TRANSPORT OF PHOSPHOENOLPYRUVATE BY THE TRICARBOXYLATE TRANSPORTING SYSTEM IN MAMMALIAN MITOCHONDRIA

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1. Introduction

At least seven different anion transporting systems have been demonstrated in rat liver mitochondria [1, 2]. No specific transporting system appears to have been described for phosphoenolpyruvate though it has been shown by Harris [3] that it behaves as a triply charged anion in distribution studies performed on rat liver mitochondria. Gamble and Mazur [4] observed that citrate added to rabbit liver mitochondria caused an efflux of phosphoenolpyruvate from the mitochondria to the suspending medium. A specific inhibitor of the tricarboxylate transporting system, benzene-1,2,3-tricarboxylate has recently been described [5] together with 2-*p*-iodobenzylmalonate which inhibits dicarboxylate and tricarboxylate transporting systems and 2-pentylmalonate which specifically inhibits the dicarboxylate transporting system. The use of these inhibitors has been combined with techniques for measuring the exchange reactions of the mitochondrial transporting systems in an effort to identify the system responsible for phosphoenolpyruvate transport.

2. Materials and methods

Preparation of liver mitochondria was performed as described previously [6]. Loading with ^{14}C-citrate and ^{14}C-L-malate was achieved as described by Robinson et al. [7] and Robinson and Williams [8] respectively. Further experimental details are given either in the text or in the figures.

3. Results

Rat liver mitochondria loaded with ^{14}C-citrate were added to 1 ml incubations of a buffer containing 125 mM KCl, 20 mM tris-chloride pH 7.4 at 10° in the presence of a variety of anions as indicated in table 1. After 2 min the mitochondria were separated by centrifugation at 18,000 *g* for 4 min, and samples of supernatant and pellet prepared for counting as

Table 1

Exchange given by a variety of anions with ^{14}C-citrate when added to mitochondria loaded with ^{14}C-citrate.

Anion	Percentage exchange given at		
	1 mM	2.5 mM	10 mM
Citrate	92.0	98.4	99.2
cis-Aconitate	90.3	92.1	94.1
threo-D_s-Isocitrate	84.5	90.0	93.2
2-Oxoglutarate	4.2	4.8	5.3
L-Aspartate	5.1	11.2	1.1
L-Glutamate	−1.4	5.3	24.0
ADP	−0.5	−1.2	−2.2
ATP	1.95	−12.8	−32.0
Acetate	0.2	6.7	14.9
Pyruvate	13.7	17.4	20.6
Phosphate	19.4	38.5	56.0
Phosphoenolpyruvate	82.5	85.5	93.9
L-Malate	26.7	79.1	92.0
Succinate	20.4	37.6	40.4

The experiment was carried out as described in the text. Mitochondrial protein was 8.7 mg per incubation and the above figures are the mean values obtained in duplicate incubations.

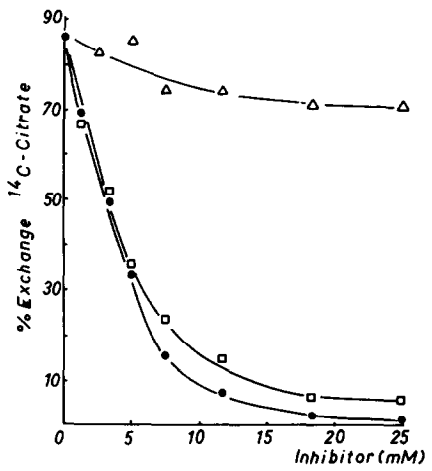

Fig. 1. The sensitivity of phosphoenolpyruvate/^{14}C-citrate exchange in rat liver mitochondria to the inhibitors benzene-1,2,3-tricarboxylate, 2-pentylmalonate and 2-*p*-iodobenzylmalonate. Rat liver mitochondria (8 mg protein aliquots) loaded with ^{14}C-citrate were added to 1 ml incubations of a medium containing 125 mM KCl, 20 mM tris-Cl and 1 mM phosphoenolpyruvate, pH 7.4 at 10°. Control incubations were carried out in the same medium in the absence of phosphoenolpyruvate. Inhibitors were included at the concentrations shown: benzene-1,2,3-tricarboxylate (●—●), 2-pentylmalonate (△—△), 2-*p*-iodobenzylmalonate (□—□). 2 min after addition of the mitochondria, the mitochondria were separated by centrifugation at 18,000 *g* for 4 min and samples of supernatant and pellet prepared for counting as described previously [7, 8].

described previously [7]. Appreciable exchange at 1 mM anion was observed only with the tricarboxylic acids and with phosphoenolpyruvate. At 10 mM, L-malate, succinate and inorganic phosphate also show appreciable exchange. Where a negative value is indicated it means that in the presence of that anion, the counts appearing in the supernatant were lower than in controls incubated in buffer only. This is especially noticeable with ATP since the presence of an energy source has been shown to restrict and even reverse L-malate/^{14}C-citrate exchange (B.H. Robinson, unpublished observations).

