Inhibition of the Chloramphenicol-induced Alternative Oxidase-Mediated Ubiquinol-15 Oxidation in *Neurospora crassa* Mitochondria by 2-Thenoyltrifluoroacetone

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Ubiquinol-15 can be used as an electron donor for both the conventional cytochrome chain and the chloramphenicol-induced alternative pathway of *Neurospora crassa* mitochondria. The oxidation of ubiquinol-15 through the chloramphenicol-induced cyanide-insensitive pathway is not inhibited by antimycin A and is strongly inhibited by salicylhydroxamic acid. In contrast, the oxidation of duroquinol by *N. crassa* mitochondria is sensitive to antimycin A under all circumstances, except for some portion which is believed to be a non-enzymic autoxidation reaction. 2-Thenoyltrifluoroacetone, a specific inhibitor of the succinate-ubiquinone oxidoreductase activity of the respiratory chain, was found to be a potent inhibitor of the chloramphenicol-induced alternative oxidase-mediated NADH and ubiquinol-15 oxidation in *N. crassa* mitochondria. It may be concluded that 2-thenoyltrifluoroacetone interacts with a specific electron carrier of the alternative oxidase, possibly an iron-sulphur centre associated with an ubisemiquinone.

INTRODUCTION

Organisms with cyanide-insensitive respiration have a branched respiratory chain (Palmer, 1976; Solomos, 1977). One branch is the conventional cytochrome chain. The second branch is of unknown composition and is called the alternative respiratory pathway or alternative oxidase. It is generally accepted that ubiquinone functions as the branchpoint for the two pathways (Hanssens *et al.*, 1978; Huq & Palmer, 1978*a*; Rich & Bonner, 1978). The alternative oxidase is insensitive to cyanide and antimycin A, the classical inhibitors of cytochrome-mediated respiration, but can be specifically inhibited by substituted hydroxamic acids such as salicylhydroxamic acid (Schonbaum *et al.*, 1971).

Various attempts have been made to isolate the mitochondrial component which catalyses the O₂-reducing step of the alternative oxidase. Huq & Palmer (1978*b*) reported the partial purification by ion-exchange chromatography of a fraction of lubrol-solubilized cyanide-insensitive mitochondria of *Arum maculatum*, which promoted salicylhydroxamic acid-sensitive oxidation of duroquinol. Rich (1978) used deoxycholate to solubilize cyanide-insensitive mitochondria of *A. maculatum*, and obtained a preparation that showed salicylhydroxamic acid-sensitive oxidation of menadiol and ubiquinol-5. In both cases, however, there were some complications, and the possibility that the activity was due to autoxidation rather than an enzyme-catalysed oxidation of the quinols could not be excluded. Moreover, the autoxidation of duroquinol and menadiol is inhibited by salicylhydroxamic acid, and therefore salicylhydroxamic acid sensitivity is not sufficient as a criterion to

distinguish simple autoxidation from enzyme-catalysed oxidation of quinols (Vanderleyden *et al.*, 1980a).

Huq & Palmer (1978c) demonstrated that duroquinol is oxidized in cyanide-insensitive mitochondria through the pathway which is antimycin A- and cyanide-insensitive and salicylhydroxamic acid-sensitive. However, Von Jagow & Bohrer (1975) reported that the oxidation of duroquinol in *Neurospora crassa* mitochondria possessing the alternative pathway is cyanide-insensitive but antimycin A-sensitive. Therefore, duroquinol is thought to react with cytochrome *b* in *N. crassa*, as was found in mitochondria of animal tissues (Slater *et al.*, 1961; Boveris *et al.*, 1971). Hence, duroquinol is not a suitable substrate for the alternative oxidase of *N. crassa* since cytochrome *b* is not an intermediate of the O₂-reducing part of the alternative oxidase, and its use in the study of the alternative oxidase so far is restricted to plant mitochondria.

