ELSEVIER

Biochimica et Biophysica Acta 1185 (1994) 153–159

BBA Biochimica et Biophysica Acta

Effect of aspartate and glutamate on the oxoglutarate carrier investigated in rat heart mitochondria and inverted submitochondrial vesicles

J.J. Hautecler, C.M. Sluse-Goffart, A. Evens, C. Duyckaerts, F.E. Sluse *

Laboratoire de Bioénergétique, Centre Interdisciplinaire de Biochimie de l'Oxygène, Institut de Chimie (B-6) au Sart-Tilman, Université de Liège, B-4000 Liège, Belgium

(Received 1 December 1993)

Abstract

Interaction of glutamate and aspartate with the oxoglutarate carrier was investigated in rat heart mitochondria or inverted submitochondrial particles. With mitochondria, glutamate and aspartate had no effect on the initial rate of oxoglutarate or malate uptake. With inverted submitochondrial vesicles, binding experiments indicated that aspartate bound to the oxoglutarate carrier on its matricial face and increased the affinity of the substrate binding site for malate but did not change the affinity for oxoglutarate. Glutamate had no effect on both substrate bindings. The dissociation constants of the binary substrate-carrier complexes on the matricial side were determined (1.28 ± 0.15 mM for oxoglutarate and 2.22 ± 0.26 mM for malate). These values, compared with those obtained previously on the cytosolic side of intact mitochondria, confirmed the asymmetry of the carrier in the native membrane (higher affinities on the cytosolic face). It is concluded that (1) aspartate and glutamate are not cytosolic effectors of the oxoglutarate carrier, (2) matricial aspartate is a positive effector of the binding of malate on the matricial side of the oxoglutarate carrier, and (3) such a characteristic may play a role in the regulation of the oxoglutarate carrier. Thus, it may be emphasized that (1) this observation is the first clear evidence of a well-defined 'sophisticated regulation' (allosteric) of a mitochondrial metabolite carrier, and (2) this regulation of the oxoglutarate carrier may have important consequences on the efficiency of reducing equivalent import in the matrix space by the malate-aspartate shuttle.

Key words: Oxoglutarate carrier; Mitochondrion; Heart; Submitochondrial vesicle; (Rat)

1. Introduction

The oxoglutarate carrier, together with the aspartate-glutamate carrier, take part in the malate-aspartate shuttle that transfers reducing equivalents through the inner mitochondrial membrane. The reducing equivalents are carried by malate^{2-} which is electroneutrally exchanged for oxoglutarate^{2-} by the oxoglutarate carrier.

The aspartate-glutamate exchange is electrogenic and under the control of the protonmotive force (Δp) that favours the exchange of external glutamate + H^+ for internal aspartate. As a result the overall process catalysed by the shuttle is under the control of Δp that favours the entry of reducing equivalents.

The kinetic properties and mechanisms of the two carriers were characterized in mitochondria and in reconstituted systems [1–9]. They follow a sequential mechanism involving a ternary complex between the protein and the two substrates that is formed by independent rapid-equilibrium binding of substrates to the internal and external binding sites. These studies on both mitochondria and proteoliposomes may be considered as examplary owing to the fact that they reinforce each other despite that (1) in mitochondria, compared with reconstituted systems, it is more difficult to vary freely the substrate concentrations on both sides of the membrane and to avoid interfering activities of other carriers and enzymes, nonspecific bindings and

* Corresponding author. Fax: +32 41 562934.

SSDI 0005-2728(93)E0214-B

possible microcompartmentation, and (2) in proteoliposomes, there are also drawbacks compared with mitochondria as "somewhat undefined state of activity after isolation and purification, the a priori unknown orientation of inserted proteins and the possibility of missing a regulatory compound" [10].

A general feature of mitochondrial metabolite carriers is the absence of clear evidence for 'sophisticated' regulations (allosteric effectors) of their activities. Concerning the oxoglutarate carrier, very old observations, obtained with liver mitochondria in such conditions that activities of several carriers and enzymes might interfere, had suggested that the entry of oxoglutarate into mitochondria was specifically inhibited by L-aspartate but that external aspartate did not influence oxoglutarate efflux from mitochondria [11]. In better conditions, but still allowing translocation interferences, aspartate and glutamate inhibited weakly the oxoglutarate uptake in liver mitochondria and stimulated its efflux but were not transported by the oxoglutarate carrier [12]. More recent studies, made with heart mitochondria where strict conditions avoided carrier and enzyme interferences and where a single exchange (external oxoglutarate/internal malate) was realized by the oxoglutarate carrier at 2°C, showed no conclusive effect of external aspartate but a stimulatory effect of internal aspartate on the initial rate of this exchange [13,14]. In reconstituted (inside-out) system (from heart) the homologous exchange oxoglutarate/oxoglutarate was not inhibited by glutamate, aspartate being not tested [15,16] and the exchange oxoglutarate/aspartate did not occur [15]. It was also shown in [17] that malonate and L-malate inhibited the oxoglutarate/oxoglutarate exchange. This observation was indeed expected as it had already been known for 20 years that in rat heart mitochondria both dicarboxylates were substrates for the oxoglutarate carrier [1,2].