The exchange of 1 mM phosphoenolpyruvate with ^{14}C-citrate was then tested for sensitivity to the inhibitors benzene-1,2,3-tricarboxylate, 2-pentylmalonate and 2-*p*-iodobenzylmalonate. At 10° in a 2 min incubation before separation as above (fig. 1) both benzene-1,2,3-tricarboxylate and 2-*p*-iodobenzylmalonate were seen to be good inhibitors while 2-pentylmalonate was ineffective. Similarly (fig. 2) it was found that the exchange of phosphoenolpyruvate with ^{14}C-L-malate in ^{14}C-L-malate loaded rat liver mitochondria was not inhibited by 2-pentylmalonate but was strongly inhibited by benzene-1,2,3-tricarboxylate and 2-*p*-iodobenzylmalonate.

Since 50 mM benzene-1,2,3-tricarboxylate was found to inhibit completely the exchange of phosphoenolpyruvate with citrate, 'inhibitor stop' experiments [8, 9] were performed using the above concentration of inhibitor to follow the time course of the exchange (fig. 3). The exchange rate of phosphoenolpyruvate at 1 mM was similar to that of citrate at 1 mM being 6.5 nmoles/min/mg protein in this experiment. The rate of exchange of 1 mM L-malate for citrate is about half of this value as measured by this technique and the extent of exchange is smaller [10].

No trace of citrate, isocitrate or *cis*-aconitate was found in the phosphoenolpyruvate used in these investigations thus ruling out and possibility of tricarboxylate exchange resulting from contamination. When low concentrations of phosphoenolpyruvate were compared with citrate in ability to exchange with ^{14}C-citrate (fig. 4) little difference was found between the two anions. Analysis of supernatant and pellet for

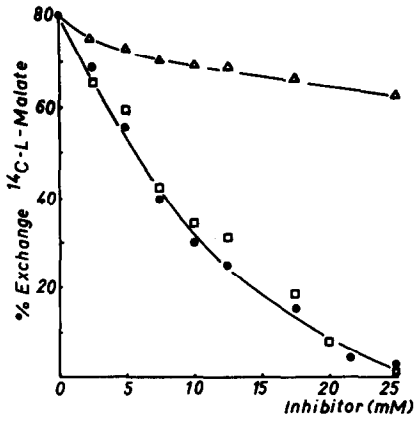

Fig. 2. The sensitivity of phosphoenolpyruvate/^{14}C-L-malate exchange in rat liver mitochondria to the inhibitors benzene-1,2,3-tricarboxylate, 2-pentylmalonate and 2-*p*-iodobenzylmalonate. The experiment was carried out as described in fig. 1 except that ^{14}C-L-malate loaded mitochondria were used and ^{14}C-L-malate counted in all samples. Benzene-1,2,3-tricarboxylate (●—●); 2-pentylmalonate (△—△); 2-*p*-iodobenzylmalonate (□—□).

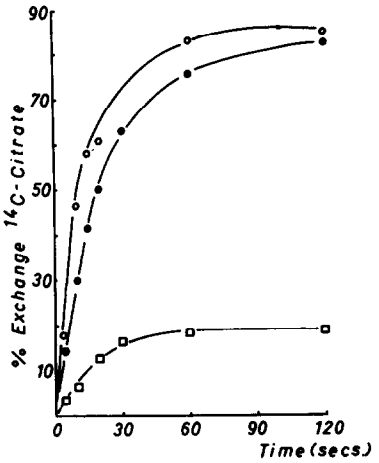

Fig. 3. Time course of citrate/^{14}C-citrate, L-malate/^{14}C-citrate and phosphoenolpyruvate/^{14}C-citrate exchanges as measured by the 'inhibitor stop' technique. Rat liver mitochondria (8.5 mg protein aliquots) loaded with ^{14}C-citrate were added to 1 ml incubation of 125 mM KCl, 20 mM tris-Cl, pH 7.4 at 10° containing 1 mM (final concentration) of either unlabelled citrate, L-malate or phosphoenolpyruvate. After time intervals of 0–2 min the exchange was terminated by the rapid addition of 50 mM benzene-1,2,3-tricarboxylate and the mitochondria separated by centrifugation. Samples of supernatant and pellet were prepared for the counting of ^{14}C-citrate as described previously [7]. 1 mM citrate (o—o); 1 mM L-malate (□—□); 1 mM phosphoenolpyruvate (●—●).