In this paper we describe the use of ubiquinol-15 as an electron donor to both the cyanide-sensitive and cyanide-insensitive pathway of *N. crassa*. In addition, we report that 2-thenoyltrifluoroacetone, earlier described as a specific inhibitor of the succinate-ubiquinone region of the respiratory chain (Takemori & King, 1964), is a potent inhibitor of the alternative oxidase-mediated oxidation of NADH and ubiquinol-15 in *N. crassa* mitochondria.

METHODS

Micro-organism and growth. The organism used was *Neurospora crassa* wild type, 74-OR23-1A (abbreviated 74A), obtained from the Fungal Genetics Stock Center (FGSC; Humboldt State University Foundation, Arcata, Calif., U.S.A.). Mycelium was grown at room temperature in liquid Vogel's medium (Vogel, 1964) as described previously (Bertrand *et al.*, 1968; Bertrand & Pittenger, 1969). Non-induced (cyanide-sensitive) mycelium was obtained after growth for 18 h in the standard Vogel's medium. Induced (cyanide-insensitive) mycelium was obtained after growth for 48 h in Vogel's medium supplemented with chloramphenicol (5 mg ml⁻¹).

Preparation of mitochondria. Mycelium was disrupted with a grinding mill (Weiss *et al.*, 1970), and mitochondria were prepared as previously described (Bertrand & Pittenger, 1969).

Reduction of ubiquinone-15. Ubiquinone-15 was reduced using the method of Rieske (1967), except that NaBH₄ was used instead of Na₂S₂O₄.

O₂ consumption. O₂ consumption of mitochondria was measured polarographically with a Clark-type oxygen electrode. Unless otherwise stated, the reaction mixture consisted of 0.44 M-sucrose, 10 mM-Tris/HCl, 10 mM-MgSO₄, 10 mM-KH₂PO₄, 1 mM-5'-GMP, 2 mM-EDTA and 0.2% (w/v) bovine serum albumin, pH 7.3.

Protein determination. Mitochondrial protein was determined by the biuret method (Gornall *et al.*, 1949) with modifications to account for absorbance due to turbidity of the sample (Busenherz *et al.*, 1953).

Ubiquinol-15 concentration. Ubiquinol-15 was made up in ethanol and the concentration was determined as described previously (Vanderleyden *et al.*, 1980c).

Materials. 5'-GMP was obtained from Sigma. 2-Thenoyltrifluoroacetone was obtained from Fisher Scientific Co., Fairlawn, N.J., U.S.A., and stored as a 500 mM solution in ethanol. Duroquinol was obtained from Pfaltz & Bauer, Stamford, Conn., U.S.A., and made up fresh as a 300 mM solution (determined gravimetrically) in ethanol. Ubiquinone-15 was a generous gift of Hoffman-la-Roche, Basel, Switzerland. The purity of the sample was checked by reversed-phase thin-layer chromatography (Ramasarma & Jayaraman, 1971).

RESULTS

Succinate and duroquinol oxidase activities of induced mitochondria of N. crassa

The succinate and duroquinol oxidation in induced mitochondria of *N. crassa* are compared in Fig. 1. Induced mitochondria contained both the conventional cytochrome chain and the alternative pathway, although the former was present to a lesser extent than in non-induced mitochondria due to inhibition of the synthesis of cytochrome *b* and cytochrome *aa₃* by chloramphenicol. The oxidation of duroquinol was insensitive to cyanide but sensitive to antimycin A, whereas the oxidation of succinate was largely insensitive to both inhibitors and to the same extent.

