# REGULATION OF CITRATE TRANSPORT IN RAT LIVER MITOCHONDRIA METABOLIZING PYRUVATE

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## 1. Introduction

Citrate and malate are the two major metabolites produced within the matrix space of rat liver mitochondria incubated in the presence of pyruvate, bicarbonate, ATP and phosphate [1]. The net transport of citrate out of the matrix is mediated by the tricarboxylate carrier which catalyzes a proton-compensated electroneutral exchange of citrate$^{3-}$ against malate$^{2-}$ across the inner membrane [2–4]. Malate is transported out of the matrix via the combined action of the dicarboxylate and phosphate carriers.

In this study it was found that the steady state distribution of citrate and malate across the inner mitochondrial membrane is asymmetric and strongly influenced by the transmembrane pH gradient. Moreover, it was found that the exchange rate of radioactive [$^{14}$C]citrate added to the medium with unlabelled citrate produced in the matrix is dependent on the distribution of citrate and malate across the inner membrane and not on the pool size of exchangeable citrate in the matrix. From these results it was concluded that the transmembrane pH gradient influences both the distribution of citrate and malate across the inner membrane as well as the kinetics of the $citrate_{ex} \rightarrow citrate_{in}$ exchange.

## 2. Materials and methods

Mitochondria were prepared as described elsewhere [1] and incubated at 37°C in a medium containing 4 mM ATP, 10 mM $MgSO_4$, 6.6 mM K-phosphate buffer pH 7.4, 6.6 mM triethanolamine buffer pH 7.4, 10 mM $KHCO_3$, 250 μg creatine kinase and 0.25 M mannitol plus 0.07 M sucrose to make a final vol of 3 ml as previously described [1,5]. Where indicated, creatine was added as a phosphoroyl acceptor. Incubated mitochondria were separated from the medium with the millipore filter technique and assayed for intra- and extramitochondrial metabolites [5]. For the determination of the pH gradients, mitochondria were separated from the medium with the silicone layer method [6]. The volume of the matrix space was estimated from the distribution of $^{3}H_2O$ and [$^{14}$C] sucrose. The pH gradients were calculated from the distribution of [$^{14}$C]5,5-dimethyl-2,4-oxazolylidenedione (DMO) between the medium and the sucrose-inaccessible space [7]. Separation of the radioactive acids with high voltage paper electrophoresis was done as previously described [1] with the exception that [$^{14}$C]citrate was first degraded with the citrate-lyase reaction when the radioactivity of [$^{14}$C]malate was measured in samples with high [$^{14}$C]citrate radioactivities. All enzymes and coenzymes were obtained from Boehringer Mannheim GmbH (Mannheim, Germany) and the radiochemicals from NEN (Dreieichenhain, Germany). Wacker silicone oil AR 100 and AR 150 was purchased from Stehelin & Co (Basel, Switzerland).

## 3. Results and discussion

Table 1 summarizes the results of an incubation of intact mitochondria with pyruvate. The accumulation of citrate and malate in the medium decreased with increasing creatine concentrations. These two metabolites account for 90% of the products formed from pyruvate via the pyruvate carboxylase and in

Table 1
Effect of creatine on the distribution of citrate and malate and on the pH gradient across the inner mitochondrial membrane

| Metabolites | Creatine (mM) 0 | 3.3 | 6.6 | 10 | 13.3 |
|---|---|---|---|---|---|
| Extramitochondrial | μmol/3 ml | | | | |
| Citrate | 1.62 | 1.11 | 0.88 | 0.80 | 0.72 |
| Malate | 1.85 | 1.19 | 0.69 | 0.43 | 0.37 |
| Intramitochondrial | nmol on filter | | | | |
| Citrate | 107.6 | 92.1 | 76.6 | 66.3 | 67.0 |
| Malate | 38.7 | 34.7 | 23.0 | 16.7 | 14.4 |
| $(cit/mal)_{ex}$ | distribution ratio | | | | |
| $(cit/mal)_{in}$ | 0.31 | 0.35 | 0.38 | 0.47 | 0.42 |
| | pH units | | | | |
| $\Delta pH_{eq}$ | 0.50 | 0.45 | 0.42 | 0.33 | 0.38 |
| $\Delta pH$ | 0.23 | 0.20 | 0.15 | 0.11 | 0.23 |

Mitochondria from 0.5 g liver containing 15 mg protein were incubated for 5 min in the standard reaction mixture and then separated from the medium as described in Materials and methods. All incubations were done in duplicates. Mitochondria from the same preparation were used for the determination of the ΔpH in a parallel incubation whereby mitochondria from 0.2 g of liver containing 6 mg protein were incubated in 1.2 ml of the reaction mixture. For the estimation of the matrix space 50 μCi $^3H_2O$ and 1 μCi [$^{14}$C]sucrose and for the measurement of the DMO distribution 50 μCi $^3H_2O$ plus 1 μCi [$^{14}$C]DMO were included in the reaction mixture. These incubations were done in triplicates. The ΔpH was calculated as the difference of the external pH at the end of the incubation (7.61 ± 0.03 pH units in all incubations) and the internal pH calculated from the DMO distribution across the inner membrane. For the calculation of $\Delta pH_{eq}$ see text.