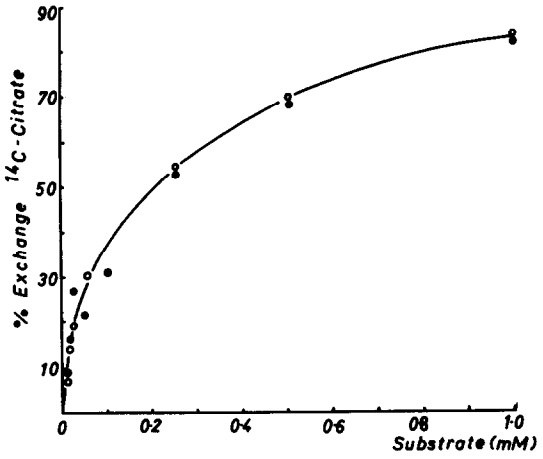

Fig. 4. The concentration dependence of the exchange of phosphoenolpyruvate and citrate for ^{14}C-citrate in rat liver mitochondria. The experiment was carried out as described for table 1 using increasing concentrations of either phosphoenolpyruvate (●—●) or citrate (o—o) as indicated.

citrate and L-malate by enzymatic assay [7, 8, 10] confirmed that the exchanges of these two anions brought about by addition of phosphoenolpyruvate were as indicated by the radioisotope data.

4. Discussion

The ability to exchange with ^{14}C-citrate extensively at low concentrations was confined to the natural tricarboxylate substrates, citrate, *cis*-aconitate and *threo*-D_s-isocitrate, except for phosphoenolpyruvate. L-Malate/^{14}C-citrate exchange is restricted because of the disequilibrium of pH set up by malate^{2-}/citrate^{2-} exchange [10], which is overcome at high concentrations of L-malate. The only other anions causing extensive exchange were phosphate and succinate at high concentrations. Succinate behaves in an analogous fashion to L-malate while the exchange of phosphate for citrate has been shown to be strongly inhibited by 2-pentylmalonate thus ruling out any possibility of a direct exchange of phosphate for citrate [10].

This exceptional ability of phosphoenolpyruvate to exchange with ^{14}C-citrate, since it is not inhibited by 2-pentylmalonate, indicates that the dicarboxylate transporting system is not involved. The inhibition of this exchange by benzene-1,2,3-tricarboxylate and 2-*p*-iodobenzylmalonate on the other hand indicates a direct involvement of the tricarboxylate transporting system. There are two possibilities; either the phosphoenolpyruvate exchanges directly with citrate on the citrate transporting system or phosphoenolpyruvate first exchanges for endogenous mitochondrial L-malate on a separate phosphoenolpyruvate transporting system, the L-malate then exchanging for ^{14}C-citrate. Three lines of experimental evidence favour the former of the two possibilities. First, the exchange of phosphoenolpyruvate with ^{14}C-L-malate is again inhibited by benzene-1,2,3-tricarboxylate and 2-*p*-iodobenzylmalonate but not by 2-pentylmalonate thus indicating that the only exchange of this type is catalysed by the tricarboxylate transporting system. Second, the rapid and complete exchange of phosphoenolpyruvate for ^{14}C-citrate shown by the 'inhibitor stop' technique could not be explained on any system involving a prior exchange for endogenous L-malate, since L-malate itself does not give such a rapid or extensive exchange with ^{14}C-citrate even at 1 mM. Third, the

almost parallel behaviour of phosphoenolpyruvate and citrate when exchanging for ^{14}C-citrate especially at low concentrations would be difficult to explain on any hypothesis other than that involving direct exchange of phosphoenolpyruvate for ^{14}C-citrate.

The ability of phosphoenolpyruvate to exchange with ^{14}C-citrate was also demonstrated in guinea-pig liver mitochondria and in rat heart mitochondria. This latter observation may indicate that the ability to transport phosphoenolpyruvate is an obligatory function of the citrate transporting system. Whether the presence of a phosphoenolpyruvate/citrate or a phosphoenolpyruvate/L-malate exchange has physiological significance either in the context of glycolytic or gluconeogenic control is an interesting question.

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