As metabolite carriers have a specific function in particular metabolic pathways, in some cases even controlling the metabolic fluxes [10], it is of the utmost importance not only to characterize their kinetic mechanism but also to describe every controlling factor of their action.

This study was undertaken to describe the putative interactions between the two amino acids involved in the malate-aspartate shuttle and the oxoglutarate carrier. We report here the effects of external aspartate and glutamate on the initial rates of malate and oxoglutarate uptakes in malate-loaded mitochondria and the effects of both amino acids on the equilibrium bindings of malate and oxoglutarate on inverted submitochondrial vesicles. Under the described conditions, we have observed that aspartate is a positive allosteric effector of the binding of malate on the matricial side of the oxoglutarate carrier.

2. Materials and methods

Mitochondrial preparation and loading

Mitochondria from rat heart ventricles were prepared according to Tyler and Gonze [18] and loaded with malate as described earlier [3,4]. Internal malate and aspartate concentrations were determined enzymatically [19,20]. Mitochondrial protein concentration was determined by the Biuret method [21] using bovine serum albumin, fraction V (BSA) as standard.

Kinetic measurements with intact mitochondria

Incubation medium, timing, equipment and requirements were described exhaustively elsewhere [3,4]. Initial rate measurements were performed at 2°C by the inhibitor-stop technique using phenylsuccinate as impermeant competitive inhibitor [12]. The external [^{14}C]oxoglutarate/internal malate and external [^{14}C]malate/internal malate exchanges were studied in the presence of external glutamate or aspartate. Eight different incubation times within 1 s (for oxoglutarate) and 3 s (for malate) from the onset of the uptake (mixing [^{14}C]substrate to the mitochondria) allowed to determine the initial rates ± S.D. by linear regression.

Inverted submitochondrial vesicle preparation

Freshly isolated mitochondria were resuspended in 0.25 M sucrose + 0.1% BSA (1 g of mitochondrial pellet + 3 ml of sucrose-BSA), frozen in liquid nitrogen and kept at −20°C until use (at most 48 h). The procedure outlined below was derived from that described by Harmon [22]. All operations were carried out at 0°C. Mitochondria were thawed slowly and homogenized gently in 30 ml of 0.25 M sucrose + 0.1% BSA. The suspension was centrifuged at 27000 × *g* for 15 min and the pellet resuspended in a total volume of 4.5 ml of sonication medium (10 mM NaH_2PO_4, 225 mM sucrose, 0.1% BSA, pH 7.4). The sonication medium contained 0.2 mM mersalyl if inverted vesicles were prepared for [^{14}C]malate binding experiments (see Principles in Binding measurements). This suspension (≈ 40 mg of protein/ml) cooled with an ice-water bath was sonicated for 30 s with a Branson B-12 sonifier (1/2-inch tip at graduation 80), diluted with 22 ml of sonication medium and centrifuged at 23500 × *g* for 10 min. The pellet was discarded and the supernatant centrifuged during 30 min at 44000 × *g*. The resulting pellet was resuspended in 10 ml of a medium containing 225 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, 0.1% BSA (pH 7.4) (STE medium). A last centrifugation at 44000 × *g* for 30 min produced a pellet (≈ 0.3 g) mainly made of inverted particles that was resuspended in the latter medium at 100 mg (wet weight) of pellet of inverted submitochondrial vesicles per ml (stock suspension). In order to check the per-

centage of inversion, the latency of the cytochrome oxidase activity was determined [23]. The rate of oxidation of cytochrome *c* at 30°C in a 0.1 M Tris-HCl buffer (pH 7.4) containing 50 μM reduced cytochrome *c* as the substrate was measured with a double beam dual-wavelength Aminco/Chance spectrophotometer at 550–540 nm in the absence and in the presence of an optimal concentration of Lubrol (after a 10-min preincubation), i.e., a concentration not inhibiting the activity of cytochrome *c* oxidase (2.6 μg per μg of vesicular protein at 0.1% Lubrol). The percentage of inversion was given by: ([activity with Lubrol − activity without Lubrol]/activity with Lubrol) × 100, and was ranging from 90 to 96%.

