

THE EFFECT OF MITOCHONDRIAL OXIDATIONS OF INHIBITORS OF THE DICARBOXYLATE ANION TRANSPORTING SYSTEM

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1. Introduction

It has been demonstrated that certain *n*-substituted malonic acids have an inhibitory effect on the oxidation of di- and tri-carboxylate anions by rat liver mitochondria [1–3]. Thus the oxidations of citrate, isocitrate, *cis*-aconitate and 2-oxoglutarate which are dependent on the addition of L-malate as an activator of mitochondrial penetration [1] were shown to be inhibited by 2-*n*-butylmalonate, the inhibition being competitive with L-malate. The oxidations of L-malate with cysteine sulphinic acid and succinate, both dependent on the penetration of the mitochondria by a dicarboxylate anion, were also inhibited by 2-*n*-butylmalonate [2]. The kinetics of the inhibition caused by butylmalonate indicated that its site of action was at the level of the membrane transporting system for dicarboxylate anions. When this inhibitor was used on rat heart mitochondria it was found to inhibit oxidations not requiring the transport of dicarboxylate anions. These difficulties were partially overcome by the use of a new inhibitor, *p*-iodobenzylmalonate.

2. Methods and materials

Rat liver mitochondria were prepared as described previously [1]. Rat heart mitochondria were prepared by the method of Tyler and Gonze [4], except the preparation medium consisted of 0.25 M sucrose, 5 mM tris HCl and 1 mM EGTA pH 7.4 and not that suggested by the above authors. Respiration was measured polarographically using a Clark oxygen electrode. Changes in intramitochondrial NAD(P) were

monitored fluorimetrically as described by Chappell and Crofts [5]. 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) was a gift from Dr. R.B.Beechey (Shell Research, Sittingbourne, Kent, UK).

Abbreviations used in this paper are TTFB as designated above and EGTA = ethylene-glycol-bis-(aminoethyl)-tetra-acetate. *p*-iodobenzylmalonic acid was purchased from K. and K. Rare Chemicals, Plainview, New York, USA. 2-*n*-butylmalonic acid was obtained from the City Chemical Corporation, New York, N.Y., USA.

3. Results

3.1. Rat liver mitochondria

Fig. 1 shows the oxidation of *cis*-aconitate by rat liver mitochondria as measured fluorimetrically by pyridine nucleotide reduction. Uncoupling agent (TTFB) was added so that the intramitochondrial NAD(P) became oxidized, followed three minutes later by antimycin to block the activity of the respiratory chain. *Cis*-aconitate caused little reduction of intramitochondrial NAD(P) until L-malate was added. When 0.8 mM butylmalonate was present the rate of reduction on the addition of L-malate was 62% inhibited and with 0.8 mM *p*-iodobenzylmalonate this rate was 72.5% inhibited. Fig. 2 shows a 1/v against inhibitor concentration plot for the inhibition by *p*-iodobenzylmalonate of isocitrate oxidation activated by two different concentrations of L-malate measured at two different isocitrate concentrations (see Legend for details). This plot indicates that *p*-iodobenzyl malonate inhibition is competitive with