Fig. 1. Succinate oxidase (left) and duroquinol oxidase (right) activities of induced mitochondria of *Neurospora crassa*. The oxidase activities were measured polarographically with succinate (Succ) or duroquinol (DHQ) as substrate. The reaction mixture (final volume 3 ml, temperature 25 °C, pH 7.3) consisted of 0.3 M-sucrose, 10 mM-*N*-tris(hydroxymethyl)methyl-2-aminomethane sulphonic acid, 5 mM-KH₂PO₄, 5 mM-MgCl₂. At the times indicated, additions were made of mitochondria (Mito) (0.25 to 0.6 mg protein), 1 mM-KCN, 3 µg antimycin A (AA), 1 mM-5'-GMP (GMP) and 2.6 mM-salicylhydroxamic acid (SHAM). The numbers above the traces represent the rates of O₂ consumption in ng-atom O min⁻¹ (mg protein)⁻¹.

5'-GMP (1 mM) was included in the oxidase assays, because the alternative oxidase activity of *N. crassa* mitochondria is stimulated by 5'-GMP (Vanderleyden *et al.*, 1980*b*). These experiments agreed with the results of Von Jagow & Bohrer (1975). The residual oxidation rate with duroquinol, in the presence of both salicylhydroxamic acid and cyanide or antimycin A, must have been due to a non-specific oxidation, including autoxidation, since it was not found with succinate or NADH (not shown).

Ubiquinol-15 as an electron donor for the respiratory pathways of N. crassa

The oxidation rates of non-induced and induced mitochondria of *N. crassa* with NADH, succinate and ubiquinol-15 as substrates were tested for sensitivity to cyanide, antimycin A and salicylhydroxamic acid. Non-induced mitochondria have only the conventional cytochrome respiratory pathway. Table 1 shows that the response to the different inhibitors of both the alternative oxidase-mediated and the cytochrome oxidase-mediated ubiquinol-15 oxidase activities was very similar to the response of the corresponding NADH and succinate oxidase activities. It is important to note that the oxidation of ubiquinol-15 by induced mitochondria was almost completely insensitive to cyanide as well as antimycin A, and to the same extent. Hence ubiquinol-15 is a more suitable quinol substrate for the alternative oxidase of *N. crassa* mitochondria than is duroquinol, since duroquinol oxidation in induced mitochondria is sensitive to antimycin A. The complete inhibition of the cytochrome oxidase-mediated ubiquinol-15 oxidation by cyanide or antimycin A was clear proof that there was no interference from non-enzymic oxidation reaction of the substrate, an observation in agreement with the properties of ubiquinol-15 described by Futami *et al.* (1979).

The amounts of mitochondrial protein used to determine the NADH and succinate oxidation rates were, respectively, 10 and 20 times higher than the amount used to determine the ubiquinol-15 oxidation rates (see legend of Table 1). When ubiquinol-15 is used as an

Table 1. *Inhibitor sensitivities of the oxidase activities of non-induced and induced mitochondria of Neurospora crassa*

The oxidase activities were measured polarographically with 1 mM-NADH, 10 mM-succinate or 1 mM-ubiquinol-15 as substrates. The reaction mixture (final volume 3 ml, temperature 25 °C, pH 7.3) consisted of 0.44 M-sucrose, 10 mM-Tris/HCl, 10 mM-KH₂PO₄, 10 mM-MgSO₄, 2 mM-EDTA, 0.2% (w/v) bovine serum albumin and 1 mM-5'-GMP. The amounts of mitochondrial protein used were 0.3 mg for the NADH oxidase assays, 0.6 mg for the succinate oxidase assays, and 0.03 mg for the ubiquinol-15 oxidase assays. The original activities [$\mu\text{g-atom O min}^{-1} (\text{mg protein})^{-1}$] of the different assays were (a) 197, (b) 177, (c) 195, (d) 410, (e) 396 and (f) 480. Abbreviations: SHAM, salicylhydroxamic acid; Succ, succinate; UQ-15, ubiquinol-15.

