INHIBITORY EFFECTS OF FLUOROCITRATES ON YEAST MITOCHONDRIA

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1. Introduction

MFC* is used extensively in studies of mitochondrial systems to inhibit the oxidation of certain metabolites by specifically blocking the tricarboxylic acid cycle. Their effect has usually been ascribed to the inhibition of aconitase by MFC investigated by Peters and his associates [1].

However, MFC has been reported to inhibit succinic dehydrogenase [2], and recent studies of its effects on pyruvate carboxylation [3] and on mitochondrial respiration rates [4] seem to indicate that the action of this analogue may be more complex than previously assumed.

We have found that MFC inhibits the oxidation of a wide range of substrates in yeast mitochondria. Similar results have been obtained with DFC. Examination of the effects of these fluoro-analogues on mitochondrial permeability suggests that they act by inhibiting the uptake of respiratory substrates.

2. Materials and methods

MFC was prepared by the modified method of Brown and Saunders [2, 5]. DFC was synthesized from its diethyl ester [6]; details will be reported elsewhere.

Yeast mitochondria were prepared from baker's yeast (D.C.L., Bristol, England) [7]. Respiration rates were measured polarographically at 30° using a modified Clark electrode [8]. Mitochondrial swelling was investigated by the ammonium swelling technique as described by Chappell and Haarhoff [9] using a Unicam SP 800 recording spectrophotometer with cuvettes in the second sample position.

Mitochondrial protein was estimated by the method of Lowry et al. [10]. Further experimental details are given in the legends to the tables.

* *Abbreviations*
MFC : synthetic monofluorocitrate
DFC : D,L-α, α-difluorocitrate
EGTA: ethylene glycol-bis (aminoethyl)-tetra acetic acid

3. Results

3.1. *The effects of fluorocitrates on substrate respiration rates*

The effects of MFC and DFC on respiration rates are shown in table 1. Uninhibited rates were similar to those reported previously for these mitochondria [7]. Results are given for mitochondria both in the absence of added ADP (state 4) and in its presence (state 3). At the concentrations of inhibitor used, the respiration of a wide variety of substrates is depressed in both state 3 and state 4.

The oxidation of pyruvate, ethanol, α-oxoglutarate, citrate and isocitrate is completely inhibited. In these mitochondria a catalytic amount of DL-malate (100 μM) has been found [7] to cause a stimulation in

Table 1
Effect of fluorocitrate on respiration rates in yeast mitochondria.

Substrate	% Inhibition of QO_2 by			
	MFC		DFC	
	State 3	State 4	State 3	State 4
Pyruvate Ethanol α-Oxoglutarate Citrate DL-Isocitrate	100	100	100	100
Pyruvate + malate Citrate + malate DL-Isocitrate + malate α-Oxoglutarate + malate	100	100	100	100
Succinate	36	38	48	52
α-Glycerophosphate	23	27	58	60
$NADH_2$	0	0	0	0

Yeast mitochondria (0.49 mg protein) were suspended to a volume of 3.6 ml in a medium of 0.25 M sucrose, 0.02 M phosphate buffer, 1 mM EGTA and 0.01 M $MgCl_2$, pH 6.8. Final concentrations of additions were a) substrates, 10 mM; b) D, L-malate, 100 μM; c) ADP, 80 mM; d) MFC and DFC, 100 μM. Respiration rates were measured polarographically at 30° and calculated as μatoms oxygen consumed/mg protein/min. At least 4 determinations were made for each substrate with mitochondria in both state 3 and 4 and are the mean values from 4 to 10 different preparations. Inhibition is expressed as a percentage of the uninhibited rate.

oxidation rates of pyruvate, citrate, isocitrate and α-oxoglutarate. It can be seen that both MFC and DFC also completely inhibit this type of respiration.

