ON THE SPECIFICITY OF THE TRICARBOXYLATE CARRIER SYSTEM IN RAT LIVER MITOCHONDRIA

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1. Introduction

The transport of trivalent anions across the inner membrane of rat liver mitochondria is mediated by a specific carrier system which catalyses an exchange of an external tricarboxylate anion or the dicarboxylate L-malate against an internal tricarboxylate anion. This translocation follows a one to one stoichiometry and shows a specific sensitivity to the inhibitor benzene-1, 2, 3-tricarboxylate (1, 2, 3-BTCA) [1–6].

Robinson was the first to report that phosphoenolpyruvate (PEP) is also transported via the tricarboxylate carrier [7, 8] which could be confirmed in our laboratory [9, 10]. Since phosphoenol-α-ketovalerate (PEV) did likewise exchange with internal citrate [9] the following experiments were performed in an attempt to elucidate the specificity of the tricarboxylate carrier system and to get more information on its possible physiological importance.

It will be shown that the tricarboxylate carrier system is rather unspecific with respect to anions transported, in that it permits not only the translocation of citrate, L-malate and PEP but also a variety of PEP-analogues, D-2-phosphoglycerate, D-3-phosphoglycerate, phosphoglycerate, and dicarboxylates like succinate, fumarate, and D-malate. The comparison with other anions which are not transported via the tricarboxylate system gives some indication for the minimum structural requirements for this translocator system.

Abbreviations:
BTCA = benzene tricarboxylate; DAP = dihydroxyacetonephosphate; PEP = phosphoenolpyruvate; PEB = phosphoenol-α-ketobutyrate; PEV = phosphoenol-α-ketovalerate; PGA = phosphoglycerate.

2. Materials and methods

Special reagents were obained from the following sources: Enzymes, coenzymes, PEP, D-2-PGA, D-3-PGA, α-ketoglutarate, DAP, fructose-1, 6-diphosphate, glucose-6-phosphate, fumarate, succinate from Boehringer Mannheim Corporation, Mannheim/ Germany, β-Glycerophosphate, β-ketoglutarate, 1, 2, 3-BTCA as well as 1, 2, 4-BTCA and 1, 3, 5-BTCA came from Schuchardt, Munchen/Germany. Antimycine A and α-D, L-glycerophosphate were obtained from Serva, Heidelberg/Germany. α-Phosphoglycolate, O-phospho-L-serine, 3-phosphohydroxypyruvate, α-ketovaleric acid, α-ketobutyric acid and D-male were derived from Sigma, St. Louis/USA. Rotenone came from the K & K Laboratories Inc., Plainview/USA and L-malate (sodium salt) from C. Roth, Karlsruhe/Germany. All other chemicals were analytical grade and were purchased from E. Merck A.G., Darmstadt/Germany.

PEB and PEV were prepared from the corresponding α-keto acids according to Clark and Kirby [11] and phenyl-PEP from phenylpyruvate according to Stubbe and Kenyon [12]. The preparations were completely free of PEP. D, L-2-Phosphoglycerate was prepared from β-glycerophosphate according to Kiessling [13] and L-2-PGA was prepared from the racemic product as will be described elsewhere [14]. The final preparation contained less than 0.5% of D-2-PGA (mole/mole).

Mitochondria were isolated from rat liver according to Myers and Slater [15]. They were preloaded with [1,5-^{14}C]-citrate (Amersham-Buchler, Braunschweig/ Germany) at 0° according to Meijer [4]. In a similar way preloading with PEP was achieved and the efflux

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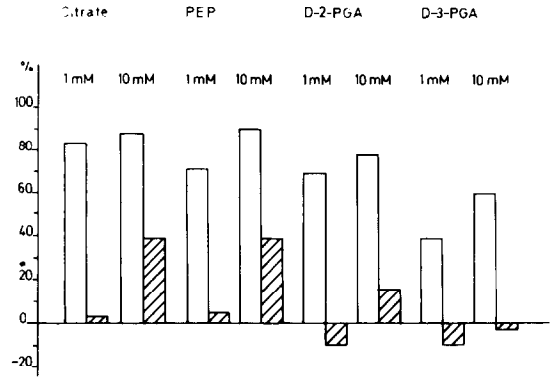

Fig. 1. Exchange of intramitochondrial 1,5-[^{14}C]-citrate with unlabeled extramitochondrial citrate, PEP, D-2-PGA, and D-3-PGA at final conc. of 1 mM and 10 mM. The experiments were performed in the absence (open columns) or presence (hatched columns) of 50 mM 1, 2, 3-BTCA (final conc.). [^{14}C]-citrate loaded mitochondria were incubated in the presence of the indicated anions (for details see Materials and Methods). After centrifugation the supernatant was immediately quenched by addition of perchloric acid. The pellet was freed from adherent supernatant and extracted by addition of perchloric acid. For determination of radioactivity an aliquot of the deproteinized sample was suspended in Insta-Gel (Packard Instrument Comp., Warrenville III., USA) and counted. The percentage of exchange was calculated as given in Materials and methods. The results were corrected for spontaneous time dependent efflux of citrate. A negative value means that in these cases the counts appearing in the supernatant were lower than in the control experiments.