the citric acid cycle [1]. Earlier studies have shown that the extra- and intramitochondrial ATP/ADP ratios decreased with increasing concentrations of the phosphoroyl acceptor creatine. Furthermore, the activity of the pyruvate carboxylase was essentially dependent on the intramitochondrial ATP/ADP ratio in the presence of saturating pyruvate concentrations as they were used here [5]. Therefore, the observed fall in the accumulation of the products is in line with these previous results. The steady state concentrations of citrate and malate in the matrix were also lowered upon the addition of creatine. The distribution of the two anions across the inner membrane was asymmetric as shown by the calculated distribution ratio in table 1. The asymmetry became less pronounced with increasing creatine concentrations up to 10 mM but increased again at 13.3 mM creatine.

Since the citrate–malate exchange via the tricarboxylate carrier is proton compensated, it can be expected that the pH gradient across the inner membrane influences the distribution of these anions. If this distribution would be in equilibrium with the transmembrane ΔpH it should follow the expression: $\Delta pH_{eq} = -\log((citrate/malate)_{ex}/(citrate/malate)_{in})$ However, from the comparison of this calculated value with the ΔpH actually measured with the DMO method in table 1 it follows that the distribution of citrate and malate is not in equilibrium with the ΔpH. The observed asymmetry in the distribution ratio is even more pronounced than would be predicted from the measured ΔpH.

Since citrate is produced within the matrix and has

to be transported out via the tricarboxylate carrier during the incubation an asymmetric distribution can also be expected if the rate-limiting step is the net transport rather than the production rate. It is important to realize that the distribution of citrate and malate is measured at a *steady state* in our experiments. In contrast, the measured distribution of the DMO represents an *equilibrium* value. Hence only the DMO distribution can be expected to follow exactly the transmembrane pH gradient. But also in studies where the *equilibrium* distribution of citrate and malate across the inner membrane has been measured it was found that this distribution was not exclusively dependent on the ΔpH but was influenced by binding of the anions to anionic sites in the matrix as well [8]. The results in table 1 show that the net transport rate of citrate out of the matrix decreased continuously with increasing creatine concentrations whereas the asymmetry of the distribution did not show such a continuous decrease. This finding supports the concept that also in the steady state the asymmetric distribution of citrate and malate across the membrane is predominantly effected by a transmembrane pH gradient and by binding of these anions to anionic sites in the matrix whereas the transport of citrate out of the matrix seems not to be limiting under the present experimental conditions.

In experiments with normoxic perfused livers an even stronger asymmetric distribution of citrate and malate between the cytosol and the mitochondrial matrix was observed than in the present experiments with isolated mitochondria [9]. Also in this context it was argued that in addition to the pH gradient also the preferential binding of citrate in the matrix could be responsible for this asymmetric distribution. All these results demonstrate that the distribution of citrate and malate across the inner membrane is strongly influenced by the pH gradient but that it is not in equilibrium with this gradient.

The decrease and subsequent increase of the pH gradient observed in our experiments after transition from near state 4 towards state 3 conditions by increasing the creatine concentration in the medium was quite unexpected. However, this peculiar behaviour of the ΔpH was always found when mitochondria were incubated with pyruvate and bicarbonate.

In the experiment depicted in fig.1 the exchange

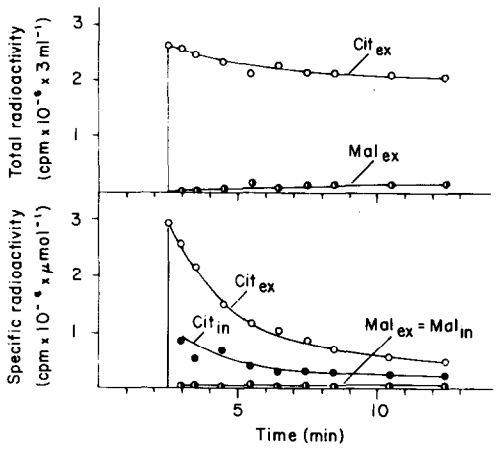


Fig.1. Distribution of $^{14}C$ in products of rat liver mitochondria after pulse labelling with 1,5-[$^{14}C$]citrate. Mitochondria from 0.5 g liver containing 16 mg protein were incubated in the standard reaction mixture as noted under Materials and methods. After 2.5 min of incubation a trace amount of 3 μCi 1,5-[$^{14}C$]citrate was added to the medium. At the times corresponding to the points in the figure the mitochondria were separated from the medium with the millipore filter technique. Both the amounts and the radioactivities of the intra- and extramitochondrial products were measured. Of the radioactive products formed in the cycle, only malate was detectable in measurable amounts on the paper strip used for the separation of the acids with high voltage electrophoresis.