Inhibitor	Percentage inhibition of maximal rate					
	Non-induced mitochondria			Induced mitochondria		
	(a) NADH-O ₂	(b) Succ-O ₂	(c) UQ-15-O ₂	(d) NADH-O ₂	(e) Succ-O ₂	(f) UQ-15-O ₂
None	0	0	0	0	0	0
KCN (1 mM)	100	100	98	7	8	8
Antimycin A (3 μg)	100	100	98	11	10	8
SHAM (2.6 mM)	0	0	0	87	80	80
SHAM (2.6 mM) + KCN (1 mM)	100	100	98	95	86	98

Table 2. *Effects of 2-thenoyltrifluoroacetone on the oxidase activities of non-induced and induced mitochondria of Neurospora crassa*

The oxidase activities were measured polarographically as indicated in the legend of Table 1. Cytochrome-mediated oxidase activities were measured with non-induced mitochondria, and alternative oxidase-mediated activities with induced mitochondria. The reaction medium for the oxidase assays was the same as indicated in the legend of Table 1, except that 1 mM-KCN or 3 μg antimycin A was included in the medium for the alternative oxidase-mediated activities. The original activities of the different assays were of the same order of magnitude as those reported in the legend of Table 1. Abbreviations: TTFA, 2-thenoyltrifluoroacetone; SHAM, salicylhydroxamic acid; Succ, succinate; UQ-15, ubiquinol-15.

Inhibitor	Percentage inhibition of maximal rate					
	Non-induced mitochondria			Induced mitochondria		
	NADH-O ₂	Succ-O ₂	UQ-15-O ₂	NADH-O ₂	Succ-O ₂	UQ-15-O ₂
None	0	0	0	0	0	0
TTFA (1 mM)	0	77	0	88	82	95
TTFA (1 mM) + KCN (1 mM)	100	100	95	88	82	95
TTFA (1 mM) + SHAM (2.6 mM)	0	75	0	92	100	100

exogenous electron donor, only a small fraction of the ubiquinol-15 added to the reaction vessel (1 mM) is soluble in water. Thus the actual concentration of ubiquinol-15 in the respiration vessel was unknown, and saturation kinetics could only be measured by adjusting the amount of mitochondrial protein with the given amount of water-soluble ubiquinol-15. In these experiments, saturation kinetics were obtained with 1 mM-ubiquinol-15 added and 30 μg mitochondrial protein.

Inhibition of the alternative oxidase activity of N. crassa by 2-thenoyltrifluoroacetone

2-Thenoyltrifluoroacetone is known to be a potent inhibitor of succinate oxidation in membrane preparations from animal and plant tissues, fungi and bacteria, acting at the

Fig. 2. Dixon plot (Dixon, 1953) of the inhibition of the alternative oxidase-mediated ubiquinol-15 oxidation in induced mitochondria of *Neurospora crassa* by 2-thenoyltrifluoroacetone. The oxidase activities were measured polarographically as indicated in the legend of Table 1. The reaction mixture for the assay was the same as indicated in the legend of Table 1, except that 1 mM-KCN was also included. Oxidase activities were measured with 0.03 mg mitochondrial protein and 1 mM-ubiquinol-15. The reaction rate (v) is expressed in $\text{ng-atom O min}^{-1} (\text{mg protein})^{-1}$.

succinate dehydrogenase–ubiquinone junction (Takemori & King, 1964). Recently (Trumpower & Simmons, 1979), this inhibitor has been used to study the mechanism by which succinate dehydrogenase transfers electrons from succinate to ubiquinone and the b and c_1 cytochromes of the respiratory chain. With ubiquinol-15 as an electron donor it is possible to determine the effect of 2-thenoyltrifluoroacetone on the O_2 -reducing part of the alternative oxidase.