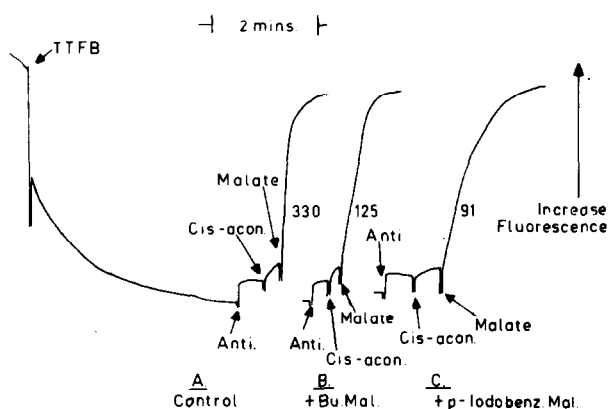


Fig. 1. The inhibition of *cis*-aconitate oxidation by butylmalonate and *p*-iodobenzylmalonate. Rat liver mitochondria (2 mg protein) were suspended in 3.0 ml of a medium containing 125 mM KCl, 20 mM tris HCl and 2 mM inorganic phosphate pH 7.4 at 30°C. Pyridine nucleotide oxidation/reduction changes were followed fluorimetrically. Only experiment A is shown in full since the initial oxidation of intramitochondrial pyridine nucleotides by uncoupling agent was the same for A, B and C. Additions were as follows: Expt. A. 4,5,6,7-tetrachloro-2-trifluoromethyl-benzimidazole (TTFB) 1 μ M; antimycin, 0.2 μ g/ml; *cis*-aconitate, 1.3 mM; L-malate 0.8 mM. Exp. B. Butylmalonate, 0.8 mM; TTFB, 1 μ M (not shown); antimycin, 0.2 μ g/ml; *cis*-aconitate, 1.3 mM; L-malate, 0.8 mM. Exp. C. *p*-iodobenzylmalonate, 0.8 mM; TTFB, 1 μ M (not shown); antimycin, 0.2 μ g/ml; *cis*-aconitate, 1.3 mM; L-malate, 0.8 mM.

respect to L-malate with a K_i of 0.15 mM. Similar studies on butylmalonate inhibition gave a K_i of 0.35 mM [2]. Butylmalonate has been shown to be a potent inhibitor of succinate oxidation in whole but not in broken rat liver mitochondria [2,6,7]. The K_i for butylmalonate was found to be 0.19 mM, but when iodobenzylmalonate was used a K_i of 0.08 mM was obtained. When oxidation of glutamate, (3 mM) by rat liver mitochondria was monitored polarographically no inhibition of respiration was caused by 2 mM butylmalonate or 2 mM *p*-iodobenzylmalonate.

3.2. Rat heart mitochondria

In the case of rat heart mitochondria the tricarboxylate anion transporting system activated by L-malate is relatively inactive [8] so this sensitive method of observing inhibition of dicarboxylate anion

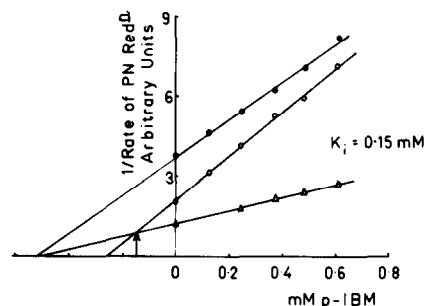


Fig. 2. The kinetics of *p*-iodobenzylmalonate inhibition of L(+)-isocitrate oxidation in the presence of L-malate. The reciprocal of the velocity of intramitochondrial NAD(P) reduction by isocitrate in the presence of L-malate is plotted against inhibitor concentration under three sets of conditions. Rat liver mitochondria (2 mg protein) were suspended in 3.0 ml of a medium containing 125 mM KCl, 20 mM tris HCl and 2 mM inorganic phosphate pH 7.4 at 30°C. 1 μ M TTFB was added and after three minutes 0.2 μ g/ml antimycin followed by *p*-iodobenzylmalonate (final concentrations shown), isocitrate and L-malate. The rate of intramitochondrial NAD(P) reduction on the addition of L-malate was monitored fluorimetrically. The three sets of conditions were: (a) 322 μ M L(+)-isocitrate, 129 μ M L-malate (\circ — \circ); (b) 322 μ M L(+)-isocitrate, 645 μ M L-malate (Δ — Δ) and (c) 60.5 μ M L(+)-isocitrate, 645 μ M L-malate (\bullet — \bullet).

entry by substituted malonates was not available. As with liver mitochondria, butylmalonate was found to inhibit succinate oxidation in whole but not in broken mitochondria. However it was also found that butylmalonate inhibited the oxidation of both pyruvate and L-acetylcarnitine by rat heart mitochondria neither oxidation involving the transport of a dicarboxylate anion for the functioning of the Krebs cycle. This inhibition could be relieved by the addition of L-malate.

