The Effects of Bathophenanthroline, Bathophenanthrolinesulphonate and 2-Thenoyltrifluoroacetone on Mung-Bean Mitochondria and Submitochondrial Particles

By PETER R. RICH, ANTHONY L. MOORE and WALTER D. BONNER, JR.
*Johnson Research Foundation, Department of Biochemistry and Biophysics,
University of Pennsylvania, Philadelphia, PA 19174, U.S.A.*

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The effects of bathophenanthroline, bathophenanthrolinesulphonate and 2-thenoyltrifluoroacetone on mung-bean mitochondria and submitochondrial particles were investigated. A variety of inhibitory effects on the oxidations of NADH, succinate and malate were observed. The results are discussed in relation to sites of inhibition and their relation to the effects on mammalian mitochondria.

The effects of a number of known inhibitors of mammalian mitochondrial electron-transport activities have been studied with mitochondria and submitochondrial particles isolated from the hypocotyls of mung beans (*Phaseolus aureus*). Permeable hydrophobic bathophenanthroline, and bathophenanthrolinesulphonate, to which the inner mitochondrial membrane is impermeable (Palmer, 1970; Harmon & Crane, 1974, 1976), have been shown to have multiple effects on mammalian mitochondria. Their major effects are thought to be on the dicarboxylate carrier of the inner membrane, on various iron-sulphur centres and on cytochrome oxidase (Tyler & Newton, 1970; Passarella *et al.*, 1973; Harmon & Crane, 1974, 1976; Phelps *et al.*, 1974; Trumpower & Katki, 1975; Lofrumento & Zannotti, 1976). 2-Thenoyltrifluoroacetone is thought to act on a component of succinate dehydrogenase (Tappel, 1960; Whittaker & Redfearn, 1963; Baginsky & Hatefi, 1969) and has been reported to have inhibitory properties on the alternative respiratory pathway of cultured sycamore cells (Wilson, 1971).

The general effects of these inhibitors on mung-bean mitochondria were found to parallel fairly closely their effects on mammalian mitochondria, although the extent of inhibition was greater than that observed with mammalian systems in some cases. Differential effects of the inhibitors on intact mitochondria and submitochondrial particles has allowed localization of their sites of action in plant mitochondria to some extent.

Materials and Methods

Preparation of mitochondria and submitochondrial particles

Mitochondria from mung-bean hypocotyls were prepared by the general method described by Bonner (1967). Mung beans were grown for 5 days at 28°C in darkness and 60% relative humidity. Submito-

chondrial particles were prepared by sonication (2×25 s sonications at 40 W power with a Branson Sonic Power Co. Sonifier, model W185) in a medium containing 250 mM-sucrose, 15 mM-MgCl₂, 1 mM-ATP and 10 mM-Tris/HCl, pH 7.5, at 10 mg of protein/ml. Submitochondrial particles were pelleted for 1 h at 4°C and 100 000g (*r*_{av}, 5.95 cm) after an initial centrifugation at 4°C and 10 000g for 10 min (*r*_{av}, 4.25 cm) to remove whole mitochondria. The yield of submitochondrial particles was increased by a second sonication of the 10 000g pellet of unbroken mitochondria followed by an identical low-speed spin and combination of supernatants for precipitation of the submitochondrial particles.

Assays

All assays were performed with a Clark-type oxygen electrode. The medium used was medium A (Bonner, 1967). Concentrations used were: ATP, 0.3 mM; succinate, 10 mM; malate, 20 mM; NADH, 1 mM; ADP, 1 mM; KCN, 1 mM. When experiments were performed with submitochondrial particles, the medium was supplemented with 0.1% bovine serum albumin and 5 μM-cytochrome *c*. After addition of 2-thenoyltrifluoroacetone, sufficient time was allowed for a constant rate to be produced. For bathophenanthrolinesulphonate and bathophenanthroline, the mitochondria were incubated with the inhibitor for 4 min before addition of substrate.