The results of the inhibition studies are summarized in Table 2. It is clear that, of the different cytochrome oxidase-mediated oxidase activities tested, only the succinate oxidase activity was inhibited by 1 mM-2-thenoyltrifluoroacetone. This agrees with the reported specificity of 2-thenoyltrifluoroacetone for the succinate–ubiquinone junction of the conventional respiratory chain (Garland *et al.*, 1966). With some mitochondrial preparations, the cytochrome oxidase-mediated NADH oxidation was stimulated by 1 mM-2-thenoyltrifluoroacetone. Since this effect appears to be unrelated to the activity of the alternative oxidase, it will not be discussed further. That 2-thenoyltrifluoroacetone did not inhibit the cytochrome oxidase-mediated ubiquinol-15 oxidation agrees with the finding of Hatefi *et al.* (1962) that 2-thenoyltrifluoroacetone does not inhibit ubiquinol–cytochrome c reductase activity. A completely different inhibition pattern was obtained with the alternative oxidase-mediated oxidations. All the activities tested were inhibited more than 80% by 1 mM-2-thenoyltrifluoroacetone, with an almost complete inhibition of the alternative oxidase-mediated ubiquinol-15 oxidation. Because a sensitivity shift was not observed when the alternative oxidase was measured in the presence of cyanide or antimycin A or both, inhibition by 2-thenoyltrifluoroacetone cannot be the result of a cyanide-induced or antimycin A-induced change of an electron carrier. Therefore, it may be concluded that 2-thenoyltrifluoroacetone interacts with a specific electron carrier of the alternative oxidase itself.

A Dixon plot (Dixon, 1953) for the inhibition of the alternative oxidase-mediated ubiquinol-15 oxidation by 2-thenoyltrifluoroacetone is shown in Fig. 2. The Dixon plot is linear from 10 to 1000 μM -2-thenoyltrifluoroacetone, a range above which 95% of the activity is inhibited. Linear Dixon plots reflect simple saturation kinetics and are consistent with the existence of a single binding site for the inhibitor. Alternatively, if there are multiple sites, their affinities for the inhibitor are indistinguishable from each other, as pointed out by

Trumpower & Simmons (1979) for the inhibition by 2-thenoyltrifluoroacetone on the succinate-ubiquinone junction of the respiratory chain.

DISCUSSION

In order to isolate and identify the O_2 -reducing component of the alternative pathway, it is necessary to find a substrate which bypasses the common electron carriers of both pathways (e.g. succinate-ubiquinone oxidoreductase) and donates electrons at or close to the alternative O_2 -consuming component. Huq & Palmer (1978*b, c*) discovered that duroquinol is an appropriate substrate for the alternative oxidase of plant mitochondria, whereas Von Jagow & Bohrer (1975) reported that the oxidation of duroquinol in cyanide-insensitive mitochondria of *N. crassa* is sensitive to antimycin A, suggesting that duroquinol donates electrons to a carrier of the conventional cytochrome chain. In the presence of cyanide, electrons are diverted to the alternative pathway. Our results confirm the finding of Von Jagow & Bohrer (1975). For this reason, it is obvious that duroquinol is not a suitable substrate for the alternative oxidase of *N. crassa* and that the electron transfer in the presence of duroquinol in plant mitochondria differs from the electron transport in *N. crassa* mitochondria.

Since ubiquinone is thought to be the branchpoint of the two pathways, short-chain ubiquinol homologues were tested as substrates for the alternative oxidase of *N. crassa*. It was found that ubiquinol-15 can be oxidized by both the cytochrome pathway and the alternative pathway, depending on the inhibitor(s) added. The inhibitor sensitivities for the oxidation of all three substrates, succinate, NADH and ubiquinol-15, by the alternative oxidase are similar. Although we did not do experiments with ubiquinone-depleted mitochondria, it is likely that ubiquinol-15 substitutes for endogenously formed ubiquinol, as demonstrated previously with beef heart mitochondria (Ambe & Crane, 1960). Ubiquinol-15 has been selected from among the short-chain ubiquinol homologues because its redox properties relate very well to those of the physiologically important ubiquinols (Futami *et al.*, 1979). There are, however, some disadvantages in the use of ubiquinone-15: its low solubility in water and its commercial unavailability.