Binding measurements

Principles. Bound [^{14}C]oxoglutarate was determined by the difference of [^{14}C]oxoglutarate contents in the pellets of inverted submitochondrial particles (ISMP) in the absence and in the presence of phenylsuccinate (for details, see Methods). Phenylsuccinate has been shown to be an impermeant competitive inhibitor of the oxoglutarate carrier of rat liver mitochondria [12] and of rat heart mitochondria [3,4]. This inhibitor has already been used to determine the specific oxoglutarate binding to the oxoglutarate carrier in heart mitochondria [4].

The absence of a significant amount of exchangeable substrate inside the ISMP was expected from the substrate content of frozen-thawed mitochondria (undetectable). This absence has been controlled by comparing the [^{14}C]oxoglutarate content of ISMP pellets obtained after incubation either with labelled oxoglutarate and phenylsuccinate added simultaneously, or with labelled oxoglutarate first followed 2 min later by the addition of phenylsuccinate. As was expected, the same values were obtained, indicating that no entry of labelled substrate had occurred by exchange during the 2-min period (data not shown). It has also been shown that the use of unlabeled oxoglutarate together with phenylsuccinate provided the same value of binding as phenylsuccinate alone. This suggests that the impermeant phenylsuccinate alone is able to remove completely the bound [^{14}C]oxoglutarate.

Bound [^{14}C]malate was similarly determined using cold oxoglutarate as competitive displacing inhibitor in order to avoid a possible release of [^{14}C]malate from other carriers (dicarboxylate carrier, for example) sharing malate as substrate (phenylsuccinate is a competitive inhibitor of the dicarboxylate carrier too). For the malate-binding experiments, the ISMP were prepared in the presence of 0.2 mM mersalyl in the sonication medium. Then, the ISMP that might contain P_i (10 mM NaH_2PO_4 in the sonication medium) were also mersalyl loaded so that external malate could not be taken up against internal P_i via the dicarboxylate carrier.

Methods. Binding experiments were carried out at 0°C. 200 μl of the stock suspension of ISMP were mixed in an Eppendorf tube with 800 μl of incubation medium (final composition: 15 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 50 mM Tris-HCl (pH 7.4), 50 mM sucrose, 1 mM arsenite, 1.4 μCi ^3H_2O, 0 or 23 mM phenylsuccinate or unlabeled oxoglutarate, various concentrations of [^{14}C]oxoglutarate or [^{14}C]malate, various concentrations of glutamate or aspartate when present). 150 μl of the mixture were sampled in 6 Airfuge tubes within 2 min and then centrifuged at 178 000 × *g* for 2 min with a Beckman Airfuge (fixed angle rotor Beckman A-100). From each Airfuge tube, 50 μl of supernatant were taken up and used to count ^{14}C and ^{3}H radioactivities (Beckman LS3801 counter with Ready Safe as scintillation medium). The tubes containing the pellets were rinsed with the STE medium and blotted rapidly. The pellets were dissolved in 150 μl of 0.1 M KOH in order to measure their radioactivity.

In a typical experiment, the radioactivity was measured in at least 12 samples (12 supernatants and 12 pellets) for each substrate concentration: 6 in the absence and 6 in the presence of the competitive inhibitor. This was the minimum number of measurements required to obtain a binding value with a reasonable standard deviation.

The different measurements allowed us to calculate the ^3H_2O volume of the inverted submitochondrial particle pellets, the free concentrations of substrates and the amounts of bound substrates.

Chemicals. Special reagents were obtained from the following sources: 2-oxo[5-^{14}C]glutarate, L-[U-^{14}C]malate, [U-^{14}C]sucrose and tritiated water from Amersham International, Amersham, UK; rotenone from Sigma Chemical Co., Saint Louis, MO, USA; mersalyl, acid form, from Mann Research Laboratories, NY, USA; phenylsuccinate from Aldrich, Milwaukee, WI, USA.

3. Results

Initial rate of malate and oxoglutarate uptake in intact mitochondria: effect of external aspartate and glutamate

External [^{14}C]oxoglutarate exchange for internal malate and [^{14}C]malate exchange for internal malate were both extensively studied by varying the concentration of the external substrate [3–6].