Solutions and chemicals

Stock solutions were: bathophenanthrolinesulphonate, 10 and 40 mg/ml of water; bathophenanthroline, 2 and 10 mg/ml of methanol; thenoyltrifluoroacetone, 40 mg/ml of ethanol. Horse heart cytochrome *c* was purchased from Boehringer (Mannheim) Corp., New York, NY, U.S.A. All other chemicals were of the highest grade commercially available.

Fig. 1. Effects of bathophenanthroline on mung-bean mitochondria and submitochondrial particles

Particles were incubated in the inhibitor for 4 min at 25°C before the substrate indicated was added. Rates are expressed as percentage of uninhibited State 3 rate. For washed mitochondria: ○, succinate+ATP; △, NADH; □, malate. For submitochondrial particles: ●, NADH; ▲, succinate+ATP. Protein concentration in the washed mitochondria was about 0.5 mg/ml, and that in the submitochondrial particles about 0.75 mg/ml (NADH) and 1.5 mg/ml (succinate).

Results

The results are expressed in terms of final concentration of inhibitor used. In case protein concentration is also a factor in some cases [cf. Harmon & Crane (1976) for bathophenanthrolinesulphonate inhibition], protein concentrations [measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard] are also given in the Figure legends.

(a) Bathophenanthroline

The results obtained with bathophenanthroline are summarized in Fig. 1. A powerful inhibition of the oxidation of exogenous NADH, malate and succinate occurred with intact washed mitochondria. When, however, submitochondrial particles were used, succinate oxidation was unaffected and a greater concentration of inhibitor was required to inhibit NADH oxidation (it should be noted that, at these very high concentrations, problems of bathophenanthroline solubility are encountered).

Contrary to the results of other workers with mammalian mitochondria and isolated complexes (Palmer, 1970; Phelps *et al.*, 1974; Trumpower & Katki, 1975), it was found that carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (1.0 μM), carbonyl cyanide *m*-chlorophenylhydrazone (1 μM) and 2,4-dinitrophenol (20 μM) were all unable to reverse the bathophenanthroline inhibition of succinate- or malate-driven State 3 respiration, although a very

Fig. 2. Effects of bathophenanthrolinesulphonate on mung-bean mitochondria and submitochondrial particles

Conditions were the same as for Fig. 1. For washed mitochondria: ○, succinate+ATP; □, malate; △, NADH. For submitochondrial particles: ●, NADH; ▲, succinate+ATP. Protein concentration in the washed mitochondria was about 0.5 mg/ml, and that in the submitochondrial particles about 0.75 mg/ml (NADH) and 1.5 mg/ml (succinate).

slight decrease (<10% decrease) of the inhibition was noticed in some cases if the bathophenanthroline was added after a 30 s incubation with uncoupler.

In mammalian systems, an inhibitory effect of bathophenanthroline on cytochrome oxidase has been reported (Phelps *et al.*, 1974). We were unable to obtain a significant inhibition by bathophenanthroline of ascorbate (10 mM) plus *NNN'*-tetramethyl-*p*-phenylenediamine (0.1 mM) oxidation. Even when the mitochondria were incubated for 5 min with 500 μM-bathophenanthroline, only a 25% inhibition of ascorbate oxidation was noted.

No effect of the inhibitor on the alternative respiratory pathway at concentrations below those required to inhibit the normal pathway could be detected.

(b) Bathophenanthrolinesulphonate

These results are summarized in Fig. 2. In intact mitochondria, a very extensive inhibition of the oxidations of succinate and malate was observed. The extent was much greater than that reported for other systems (Harmon & Crane, 1976). The inhibition was the same whether the mitochondria were in State 3 or in an uncoupled State 3 (1 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone or 20 μM-2,4-dinitrophenol). Inhibition of the oxidation of exogenous NADH was achieved only at much higher concentrations of bathophenanthrolinesulphonate. In submitochondrial particles, this inhibitor did not significantly affect succinate oxidation, but had an inhibitory effect on NADH oxidation at high con-

Fig. 3. Effects of 2-thenoyltrifluoroacetone on mung-bean mitochondria and submitochondrial particles