The oxidations of succinate, α-glycerophosphate, and $NADH_2$ show somewhat different responses to MFC and DFC. At the concentrations used, only partial inhibition was observed with succinate and α-glycerophosphate. Respiration rates for $NADH_2$ were unaffected in both states 3 and 4. The slight differences in the degree of inhibition between state 3 and 4 shown with both succinate and α-glycerophosphate may not be significant but it is clear that DFC is a more effective inhibitor than is MFC, for both substrates.

Table 2
Effects of MFC and DFC on substrate induced swelling of yeast mitochondria.

Substrate	Decrease in absorbance		
	No inhibitors	+ MFC	+ DFC
Control	0.000	0.004	0.004
Pyruvate	0.012	0.003	0.003
Citrate	0.012	0.004	0.004
Pyruvate + malate	0.016	0.004	0.004
Citrate + malate	0.021	0.004	0.005
Succinate	0.015	0.010	0.007
α-Glycerophosphate	0.012	0.009	0.006

Both reference and experimental cuvettes contained yeast mitochondria (0.57 mg protein) suspended in the medium previously described (table 1) to a total volume of 3.0 ml. Final concentrations were a) substrates, 10 mM (added last); b) malate, 100 μM; c) antimycin A, 4.0×10^{-9} M; d) MFC and DFC, 0.1 mM. Results are average values from 3–5 separate mitochondrial preparations and represent the decrease in absorbance at 520 nm recorded one minute after addition of substrate.

3.2. *The effects of fluorocitrates on substrate permeation*

The effects of MFC and DFC on the penetration of the ammonium salts of certain respiratory substrates into yeast mitochondria are shown in table 2. Respiration was blocked with antimycin A. In these experiments the decrease in absorbance at 520 nm of the mitochondrial suspensions is a measure of anion permeability [see 9].

Both MFC and DFC themselves cause a small degree of swelling, but both completely inhibit the swelling induced by pyruvate and citrate. A low concentration of malate (100 μM) augments both pyruvate- and citrate-induced swelling (no swelling was observed in the presence of malate alone at this concentration [see 7]). These swellings were also completely abolished in the presence of either MFC or DFC, which correlates with the observed effects on oxidation rates.

In the case of succinate and α-glycerophosphate, swelling is partially inhibited by either fluorocitrates, DFC being more effective. Moreover, the degree of

inhibition of swelling with either substrate and either fluoro-compound is quantitatively similar to the corresponding effects seen in the respiration experiments.

4. Discussion

Inhibition of the respiration rates of citrate, pyruvate and possibly ethanol, together with the malate-stimulated rates of citrate and pyruvate, could be ascribed to an inhibition by the fluorocitrates of aconitase. However, it is unlikely that such a single site of action could explain the marked inhibitions seen with isocitrate, α-oxoglutarate, succinate and α-glycerophosphate.

The inhibition of respiration of such a wide range of substrates could be explained by a site of inhibition at some intermediate stage common to the respiratory mechanisms of all the substrates examined. The resistance of $NADH_2$ respiration to inhibition argues against a site of action at an electron transport carrier subsequent to flavoprotein. Inhibition of respiratory carriers prior to flavoprotein would require multiple sites of action and this remains a possibility. In this respect it is significant that the oxidations of $NADH_2$, α-glycerophosphate and succinate are those least affected by these inhibitors. The primary dehydrogenases for these substrates are those which have been shown to be firmly attached to the inner membrane [see e.g. 11–13].

Changes in the permeability of this inner membrane might well offer a site of action for the fluorocitrates which would explain the wide range of respiratory inhibitions observed. The inhibition of mitochondrial swelling observed is evidence that substrate uptake is completely inhibited in the case of those substrates whose respiration is completely inhibited. Furthermore, the close correlation between the degree of respiratory inhibition and the degree of inhibition of swelling for both succinate and α-glycerophosphate, as well as the correlation in the effectiveness of MFC and DFC with respect to these substrates, confirms that these inhibitors influence inner membrane permeability. Whether MFC and DFC exert similar effects upon mitochondria from other sources remains to be established and this is currently being investigated.

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