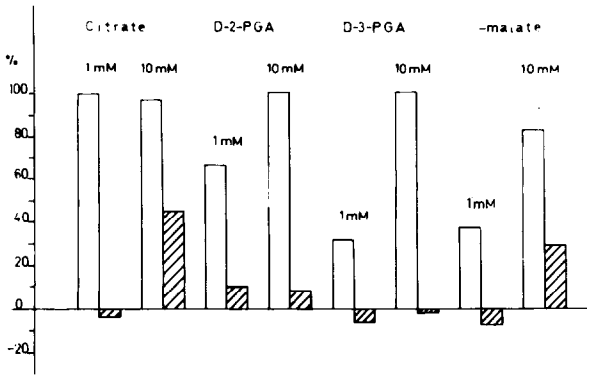

Fig. 2. Exchange of intramitochondrial PEP with extramitochondrial citrate, D-2-PGA, D-3-PGA and L-malate at final concentrations of 1 mM and 10 mM. The experiments were performed in the absence (open columns) and presence (hatched columns) of 50 mM 1, 2, 3-BTCA (final conc.). Otherwise the experimental conditions were the same as given in the legend to fig. 1 and under Materials and methods, except that the mitochondria were preloaded with PEP and the distribution of this metabolite was measured enzymatically in the neutralized samples.

of PEP resp. citrate was followed at 25° in the presence of rotenone and antimycine A. After 1 min of incubation the mitochondria (4–6 mg protein) were separated from the medium by rapid centrifugation in an Eppendorf microcentrifuge [4].

The standard incubation medium contained (final conc.): 0.2 M mannitol, 25 mM triethanolamine buffer pH 7.4, 5 mM n-butyl-malonate, 2 μg/ml rotenone, 0.5 μg/ml antimycine A and between 22.5 and 25 mM sucrose. Radioactivity was determined in a Packard liquid scintillation spectrometer model 3380 with automatic quench correction. Metabolite determinations were performed either spectrophotometrically or fluorometrically, dependent on the experimental condition (Eppendorf-Photometer with fluorometric assembly) [16, 17]. Protein was determined with the biuret method [18] using serum albumin as a standard. All substrates used in the exchange experiments were taken as sodium salts. The percentage of exchange was calculated according to the following equation:

$$\text{Percent exchange} = \frac{100\left(\frac{x}{y+x} - \frac{a}{b+a}\right)}{1 - \frac{a}{b+a}}$$

where x is the total radioactivity (dpm) in the supernatant, y the total radioactivity (dpm) in the pellet of the experimental probe, and a the total radioactivity (dpm) in the supernatant, b the total radioactivity (dpm) in the pellet of the respective control. In experiments where the mitochondria had been loaded with an unlabeled substrate, the amount (nmoles) of this substrate in the various fractions instead of radioactivity was used for the calculation. Further experimental details are given in the legends of the tables and figures.

3. Results

3.1. *Exchange of intramitochondrial citrate with various extramitochondrial anions*

The results are summarized in fig. 1. At a concentration of 10 mM not only citrate and PEP did exchange with internal [^{14}C]-citrate but also D-2-PGA and D-3-PGA.

