TRANSPORT OF CITRATE IN SUBMITOCHONDRIAL PARTICLES

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1. Introduction

The efflux of citrate from mitochondria provides precursors for biosynthetic processes such as fatty acid synthesis. It also provides a mean for transporting reducing equivalents from the matrix to the cytoplasm. There is evidence, in RLM, that the transport of citrate is mediated by a specific transport system, known as the tricarboxylate carrier, which catalyzes an exchange between citrate, malate and PEP [1]. The exchange is inhibited by 1,2,3-benzenetricarboxylate, and less specifically by *p*-iodobenzylmalonate [1,2]. The tricarboxylate carrier is virtually absent in mitochondria from heart [3], where the fatty acid synthesis is very low. In this report it is shown that SMP from RLM retain the transport system for citrate, but with a decreased activity. Part of this work has been communicated [4].

2. Materials and methods

Mitochondria from rat liver and beef heart were isolated with standard procedures. The medium for the preparation of SMP consisted of 240 mM sucrose, 10 mM $MgCl_2$, 10–20 mM citrate–Tris, pH 7.4 and 20–40 mg mitochondrial protein/ml. Formation of sonic SMP was achieved by sonication for 3 × 10 s. Where indicated, SMP prepared by nitrogen decompression [5] were used. After centrifugation for 10 min at 27 000 × *g* to remove the unbroken mitochondria, the supernatant was centrifuged for 45 min at 145 000 × *g*. The particles were washed once and suspended in 120 mM KCl and 20 mM Hepes–Tris (15–25 mg/ml). They contained 3–10 μmol citrate/g protein.

The activity of citrate transport was assayed by addition of labeled (forward exchange) or unlabeled substrate (back exchange). In the latter case, the citrate trapped within the particles was first labeled by adding carrier-free [^{14}C]citrate (1.25 μCi/ml) to the SMP suspension, which was kept at 0°C for 1 h before washing. The %-exchange was calculated according to the equation

$$\% \text{ exchange} = 100(C_a - C_p)/C_a,$$

where C_p and C_a represent the radioactivity in the particles, in the presence and absence of external anions. Protein was measured by the biuret method [2]. In some experiments citrate was assayed enzymatically with citrate lyase and malate dehydrogenase [6].

[^{14}C]Citrate was purchased from Amersham, 1,2,4- and 1,3,5-benzenetricarboxylic acids from Schuchardt, *p*-iodobenzylmalonic acid from K and K, and *n*-butylmalonic acid from Aldrich. Other special chemicals were obtained as described [2].

3. Results and discussion

When SMP loaded with [^{14}C]nitrate are incubated

Abbreviations: RLM, rat liver mitochondria; SMP, submitochondrial particles; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate; PEP, phosphoenolpyruvate; BTA, benzenetricarboxylate; 1,2,3-TAA, 1,2,3-propanetricarboxylate (tricarballylate); 1,3,5-PTA, 1,3,5-pentanetricarboxylate

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Table 1
Exchange of [^{14}C]citrate with externally added anions in citrate-loaded SMP

Additions	% Exchange	Additions	% Exchange
Citrate	70, 69	Malonate	5, 8
cis-Aconitate	64, 66	Oxalate	9, 3
trans-Aconitate	10, 18	Glutarate	12, 8
1,2,3-TAA	67, 62	PEP	68, 70
1,3,5-PTA	9, 14	P_i	5, 1
1,2,3-BTA	12, 13	Pyruvate	14, 10
1,2,4-BTA	8, 5	ADP	13, 8
1,3,5-BTA	3, 7	2-Oxoglutarate	9, 4
Malate	61, 57	Glutamate	−4, −3
Succinate	46, 59	Aspartate	−5, −2

[^{14}C]Citrate-loaded SMP (1.5 mg protein) were incubated at 0°C in 1 ml medium consisting of 120 mM KCl, 20 mM Hepes–Tris, pH 7.0, and 1 mM of the anions indicated in the table (except in the control samples). After 20 min incubation, the particles were separated by centrifugation at 105 000 × *g* for 1 h. Duplicate values from a representative experiment are reported

in the absence of counteranions, there is no significant efflux of the labeled metabolite (approx. 10% after 20 min incubation at 0°C). The addition of citrate to the reaction mixture elicits efflux of internal [^{14}C]citrate. The specificity of citrate transport was investigated by studying the ability of a variety of anions to cause the efflux of [^{14}C]citrate. The results (table 1) show that the substrates capable of exchanging for citrate must have at least two carboxyl groups (or a carboxyl and a phosphoryl group as in PEP). The carboxyl groups must be in *cis* configuration, as demonstrated by the different ability of *cis*- and *trans*-aconitate to exchange. The distance between the carboxyl groups is also important, since succinate, but not oxalate, malonate and glutarate, is transported. Similarly 1,2,3-propanetricarboxylate is transported, in contrast to 1,3,5-pentanetricarboxylate. None of the three isomer benzenetricarboxylates exchanges with internal citrate, although in 1,2,3-benzenetricarboxylate the carboxyl groups may be the right distance apart. In fact, the latter compound interacts with the transport system (see below), but the presence of the aromatic group may prevent transport due to steric hindrance.

Besides the specificity of transport, another evidence for the existence of a carrier is the finding of specific inhibitors. The inhibition of the citrate/citrate exchange was therefore tested by measuring forward and back exchange, at two externally added citrate concentrations. As shown in table 2, the exchange is inhibited by 1,2,3-benzenetricarboxylate and p-iodobenzylmalonate very effectively. As in mitochondria [2], butylmalonate and phenylsuccinate exhibit a slight inhibitory effect. No inhibition is observed with 1,3,5-benzenetricarboxylate.