In the present study, different non-saturating concentrations were chosen for each substrate. Table 1 presents a selection from several kinetic experiments. In each experiment, everything was kept constant except the external concentration of the tested amino acid. The mitochondria could differ from one experiment to another by their matricial content in malate and aspartate (see legend of Table 1), but owing to the

Table 1
Initial rate of malate (Mal) and oxoglutarate (OG) uptake in malate-loaded mitochondria: effect of external aspartate (Asp) and glutamate (Glu)

Expt.	External substrate		External amino acid		$\nu \pm$S.D. pmol s^{-1} (mg protein)$^{-1}$
	[OG] μM	[Mal] μM	[Asp] mM	[Glu] mM	
1	0.4	–	0	–	10.2± 0.7
	0.4	–	0.5	–	9.0± 0.8
	0.4	–	2	–	9.6± 0.4
2	1.3	–	–	0	19.8± 2.5
	1.3	–	–	1	22.4± 1.7
	1.3	–	–	5	23.0± 1.7
3	–	10	0	–	21.7± 0.6
	–	10	1	–	21.1± 0.8
	–	10	5	–	19.9± 1.0
4	–	19	–	0	28.7± 2.5
	–	19	–	1	27.9± 2.3
	–	19	–	5	29.8± 1.6
5	12	–	0	–	186 ±15
	12	–	0.5	–	176 ±18
	12	–	2	–	178 ±11
6	6.5	–	–	0	89 ±10
	6.5	–	–	1	93 ± 6
	6.5	–	–	5	90 ± 6
7	–	250	0	–	256 ±34
	–	250	2	–	232 ±31
	–	250	4	–	247 ±25
8	–	190	–	0	176 ±12
	–	190	–	1	211 ±29
	–	190	–	5	204 ±28
9	50	–	0	–	428 ±13
	50	–	0.5	–	428 ±35
	50	–	2	–	415 ±21

Internal malate concentrations ranged from 4.7 to 6.8 mM and internal aspartate concentrations from 7.6 to 9.7 mM. $\nu \pm$S.D. were determined as described in Section 2.

internal/external independence evidenced previously [1–3,7] the matricial content is not expected to influence the behaviour of an external effector. As shown in Table 1, whatever the external substrate and its concentration, the three initial rates determined in each experiment were not significantly different: neither aspartate nor glutamate had any effect. The absence of effect on the initial rates of the exchanges implies that neither the substrate binding to the oxoglutarate carrier nor the catalytic rate constants of the exchanges are modified in the presence of both amino acids. The studies of possible effects of aspartate and glutamate on oxoglutarate and malate binding on the cytosolic side of the carrier could not bring additional information.

Binding of oxoglutarate and malate to the inverted submitochondrial vesicles: effect of aspartate and glutamate

The method used for equilibrium binding measurements led to the determination of the total binding for oxoglutarate and part of the malate binding that could be removed by oxoglutarate (see Section 2). In both cases, the estimated binding included the binding to the matricial sites of the oxoglutarate carrier which were exposed at the external surface of the submitochondrial vesicles.

Data obtained in the absence of aspartate and glutamate are shown in Fig. 1 (double-reciprocal plots) and the binding parameters determined by linear regres-

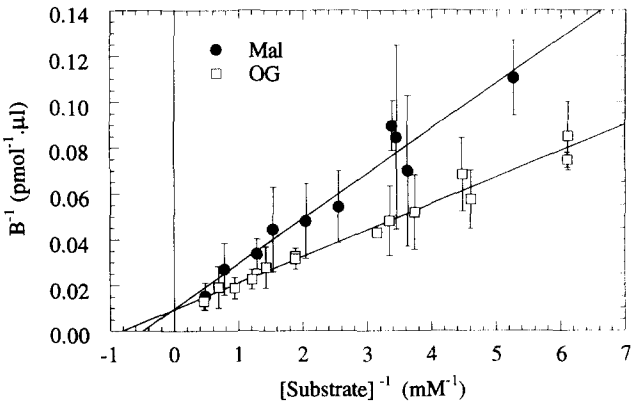

Fig. 1. Reciprocal of malate (●) and oxoglutarate (□) equilibrium bindings as a function of the reciprocal of their free concentrations. Inverted submitochondrial particles (around 2 μl of pellet) are prepared as described in Section 2. Each point is the mean ± S.D. (vertical bar) of 6 binding determinations made with the same preparation. Straight lines are obtained by linear regression.

