al.*, 1981) is rapidly oxidized to ubiquinone by *b* cytochromes. Hence the steady-state concentration of $\text{QH}^{\bullet}$ and the rate of its oxidation should be very low. When electron transfer via cytochromes *b*-566 and *b*-562 is inhibited by antimycin A, cytochrome *b*-566 becomes highly reduced in the aerobic steady state and can no longer compete with oxygen for $\text{QH}^{\bullet}$. The consequence is that auto-oxidation of $\text{QH}^{\bullet}$ will be greatly enhanced and $\text{O}_2^{\bullet-}$ will be generated. If KCN is also present, antimycin-stimulated $\text{O}_2^{\bullet-}$ production will be abolished, as KCN blocks oxidation of QH_2 to $\text{QH}^{\bullet}$ via the cytochrome chain (Cadenas & Boveris, 1980; Ksenzenko *et al.*, 1983).

The present results are compatible with the above hypothesis with regard to $\text{O}_2^{\bullet-}$ generation during dihydro-orotate oxidation by the membrane-bound DHO-DHase. As evident in Table 4, when cytochrome *c* reduction was blocked by antimycin A (in the presence of KCN) $\text{O}_2^{\bullet-}$ production was abolished. On the other hand, when DHO-DHase activity was only partially inhibited by TTFA the extent of inhibition in cytochrome *c* reduction (44%) was approximately equal to the inhibition of superoxide dismutase-resistant cytochrome *c* reduction (48%). These results indicate that inhibition of $\text{O}_2^{\bullet-}$ production by TTFA is a consequence of the overall blockade of dihydro-orotate oxidation resulting from the inhibition of DHO-DHase, whereas that by antimycin A is due to the inhibition of $\text{QH}^{\bullet}$ generation resulting from the simultaneous blockade of electron transfer at cytochrome *b*-*c*₁ and the oxidation of QH_2 . Thus it is assumed that $\text{O}_2^{\bullet-}$ produced during DHO-DHase reaction is at the cytochrome *b*-*c*₁ segment and not at a site in the primary enzyme.

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