The kinetics of the citrate/citrate exchange in SMP may be measured adequately by the millipore filtration method, since the rates of both efflux and uptake of citrate are constant for about 40 s at 10°C. At 20°C the rates are about 3 times higher. The Lineweaver-Burk analysis of [^{14}C]citrate uptake (fig.1) reveals hyperbolic saturation characteristics with an apparent K_m of 1.2 mM and an apparent V_{max} of 1.9 μmol/min · g protein (1.2 ± 0.2 mM and 2.0 ± 0.4 μmol/min × g at 10°C in 5 exp.). Similar data are obtained from back exchange experiments, in the presence of different external substrate concentrations. Figure 1 also shows that 1,2,3-benzenetricarboxylate and *p*-iodobenzylmalonate increase the K_m without changing the V_{max} of citrate uptake, demonstrating that their inhibition is of the competitive type. The SMP isolated from beef heart mitochondria, which do not contain the tricarboxylate carrier, also do not exhibit detectable exchange with [^{14}C]citrate (5 measurements at 20°C). The kinetics of the citrate/citrate exchange were also measured in SMP prepared by nitrogen decompression [5]. By using the same criterion described by Fleischer et al. [5], we have

Table 2
The inhibition of the citrate/citrate exchange by various tricarboxylates and dicarboxylates in SMP

Additions	% Inhibition			
	With 0.25 mM citrate		With 0.5 mM citrate	
	Efflux	Uptake	Efflux	Uptake
1,2,3-Benzenetricarboxylate	100	87	40	47
p-Iodobenzylmalonate	76	76	31	38
Butylmalonate	–	24	–	2
Phenylsuccinate	–	18	–	15
1,3,5-Benzenetricarboxylate	0	4	2	0

Twenty μl [^{14}C]citrate-loaded (0.33 mg protein) or citrate-loaded particles (0.36 mg protein), in back and forward exchange, respectively, were added to 0.1 ml reaction medium containing 120 mM KCl, 20 mM Hepes–Tris, pH 7.0, 4 mM of the indicated compounds (except in the control samples) and unlabeled or labeled citrate at the concentrations indicated. Temperature 20°C. After 2 min (efflux) or 30 s (uptake), the reaction was terminated by 20-fold dilution with ice-cold KCl–Hepes medium, immediately followed by millipore filtration. The dash indicates that it was not determined. The values given for the efflux were corrected for the small release of citrate caused by the inhibitors alone

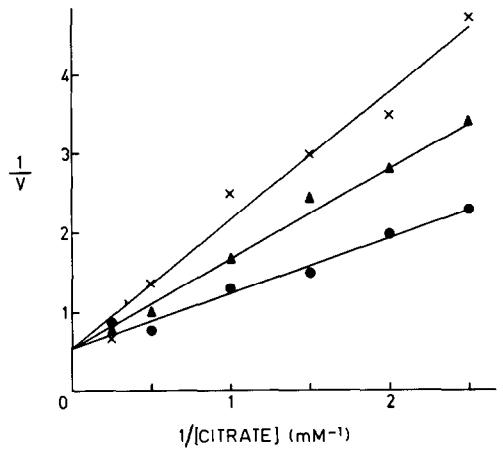

Fig.1. Dependence of the rate of citrate uptake on substrate concentration and competitive inhibition by 1,2,3-benzenetricarboxylate and *p*-iodobenzylmalonate. Twenty μl citrate-loaded SMP (0.36 mg protein) were added to 100 μl medium consisting of 120 mM KCl, 20 mM Hepes–Tris, pH 7.0, and the indicated concentrations of [^{14}C]citrate, at 10°C. Where indicated, 2 mM 1,2,3-benzenetricarboxylate (×) and 2 mM *p*-iodobenzylmalonate (▲) were also present in the reaction mixture. After 30 s incubation, the reaction was terminated by 20-fold dilution with ice-cold KCl–Hepes medium, immediately followed by millipore filtration. The results are the mean values of triplicate assays. V is expressed in μmol/min · g protein.

confirmed that these vesicles are more than 95% inside-out, whereas sonic SMP are found to be 60–70% inverted. The K_m and V_{max} of citrate transport are 1.1 mM and 2.6 μmol/min · g protein (average of 3 experiments), i.e., they do not significantly differ from those found in sonic SMP.

Although citrate transport in SMP exhibits properties of specificity and inhibitor sensitivity which are distinctive features of the tricarboxylate carrier in mitochondria [2,7], the rate of transport is much lower in SMP than in mitochondria (in RLM V_{max} 22.5 μmol/min · g and K_m 0.12 mM at 9°C [2]. The possibility that the marked decrease in the translocation rate is due to a significant portion of right side-out particles, which have not trapped the substrate inside, is ruled out by the finding that nitrogen decompression particles, more than 95% inverted, have also a very low activity. A possible interpretation is that the perturbation of the membrane occurring during the formation of the vesicles induces the inactivation of a large portion of the carrier sites. This view is supported by a decrease of about 4 times in the specific binding of 1,2,3-[^{3}H]benzenetricarboxylate to SMP with respect to RLM [8,9]. For the higher K_m of citrate transport found in

SMP the possibility has also to be considered that the carrier sites facing the inside in the mitochondria, and the outside in SMP, have a lower affinity for their substrates. This is in agreement with the reported higher K_m values for intra- than for extramitochondrial citrate and oxoglutarate (K_m for internal citrate 3 mM [10]; K_m for internal oxoglutarate 1 mM [11]).

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