Table 2
Binding parameters

Substrate	Aspartate (mM)	B_{max} [a] ± S.D. (pmol μl^{-1})	$K_{d(app)}$ [b] ± S.D. (mM)	B_{max} [b] ± S.D. (pmol μl^{-1})	$K_{d(app)}$ [b] ± S.D. (mM)
OG	–	107 ± 20	1.24 ± 0.24	111 ± 12	1.28 ± 0.15
Mal	–	104 ± 50	2.04 ± 0.99	111 ± 12	2.22 ± 0.26
Mal	1.5	111 ± 9	1.11 ± 0.10	111 ± 12	1.11 ± 0.13
Mal	3.3	132 ± 24	0.76 ± 0.15	111 ± 12	0.59 ± 0.08
Mal	10	111 ± 25	0.61 ± 0.16	111 ± 12	0.61 ± 0.09
Mal	20	113 ± 15	0.60 ± 0.10	111 ± 12	0.58 ± 0.08

B_{max} = maximal binding expressed in pmol per μl of vesicles in the pellet, $K_{d(app)}$ = dissociation constant of the translocator-substrate complex or its apparent value in the presence of aspartate.

[a] Values obtained from individual linear regressions (Figs. 1 and 3).

[b] Values obtained when the data are treated together as belonging to a set of straight lines with a common point (unknown a priori) on the ordinate.

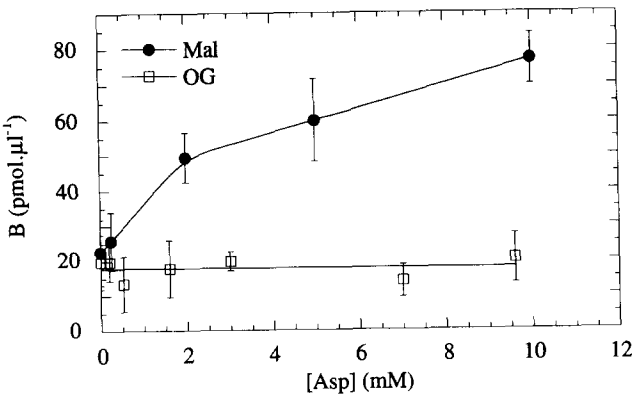

Fig. 2. Effect of aspartate on malate (●) and oxoglutarate (□) equilibrium bindings to inverted submitochondrial particles. Malate-free concentration: 0.65 mM, oxoglutarate-free concentration: 0.32 mM. For other details see Section 2 and the legend of Fig. 1. Straight line parallel to X-axis corresponds to the mean value of oxoglutarate equilibrium bindings for the various aspartate concentrations.

sion are given in Table 2. The maximal binding values for oxoglutarate and for malate were not significantly different from each other, suggesting that a single type of binding site without interaction was involved, likely that of the oxoglutarate carrier (see Section 4). The dissociation constant (K_d) values were of the same order of magnitude (mM) for oxoglutarate and malate.

In experiments performed at a non-saturating concentration of [^{14}C]oxoglutarate or [^{14}C]malate and at various concentrations of glutamate, no significant effect of glutamate was observed on both bindings (data not shown). Fig. 2 shows the results of the same experiments performed at various concentrations of aspartate. The oxoglutarate binding was not modified (lower curve of Fig. 2) but the malate binding was increased in the presence of increasing concentrations of aspartate (upper curve of Fig. 2). The effect of aspartate was further investigated by varying the malate concentration for different fixed aspartate concentrations (Fig. 3). The maximal malate binding remained constant while the apparent dissociation constant sharply decreased till an almost constant value when the aspartate concentration was increased (Table 2).

The individual maximal bindings ($B_{max}{}^{a}$ in Table 2) were not significantly different and a common value was better estimated ($B_{max}{}^{b}$) by a least squared method taking into account the whole set of oxoglutarate and malate binding data [2]. This procedure provided K_d values that were only slightly modified and had in most cases a lower standard deviation.

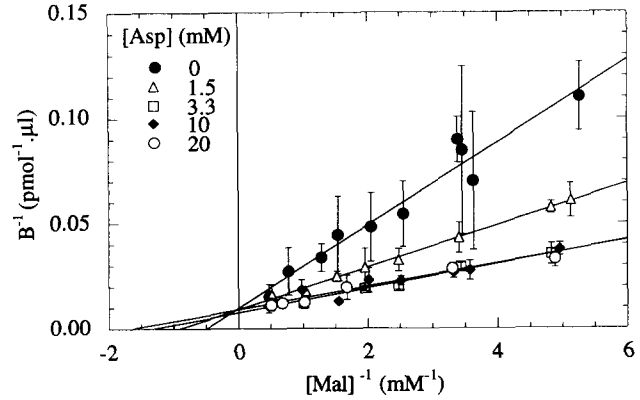

Fig. 3. Reciprocal of malate equilibrium binding as a function of the reciprocal of its free concentration: effect of aspartate between 0 and 20 mM. Linear regression is calculated for each aspartate concentration. For other details see Section 2 and legend of Fig. 1.