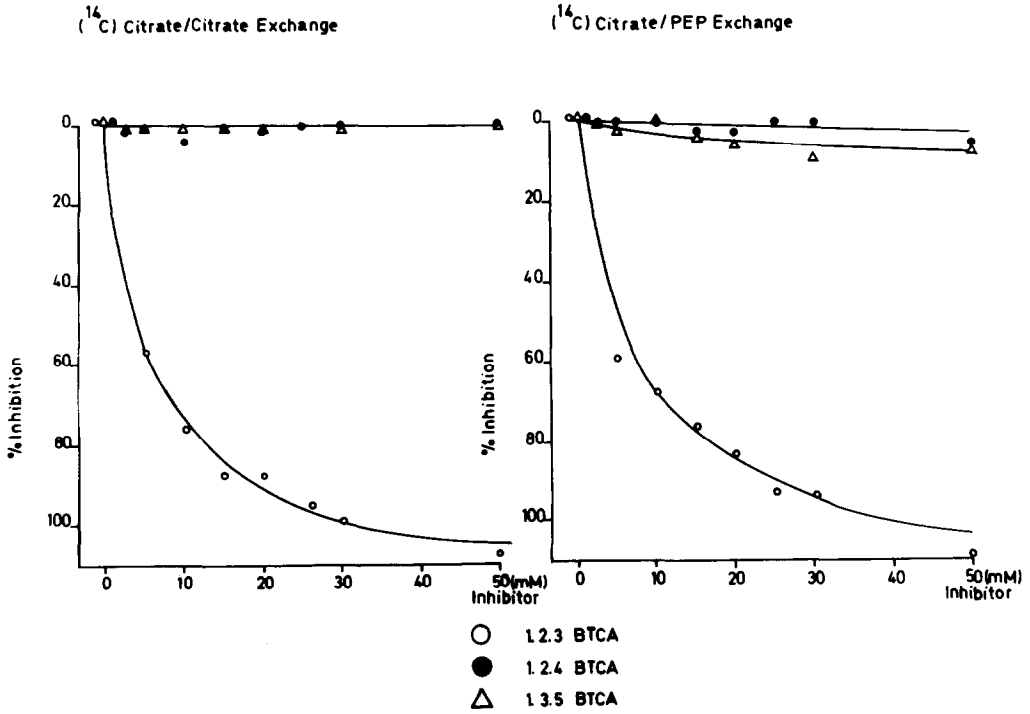

Fig. 3. Effects of various benzene tricarboxylates on the exchange of intramitochondrial [^{14}C]-citrate with extramitochondrial citrate or PEP. The concentration of the inhibitors are given on the abscissa. Citrate or PEP was added at a final concentration of 1 mM. The exchange measured in absence of any inhibitor was taken as 100%. For further details see fig. 1 and Materials and methods.

The rate of exchange with D-3-PGA and D-2-PGA was lower than with citrate and PEP (fig. 1). At a concentration of 1 mM the rate of exchange increased in the following order: D-3-PGA < D-2-PGA < PEP < citrate (fig. 1). At this concentration the exchange was completely abolished by 50 mM 1, 2, 3-BTCA, a competitive inhibitor of the tricarboxylate carrier system [2]. In accordance with the competitive type of inhibition, the exchange was not completely inhibited by 1, 2, 3-BTCA when the concentration of PEP or citrate was raised to 10 mM (fig. 1)

3.2. *Exchange of intramitochondrial PEP with various extramitochondrial anions*

With PEP as internal counteranion the results were similar to those obtained with citrate loaded mitochondria (fig. 2). Again the rate of exchange with D-3-PGA and D-2-PGA was lower than with citrate. L-Malate which is partially transported via this carrier system exhibits about the same rate of exchange as D-3-PGA. In analogy to fig. 1 the same concentration dependency with respect to the inhibition of exchange by 1, 2, 3-BTCA was observed (fig. 2).

3.3. *Concentration dependency of the inhibitory action of various benzene tricarboxylates*

A complete inhibition of the PEP/[^{14}C]-citrate and the citrate/[^{14}C]-citrate exchange was obtained with 25 mM 1, 2, 3-BTCA, which is in according with earlier observations [2, 7]. 1, 2, 4-BTCA and 1, 3, 5-BTCA were without any inhibitory effect up to a concentration of 50 mM (fig. 3). This corresponds well with recent data of Palmieri et al. [6], who found a strong inhibition with 1, 2, 3-pentene tricarboxylate but not with 1, 3, 5-pentene tricarboxylate.

3.4. *Further anions exchanging with intramitochondrial citrate*

At a final concentration of 1 mM also analogues of PEP like PEB, PEV and phenyl-PEP exchanged with

Table 1
Exchange of intramitochondrial [^{14}C]-citrate with various extramitochondrial anions.

Anion	Percent exchange	Percent exchange in the presence of 50 mM 1, 2, 3-BTCA
Citrate	83.7	2.52
PEP	71.3	4.58
D-2-PGA	69.1	−9.40
L-2-PGA	59.7	−3.86
Phosphoglycolate	59.0	−6.67
Phenyl-PEP	38.6	9.34
D-3-PGA	38.2	−10.1
PEB	35.0	6.61
L-Malate	29.6	3.44
PEV	24.6	10.6
D-Malate	17.2	−0.46
Succinate	16.6	−4.50
Fumarate	12.1	−6.92
Phosphate	4.25	−2.71
DAP	3.25	−2.50

The experiments were carried out as described in the legend to fig. 1 and in Materials and methods. The final conc. of all external anions added was 1 mM. The figures given are mean values of at least two separate experiments in each group.