The inhibitor was added to particles respiring maximally in State 3, and sufficient time was allowed for a steady rate to be achieved. For washed mitochondria: ○, succinate+ATP; △, NADH; □, malate; ■, NADH+KCN. For submitochondrial particles: ●, succinate+ATP; ▲, NADH. Protein concentration in the washed mitochondria was about 0.5 mg/ml, or 2 mg/ml for NADH+KCN. Protein concentration in the submitochondrial particles was about 0.75 mg/ml (NADH) and 1.5 mg/ml (succinate).

centrations. No effect of bathophenanthroline-sulphonate on ascorbate/*NNN'*-tetramethyl-*p*-phenylenediamine oxidation was detected and no uncoupler reversal of the bathophenanthroline-sulphonate inhibition was found, whether the uncoupler was added before or after inhibitor.

(c) 2-Thenoyltrifluoroacetone

The results obtained with 2-thenoyltrifluoroacetone are summarized in Fig. 3. Those obtained with intact washed mitochondria are similar to the results of Wilson (1971) with sycamore-cell cultures, i.e. a potent inhibition of succinate oxidation but little effect on the oxidations of malate and exogenous NADH. A similar inhibition of succinate oxidation was obtained when submitochondrial particles were used, but NADH oxidation was inhibited only at very high concentrations.

In further agreement with Wilson (1971), an effect of the inhibitor on the alternative pathway of oxidation of NADH (i.e. in the presence of 1 mM-KCN) was noted at concentrations above those required to inhibit succinate oxidation and below those required to affect NADH oxidation by the main pathway.

Discussion

The effects of these inhibitors on mung-bean mitochondria are generally similar to those obtained with mammalian systems. A possible interpretation of these results is as follows: (1) bathophenanthroline and bathophenanthroline-sulphonate inhibit the dicarboxylate carrier of the inner membrane, and hence inhibit succinate and malate oxidations in whole mitochondria, but not succinate oxidation in submitochondrial particles; (2) exogenous NADH oxidation can be inhibited at a unique hydrophobic site readily available to bathophenanthroline, but not so readily available to hydrophilic bathophenanthroline-sulphonate; (3) 2-thenoyltrifluoroacetone inhibits succinate dehydrogenase directly, and hence inhibits succinate oxidation in both whole mitochondria and submitochondrial particles, yet does not affect the oxidations of malate and exogenous NADH; (4) the alternative respiratory pathway is inhibited by 2-thenoyltrifluoroacetone, but not by bathophenanthroline. Bathophenanthroline-sulphonate had some effect on the alternative pathway in washed mitochondria, but not in submitochondrial particles, as judged by the slightly higher sensitivity to bathophenanthroline-sulphonate of exogenous NADH oxidation in the presence of 1 mM-KCN. Since, however, bathophenanthroline did not give a similar response, and our current theoretical (Rich & Moore, 1976) and electron-paramagnetic-resonance (Moore *et al.*, 1976) data indicate a matrix-side location of the alternative pathway close to succinate dehydrogenase, we feel that this bathophenanthroline-sulphonate effect is trivial, and could be, for example, a detergent-type action of this compound; (5) both bathophenanthroline and bathophenanthroline-sulphonate have inhibitory properties on site I (i.e. inhibition of submitochondrial-particle NADH oxidation) only at very high concentrations.

In contrast with the results of other workers with mammalian systems (Palmer, 1970; Phelps *et al.*, 1974; Trumpower & Katki, 1975), the inhibitory effects of bathophenanthroline on succinate and malate oxidations were not significantly reversible by uncoupler and were the same both in State 3 and the uncoupled State 3. This is consistent with their site of action on the dicarboxylate carrier only and not on sites close to those involved in energy conservation. Trumpower & Katki (1975) suggested that the site of uncoupler-reversible bathophenanthroline inhibition in bovine heart succinate-cytochrome *c* reductase might be Rieske's iron-sulphur centre (Rieske *et al.*, 1964). It is noteworthy that this centre is apparently absent from higher-plant mitochondria (W. J. Ingledew, A. L. Moore, R. C. Prince & P. R. Rich, unpublished work).

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