4. Discussion

Extensive studies of kinetic and ligand binding behaviours of the oxoglutarate carrier have been performed with rat heart mitochondria. Within a large concentration range (0.05 to 850 μM) malate was a Michaelian substrate towards the cytosolic side of the carrier [5]. By contrast, oxoglutarate exhibited multi-jump saturation curves for initial rate kinetics [3] and for equilibrium binding [4]. However, both substrates were demonstrated to share the same sites on the carrier [5]. In order to rationalize these observations it has been suggested that the carrier had an oligomeric structure with only one kind of subunit, and that subunit-conformational changes were induced by oxoglutarate binding but not by malate binding [5,24]. Results were also obtained regarding the matricial side of the carrier: the kinetics seemed to be Michaelian for oxoglutarate [14] but not for malate [13] (the data for malate were only available in the presence of matricial aspartate). These results, for the matricial side, have to be considered with caution because, if it was not too difficult to control and vary the internal substrate concentration, it was impossible to control in the same time the concentrations of other molecules that might be potential effectors of the carrier.

When purified from bovine heart mitochondria and reincorporated into a liposome membrane, the oxoglutarate carrier has shown a Michaelian behaviour on both sides of the membrane and for both substrates, malate and oxoglutarate [7]. Even if the concentration ranges were narrower than in the studies with mitochondria, the results and conclusions with reconstituted systems are reliable. One explanation of the discrepancy between both studies may be due to the fact that the carrier is isolated in its monomeric form and reinserted in a very few copies per liposome. It is therefore not astonishing that there is no evidence for the presence of oligomeric forms in the proteoliposomes, and it can be proposed that the behaviour of the reconstituted carrier regarding cytosolic oxoglutarate may be a drawback of the system used. As

already mentioned in the Introduction, aspartate was not tested as putative effector on homologous or heterologous exchanges with proteoliposomes.

In contrast to mitochondria, inverted submitochondrial vesicles have the advantage to expose the matricial face of the translocator to the suspension medium, the composition of which can be easily controlled, and in contrast to proteoliposomes, they have the advantage of containing the translocator in its native form, avoiding the drawbacks detailed in [10]. It is clear, however, that the three approaches are necessary and complement each other.

Oxoglutarate and malate binding

The binding data obtained with inverted submitochondrial vesicles apparently obey Michaelian-type equations (linear reciprocal plots, Fig. 1). The absence of cooperative phenomena in malate binding as well as in oxoglutarate binding is supported by the fact that extrapolation to infinite substrate concentration leads to the same maximal binding value in any case (Table 2). Furthermore, there is an excellent agreement between the K_d value of oxoglutarate (1.28 ± 0.15 mM) and the K_m value of matricial oxoglutarate obtained from transport kinetic experiments with mitochondria (1.29 ± 0.10 mM) [14]. Such an agreement was expected because of the rapid-equilibrium kinetics of the exchanges catalysed by the oxoglutarate carrier and of the internal/external independence, provided no cooperative phenomena would interfere.

The present data for malate ($K_d = 2.22$ mM) are at variance with the results obtained for matricial malate with mitochondria, at least apparently. Indeed with mitochondria, the data for matricial malate were only available in the presence of matricial aspartate (contrary to the data for oxoglutarate for which matricial aspartate is absent [13]) and exhibited a negative cooperativity: at 5.4 mM internal aspartate, the apparent K_m value for internal malate was smaller than 0.2 mM in the low concentration range of malate (< 2 mM) and around 1.5 mM for malate concentrations higher than 2 mM [13]. In ISMP, at 5 mM aspartate, the binding apparent K_d for malate is 0.6 mM. It looks plausible that with mitochondria, the negative cooperativity observed at each matricial aspartate concentration is due to an unknown matricial effector, as already suggested [13].

From the analysis of the binding data described here, it can be concluded that the measured values of binding correspond to the matricial sites of the oxoglutarate carrier and that no substrate-induced conformational changes of interacting subunits are evidenced on the matricial side of the carrier, in contrast to the cytosolic side.