of added radioactive 1,5-[$^{14}C$]citrate with unlabelled citrate produced in the matrix was measured. It is important to note that every molecule of 1,5-[$^{14}C$]citrate metabolized in the citric acid cycle loses one of its labels as $^{14}CO_2$, whereas the other is incorporated into the products of the cycle. The slow disappearance of the citrate radioactivity from the medium and the slow incorporation of the $^{14}C$-label in malate (fig.1, upper panel) indicate that the exchange of extra- with intramitochondrial citrate is a slow process. The accumulation in the medium of unlabelled citrate produced from pyruvate was essentially linear during the incubation period [1,5]. Therefore, the specific radioactivity of the added [$^{14}C$]citrate was constantly diluted (fig.1, lower panel). The fact that the specific radioactivities of the intra- and extramitochondrial citrates did not equilibrate during the incubation period also suggests that the $citrate_{ex} \rightarrow citrate_{in}$ exchange is a slow process.

The experimental procedure illustrated in fig.1

Table 2
Effect of creatine on the $citrate_{ex} \rightarrow citrate_{in}$ exchange in incubated mitochondria

| Specific radioactivity of citrate | Creatine (mM) | | | | |
|---|---|---|---|---|---|
| | 0 | 3.3 | 6.6 | 10 | 13.3 |
| | cpm × $10^{-3}$ × $\mu mol^{-1}$ | | | | |
| Initial | 766.2 | 933.6 | 1366.0 | 1926.6 | 1949.1 |
| $Final_{calc.}$ | 255.4 | 311.2 | 455.3 | 642.2 | 649.7 |
| $Final_{meas.}$ | 205.2 | 245.1 | 349.2 | 462.1 | 487.0 |
| $\frac{Final_{calc.} - final_{meas.}}{Initial}$ | relative rate | | | | |
| | 0.065 | 0.071 | 0.078 | 0.093 | 0.083 |

Mitochondria from 0.5 g liver containing 15 mg protein were incubated as in the experiment in fig.1. The mitochondria were separated from the medium with the millipore filter technique after 2.5 min of incubation immediately after the addition of 1 μCi 1,5-[$^{14}$C]citrate and after 10 min of incubation. The initial and the $final_{meas.}$ specific radioactivities were calculated from the measured amounts and radioactivities of the citrate in the filtrate. The specific radioactivity resulting from dilution with unlabelled citrate alone ($final_{calc.}$) was calculated by dividing the total radioactivity of the added [$^{14}$C]citrate by the amount of citrate found after 10 min. For the calculation of the relative exchange rates from these values see text.

was used to measure the relative exchange rates of extra- with intramitochondrial citrate shown in table 2. This rate can be estimated from the difference of the calculated specific radioactivity of citrate which would result from dilution with unlabelled citrate alone ($final_{calc.}$) and that actually measured which is the result of both the dilution and the uptake ($final_{meas.}$). Since the rate of citrate production in the matrix, though it is constant with time, is different with different creatine concentrations (table 1), the difference of the specific radioactivities has to be corrected for the initial specific radioactivity of the added [$^{14}$C]citrate. Thus, the difference of the specific radioactivities ($final_{calc.} - final_{meas.}$) divided by the initial specific citrate radioactivity is a relative measure of the velocity of the citrate exchange.

The comparison of these rates in table 2 with the intramitochondrial steady state levels of citrate in table 1 shows that the exchange rate is not correlated with the pool size of exchangeable intramitochondrial citrate. On the other hand, a strict parallelism between the exchange rates and the distribution ratios $(citrate/malate)_{ex}/(citrate/malate)_{in}$ in table 1 was observed. The exchange rate was enhanced by lowering the asymmetry of the distribution and vice versa. Therefore this exchange shows not the characteristics of simple exchange diffusion.

From these results it can be concluded that the pH gradient across the inner membrane influences both the distribution of citrate and malate as well as the kinetics of the $citrate_{ex} \rightarrow citrate_{in}$ exchange. In this context it is interesting to note that the translocation of ATP and ADP via the adenine-nucleotide translocase shows also asymmetric functions both with respect to distribution and exchange kinetics of ATP and ADP across the inner membrane [2]. In contrast to the citrate-malate translocation the asymmetric behaviour of adenine-nucleotide translocation is due to the transmembrane electrical potential rather than to the transmembrane pH gradient. These two transport systems are therefore different examples to illustrate how asymmetric membrane functions can result from electrical or chemical gradients across the membrane.

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