p-Iodobenzylmalonate did not inhibit pyruvate and L-acetyl carnitine oxidation to the same extent as butylmalonate when used at concentrations capable of inhibitory succinate oxidation to the same extent as butylmalonate, table 1. Thus, while 2 mM butylmalonate inhibited succinate oxidation to the same extent as 0.75 mM *p*-iodobenzylmalonate, it inhibited pyruvate oxidation 68% while 0.75 mM *p*-iodobenzylmalonate inhibited pyruvate oxidation 14%. On this basis of comparing other effects in relation to inhibitory potency to dicarboxylate transport butylmalonate inhibits succinate oxidation in broken mitochondria 7% and *p*-iodobenzylmalonate 3.5%.

Table 1
The inhibition of rat heart mitochondrial oxidations by 2-*n*-butylmalonate and *p*-iodo-benzylmalonate.

Substrate	2- <i>n</i> - Butylmalonate		<i>p</i> -Iodobenzylmalonate	
	Concn. (mM)	% Inhibition	Concn. (mM)	% Inhibition
Expt. A				
2.5 mM Pyruvate	2	68%	0.75	14%
2.5 mM Acetylcarnitine	2	87%	0.75	15%
3 mM Succinate	2	58%	0.75	54%
Expt. B				
5 mM Succinate	2.5	66%	0.91	71%
Broken mitochondria				
5 mM Succinate	2.5	7.0%	0.91	3.5%

Rat heart mitochondria (2.5 mg protein) were added to 2 ml of a medium containing 125 mM KCl, 20 mM tris HCl and 5 mM inorganic phosphate pH 7.4 at 25°C. The substrate was added at the concentrations shown followed by 1 mM ADP. When a linear rate had been obtained the inhibitor was added at the concentrations shown. The linear rate achieved after the addition of inhibitor was measured and used to calculate the percentage inhibition above. In experiments with succinate, 1 μ M rotenone was used. For the broken mitochondrial experiments 0.06% Triton-X-100 was added to the mitochondrial suspension and 0.1 mg/ml phenazine methosulphate as an autoxidisable electron acceptor from succinate dehydrogenase.

The K_i values for inhibition of succinate oxidation in whole rat heart mitochondria were found to be 0.15 mM and 0.08 mM respectively for butylmalonate and *p*-iodobenzylmalonate.

4. Discussion

The lower K_i values found for *p*-iodobenzylmalonate with respect to L-malate activation of tricarboxylate anion oxidation and with respect to succinate oxidation indicate that this is a better inhibitor of dicarboxylate anion transport than 2-*n* butylmalonate. The K_i values for the above mentioned oxidations may also indicate that the dicarboxylate anion transporting system in both liver and heart mitochondria has more affinity for L-malate than for succinate since lower K_i values are evident for succinate oxidation than for L-malate activated isocitrate oxidation.

The inhibition of pyruvate and acetylcarnitine oxidation in heart mitochondria by butylmalonate was probably a result of competition of the inhibitor with succinate for succinate dehydrogenase. Since these oxidations are dependent on a supply of four carbon skeletons to citrate synthase, inhibition of succinate dehydrogenase would limit the flow through the

Krebs cycle to malate and oxaloacetate thus preventing the oxidation. This would be relieved by the addition of L-malate. Although butylmalonate is essentially a mitochondrial non-penetrant [2] and a poor inhibitor of succinate dehydrogenase small amounts getting into heart mitochondria might cause an inhibition of succinate dehydrogenase significant enough to prevent the Krebs cycle from turning at its normal rate with these substrates. *p*-Iodobenzylmalonate, although having a superior affinity for the transporting system compared to butylmalonate does not prevent the cyclical oxidation of pyruvate and acetylcarnitine when used at concentrations inhibitory to dicarboxylate anion transport. Since butylmalonate is now being tested on perfused tissues [10], we thought it useful to point out some of the disadvantages of the use of butylmalonate.

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