The dissociation constants of the translocator-substrate complexes on the matricial face of the oxoglutarate carrier are now determined in a safer way. Their values (1.28 ± 0.15 mM for oxoglutarate and 2.22 ± 0.26 mM for malate) compared with $S_{0.5}$ for oxoglutarate (≈ 0.01 mM determined from kinetic and binding data [4]) and K_d for malate (0.21 ± 0.01 mM determined from kinetic data [5]) on the cytosolic side confirm the general observations that the anion mitochondrial carriers have higher affinities (a factor of 10 at least) for their substrates on the cytosolic side. For the glutamate/aspartate translocator such an asymmetry also holds true in the native membrane and in the liposomal membrane: there is a good agreement for aspartate K_m in both types of membrane according to a right-side-out orientation of the protein in the reconstituted system [8,9]. For the oxoglutarate carrier, the asymmetry appears surprisingly very slight in the reconstituted system [7].

Aspartate and glutamate as possible effectors

Results reported in Table 1, contrary to the single observation made in [13] for the lowest (0.5 μM) external oxoglutarate concentration, indicate clearly that external aspartate and glutamate have no effect on the activity of the oxoglutarate carrier in mitochondria and thus on the binding of substrates. Therefore it may be concluded that these two amino acids would not act as cytosolic effectors.

The difficulties encountered in the manipulation of the matricial content of mitochondria are such that no information was available regarding the regulation of the oxoglutarate carrier on the matricial side, except our observation that a decrease in the aspartate mitochondrial content of rat heart mitochondria was accompanied by a decrease in the exit rate of malate in exchange with external oxoglutarate. The matricial aspartate appeared to increase the affinity of the carrier for malate but to decrease the maximal rate of the exchange reaction [13]. The results obtained with inverted submitochondrial vesicles (Figs. 2 and 3) confirm that aspartate enhances malate binding to the matricial sites of the oxoglutarate carrier. This effect implies the existence of a specific site for aspartate and the formation of a ternary complex carrier-malate-aspartate. A binary complex carrier-aspartate is also formed. Indeed, the apparent K_d for malate (2.2 mM in the absence of aspartate) seems to reach a limit value around 0.6 mM instead of going down to zero when the aspartate concentration is increased (Table 2). The ratio 2.2/0.6 suggests that the affinity of the binary complex carrier-aspartate for malate is about 4-times greater than the affinity of the aspartate unloaded carrier. Thermodynamics impose that bound malate also increases 4-fold the affinity of the carrier for aspartate. Indeed binding of malate first and aspartate second, or binding of aspartate first and malate second, are two ways of going from the same initial

state to the same final state, the product of the two equilibrium constants of one way necessarily being equal to that of the other way. The affinity of the carrier for aspartate looks rather high as saturation by aspartate is already reached at 3.3 mM, a concentration that is currently found in mitochondria.

Surprisingly, aspartate does not modify the oxoglutarate binding (Fig. 2). However, it has been concluded above that aspartate is able to bind to the translocator even in the absence of malate. It is thus necessary to admit that the carrier and the carrier-aspartate complex have the same affinity for oxoglutarate so that oxoglutarate and aspartate bind independently. This would not exclude an aspartate effect on the catalytic rate constant of the oxoglutarate transport (pure noncompetitive effect). An effect of matricial glutamate on the transport rates would also remain possible although it acts neither on the oxoglutarate nor on the malate bindings. It is clear that it would be useful to have data for the kinetic effects of aspartate and glutamate on oxoglutarate and malate transport at the matricial side of the carrier. Unfortunately, proper conditions to perform kinetic measurements with inverted submitochondrial vesicles have not yet been obtained so that kinetic experiments with the inside-out reconstituted system would be necessary to clarify this aspect. In any case, it must be pointed out that an increase in the aspartate concentration will diminish the oxoglutarate binding on the matricial side of the carrier (and this will affect the rate of oxoglutarate exit from mitochondria), despite the absence of a direct effect if malate is also present, as both substrates are competitive. In fact malate and oxoglutarate as well as aspartate are always present together in the mitochondrial matrix, as is the case in freshly isolated heart mitochondria: [OG] $\approx$ 0.1 mM, [Mal] $\approx$ 2 mM, [Asp] $\approx$ 6 mM and [Glu] $\approx$ 2.5 mM.

Thus a very clear conclusion emerges from the results described in this paper: matricial aspartate plays a role in the regulation of the oxoglutarate carrier and may modulate the efficiency of reducing equivalent import in the matrix by the malate-aspartate shuttle. Moreover, it may be emphasized that our results are the first clear evidence of a well-defined 'sophisticated regulation' (allosteric) of a mitochondrial metabolite carrier. How complex is this allosteric regulation depends upon the existence of kinetic effects on the matricial side of the carrier of one or both amino acids.