intramitochondrial citrate (table 1). The unphysiological stereoisomer L-2-PGA did exchange at a rate comparable to that seen with D-2-PGA. Moreover another phosphorylated carboxylic acid namely phosphoglycolate was transported in exchange for internal citrate (table 1). The rate of exchange with D-malate was about half of that observed with L-malate. Similar results were obtained with succinate and fumarate. These dicarboxylates exhibited about the same rates as D-malate. At a final concentration of 1 mM the rate of exchange with inorganic phosphate or dihydroxyacetonephosphate (DAP) was very low but significantly higher than the leakage measured in control experiments (table 1). The rates of exchange with both substrates at a final concentration of 10 mM increased to values above 10% (not shown). The exchange with all these anions was strongly inhibited by 1, 2, 3-BTCA (table 1). For the following anions (tested at a concentration of 1 mM) no significant exchange via the tricarboxylate carrier was obtained: O-phospho-L-serine, 3-phosphohydroxypyruvate, α-D, L-glycerophosphate, β-glycerophosphate, α-ketoglutarate, β-ketoglutarate, 2, 3-diphosphoglycerate, fructose-1, 6-diphosphate and glucose-6-phosphate.

4. Discussion

The ability to exchange at low concentrations with intramitochondrial tricarboxylates via the tricarboxylate carrier system was thought to be confined to citrate, threo-D_3-isocitrate, PEP and the dicarboxylate L-malate. Several attempts have been made to elucidate the nature of translocation [1–6] via this carrier system. The exchange with L-malate seems to be restricted by a disequilibrium of either charge or pH across the inner mitochondrial membrane [3]. Although there is some evidence at least for the case of malate/citrate exchange supporting an electroneutral transport of charged anions coupled to proton translocation, an electroneutral transport of the undissociated acids seems to be more likely [19]. In any case the tricarboxylate carrier seems to be tightly linked to the malate/P_i exchange system and thus finally to the P_i/OH system [20].

The data presented in this paper show that the tricarboxylate carrier system of rat liver mitochondria is extremely unspecific. In addtion to the metabolites mentioned above, D-2-PGA and D-3-PGA and even phosphoglycolate were found to be transported under the same conditions and at a comparable rate. Moreover the unphysiological stereoisomer L-2-PGA exchanged with citrate, which is in contrast to the earlier proposal of stereospecificity, which was based on the finding that D-malate was ineffective in stimulating tricarboxylate translocation [21]. This is in contrast to our data, which show that D-malate is at least as effective as succinate or fumarate. The rate of exchange with D-malate was about half of that with L-malate. This is in good accordance with data from Ferguson et al. [22], who showed that the increased utilization of isocitrate by rat liver mitochondria following the addition of D-malate was about 50% of that measured with L-malate. Whether the small but significant exchange with phosphate indicates a direct involvment of this substrate in the transport via the tricarboxylate carrier under physiological conditions is under investigation.

In accordance with recent data of Palmieri et al [6] our findings support and extend the knowledge about the structural requirements of the tricarboxylate carrier

system: At least two carboxyl groups (malate, succinate) or three neighbouring carboxyl groups (citrate, isocitrate) or one carboxyl group and a phosphate group (PEP, 2-PGA, 3-PGA, phosphoglycolate, PEP-analogues) are required. In most substrates with carboxyl and a phosphate group present, the carboxyl group and the phosphate group are separated by only one carbon atom (phosphoglycolate, PEP, PEV, PEB, phenyl-PEP and 2-PGA), whereas the rest of the molecule seems to be of minor importance as demonstrated with the various PEP-analogues. Moreover one can state that in this case a carbonyl group in C_2-position like in 3-phosphohydroxypyruvate prevents transport, whereas a hydroxyl group in the same position like in 3-PGA permits transport. Assuming that a certain spatial arrangement of the phosphate and the carboxyl group is needed to fit a hypothetical binding site or "pore", enolisation occurring in 3-phosphohydroxypyruvate could inhibit the development of this special configuration of the molecule. The fact that 1, 2, 3-BTCA did inhibit the exchange of all substrates tested so far, seems to indicate that we are dealing with the same carrier although it has not been excluded completely that more than one 1, 2, 3-BTCA sensitive carrier system exists. Preliminary results of competition experiments point to the direction of a single carrier system.

The possible physiological importance of the transport of some of the phosphocarboxylates will be discussed elsewhere [10]. In view of the lack of substrate specificity one is tempted to speculate that the ability of mitochondria from heart [4, 23] or insect flight muscle [24] to prevent loss of intramitochondrial citrate is far more "specific" than the tricarboxylate carrier system in mitochondria from liver and skeletal muscle.

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