A major step forward in the study of the alternative oxidase was the finding of Schonbaum *et al.* (1971) that substituted hydroxamic acids specifically inhibit the alternative oxidase activity. The inhibition mechanism of these compounds is not well understood, however. Several mechanisms have been proposed by Schonbaum *et al.* (1971), among which are chelation of a transition metal ion, most probably ferric ion, and polyfunctional hydrogen bonding. Interaction of hydroxamic acids with an iron-sulphur centre as the possible inhibition mechanism has been favoured in the past (Henry *et al.*, 1973), but Rich *et al.* (1978) proposed that mechanisms involving either hydrogen bonding or formation of charge-transfer complexes are more feasible. Indeed, Moore & Rupp (1978) have observed an interaction of salicylhydroxamic acid and semiquinones.

Since both ubiquinone and iron-sulphur centres seem to be required for alternative oxidase activity, and because 2-thenoyltrifluoroacetone is known to disrupt an interaction between ubiquinone and the iron-sulphur centre of succinate dehydrogenase (Nelson *et al.*, 1971), the effect of this compound on the alternative oxidase was tested. The results obtained from those experiments reveal that 2-thenoyltrifluoroacetone is a potent inhibitor of the alternative oxidase activity (95 % inhibition of the alternative oxidase-mediated ubiquinol-15 oxidation by 1 mM-2-thenoyltrifluoroacetone), but does not affect the cytochrome oxidase-mediated NADH and ubiquinol-15 oxidase in *N. crassa* mitochondria. Wilson (1971) first reported the effect of 2-thenoyltrifluoroacetone on the alternative oxidase-mediated NADH and malate oxidation of *Acer pseudoplatanus* mitochondria. He noted the inhibition of NADH and malate oxidation by 2-thenoyltrifluoroacetone through both pathways, but the inhibitory effect was enhanced by the addition of cyanide to the reaction mixture. Rich *et al.* (1977) did the same

experiments with *Phaseolus aureus* mitochondria, and reported different percentages of inhibition by 2-thenoyltrifluoroacetone of the alternative oxidase-mediated and cytochrome oxidase-mediated NADH oxidation, although a difference of only about 30% was observed. Ziogas & Georgopoulos (1979) showed that 2-thenoyltrifluoroacetone inhibits NADH oxidation by the alternative pathway but not by the cytochrome pathway in *Ustilago maydis* mitochondria. With our experiments on NADH and ubiquinol-15 oxidase activities in *N. crassa* mitochondria, it may be concluded that 2-thenoyltrifluoroacetone, at the concentration used, acts on a specific electron carrier which is a component of the alternative oxidase, because there is no inhibition at the NADH-ubiquinone junction and the inhibition of the transfer of electrons from exogenously added ubiquinol-15 to the endogenous ubiquinone pool can be excluded, since the inhibitor does not interfere with the oxidation of ubiquinol-15 by the cyanide-sensitive pathway.

The use of 2-thenoyltrifluoroacetone as an inhibitor of the alternative oxidase is very attractive, since the mechanism of inhibition of the succinate-ubiquinone oxidoreductase by this compound has been recently elucidated. It is believed that 2-thenoyltrifluoroacetone inhibits the reduction of ubisemiquinone to ubiquinol by intercalating between the high potential iron-sulphur centre S-3 of succinic dehydrogenase and the spin-coupled ubisemiquinone pair associated with this centre (Trumpower & Simmons, 1978, 1979). Hence, the inhibition of the alternative oxidase by 2-thenoyltrifluoroacetone confirms the importance of ubisemiquinone and the probable involvement of at least one iron-sulphur centre in the activity of the inducible alternative respiratory pathway of *N. crassa* mitochondria.

The results described here have been confirmed with mitochondria of chloramphenicol-induced *Moniliella tomentosa* (Vanderleyden *et al.*, 1980a), which also oxidize ubiquinol-15 through both pathways. The oxidation through the cytochrome chain is insensitive to 1 mM-2-thenoyltrifluoroacetone but is almost completely blocked through the alternative pathway by this compound.

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