5. Acknowledgements

We wish to thank Mrs. Laurette Bertrand for skilful secretarial assistance. Our thanks are also due to professor F. Palmieri (Bari, Italy) for his financial support at the beginning of this work and for his competent and fruitful advice. This work was supported by grants from the Belgian Fonds National de la Recherche Scientifique (contrats FRFC 2.4503.91 and FRSM Loterie Nationale 9.4578.91) and the Fonds Spécial de la Recherche dans les Universités. A.E. is a fellow of IRSIA.

6. References

[1] Sluse, F.E., Ranson, M. and Liébecq, C. (1972) Eur. J. Biochem. 25, 207–217.
[2] Sluse, F.E., Goffart, G. and Liébecq, C. (1973) Eur. J. Biochem. 32, 283–291.
[3] Sluse, F.E., Sluse-Goffart, C.M., Duyckaerts, C. and Liébecq, C. (1975) Eur. J. Biochem. 56, 1–14.
[4] Sluse, F.E., Duyckaerts, C., Liébecq, C. and Sluse-Goffart, C.M. (1979) Eur. J. Biochem. 100, 3–17.
[5] Sluse-Goffart, C.M., Sluse, F.E., Duyckaerts, C., Richard, M., Hengesch, P. and Liébecq, C. (1983) Eur. J. Biochem. 134, 397–406.
[6] Duyckaerts, C., Sluse-Goffart, C.M., Sluse, F.E., Gosselin-Rey, C. and Liébecq, C. (1984) Eur. J. Biochem. 142, 203–208.
[7] Indiveri, C., Dierks, T., Krämer, R. and Palmieri, F. (1991) Eur. J. Biochem. 198, 339–347.
[8] Dierks, T., Riemer, E. and Krämer, R. (1988) Biochim. Biophys. Acta 943, 231–244.
[9] Sluse, F.E., Evens, A., Dierks, T., Duyckaerts, C., Sluse-Goffart, C.M. and Krämer, R. (1991) Biochim. Biophys. Acta 1058, 329–338.
[10] Krämer, R. and Palmieri, F. (1992) in Molecular Mechanism in Bioenergetics (Ernster, L., ed.), pp. 359–384, Elsevier, Amsterdam.
[11] McGivan, J.D., Bradford, N.M. and Chappell, J.B. (1969) FEBS Lett. 4, 247–250.
[12] Palmieri, F., Quagliariello, E. and Klingenberg, M. (1972) Eur. J. Biochem. 29, 408–416.
[13] Sluse, F.E., Duyckaerts, C., Sluse-Goffart, C.M., Fux, J.P. and Liébecq, C. (1980) FEBS Lett. 120, 94–98.
[14] Sluse, F.E., Sluse-Goffart, C.M. and Duyckaerts, C. (1989) in Anion Carriers of Mitochondrial Membranes (Azzi, A. et al., eds.), pp. 183–195, Springer, Berlin.
[15] Bisaccia, F., Indiveri, C. and Palmieri, F. (1985) Biochim. Biophys. Acta 810, 362–369.
[16] Nalecz, M.J., Nalecz, K.A., Broger, C., Bolli, R., Wojtczak, L. and Azzi, A. (1986) FEBS Lett. 196, 331–336.
[17] Bolli, R., Nalecz, K.A. and Azzi, A. (1989) J. Biol. Chem. 264, 18024–18030.
[18] Tyler, D.D. and Gonze, J. (1967) Methods Enzymol. 10, 75–77.
[19] Schoner, W. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), Vol. 3, 2nd English Edn., pp. 1596–1599, Academic Press, New York.
[20] Bergmeyer, H.U., Bernt, E., Möllering, H. and Pfleiderer, G. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), Vol. 3, 2nd English Edn., pp. 1577–1580, Academic Press, New York.
[21] Von Beisenherz, G., Boltze, H.J., Bücher, T., Czok, R., Garbade, K.H., Meyer-Arendt, E. and Pfleiderer, G. (1953) Z. Naturforsch. Teil B 8, 555–577.
[22] Harmon, H.J. (1982) J. Bioenerg. Biomembr. 14, 377–386.
[23] Möller, I.M., Bergman, A., Gardeström, P., Ericson, I. and Palmer, J.M. (1981) FEBS Lett., 126, 13–17.
[24] Sluse-Goffart, C.M. and Sluse, F.E. (1986) in Dynamics of Biochemical Systems (Damjanovich, S., Keleti, T. and Tron, L., eds.), pp. 521–535, Elsevier, Amsterdam.