# Conformational Changes and Possible Structure of the Oxoglutarate Translocator of Rat-Heart Mitochondria Revealed by the Kinetic Study of Malate and Oxoglutarate Uptake

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Initial rates of the exchanges [$^{14}$C]malate$_{out}$ : malate$_{in}$, ([$^{14}$C]oxoglutarate + malate)$_{out}$ : malate$_{in}$ and ([$^{14}$C]malate + oxoglutarate)$_{out}$ : malate$_{in}$ catalysed by the oxoglutarate carrier of rat-heart mitochondria have been studied under conditions where internal and external substrates may be varied.

It is shown that contrary to external oxoglutarate which induces a conformational change of the translocator subunit to which it binds, external malate does not induce conformational changes during its binding and is a Michaelian substrate.

The study of the effect of external malate on the rate of oxoglutarate uptake shows that external malate and external oxoglutarate are competitive.

External oxoglutarate affects the catalytic rate constant of malate uptake in a modulated way.

After substrate binding, the exchange reaction between an external dicarboxylate and an internal dicarboxylate is accompanied by conformational changes. The particular form of the rate equation strongly suggests that during a first step the external substrate bound to an external binding subunit at the external surface of the membrane, and the internal substrate bound to an internal binding subunit at the internal surface of the membrane, are transferred to a catalytic subunit (channel?) deeper in the membrane. Two models, one with a single channel, and the other with several associated channels, are proposed.

It is demonstrated that a binding subunit which has transferred its substrate to a catalytic subunit is left in a conformation which does not depend on the substrate that has 'passed through it'. It is also demonstrated that all the catalytic subunits are identical. These theoretical deductions allow a simple description of the complicated effect that external oxoglutarate has on the rate of malate uptake.

The fact that all the external binding subunits are equivalent regarding external malate binding and that all the catalytic subunits are identical support the view that the mitochondrial preparation contains a single species of oxoglutarate translocator and not an isozymic mixture.

The oxoglutarate translocator present in rat-heart mitochondria [1] is located in the inner membrane and performs one-to-one exchanges between an external and an internal dicarboxylate anion as shown explicitly in [2].

The oxoglutarate translocator is a component of the malate-aspartate shuttle which transfers reducing equivalents produced in the cytosol to the respiratory chain located in the mitochondria. This shuttle is not only the main transfer device in heart but is also strictly necessary in working heart preparations metabolizing glucose, since aminooxyacetate, an inhibitor of the transamination steps of the shuttle, causes left-ventricular failure, decreases myocardial respiration and increases lactate production [3]. By recycling the glycolytic NADH, the malate-aspartate shuttle thus protects pyruvate from reduction allowing its vital oxidation by the Krebs cycle.

The glycolytic activity in the sarcoplasm and the oxidative phosphorylation in the mitochondria are linked by transmembrane translocators: the pyruvate, adenine-nucleotide, oxoglutarate, aspartate-glutamate and phosphate carriers. It is therefore of interest to know the kinetic properties of these translocators, the nature of the regulations they are submitted to and whether or not they are limiting steps in the overall process of energy supply.

Kinetic and binding studies [4–9] have shown that the oxoglutarate translocator of rat-heart mitchondria is an oligomer or a mixture of oligomers [7, 8]. Each subunit of the translocator is accessible to both internal and external substrates and binds them independently. The kinetics is of the rapid-equilibrium type in the given experimental conditions and at 2 °C.

The initial rate equation for the exchange between an external dicarboxylate ion A, and an internal dicarboxylate ion B, is of the form:

$$v_{A:B} = V_{A:B}\, f_A([A])g_B([B]) \qquad (1)$$

where $V_{A:B}$ is the maximal rate of the exchange, [A] is the concentration of the external substrate and [B] is the concentration of the internal substrate. The factor $f_A([A])$ is a function of the external-substrate concentration which contains coef-

*Abbreviations.* Mal, malate; OG, 2-oxoglutarate; SEM, standard error of the mean; SD, standard deviation.

ficients characteristic of A only, so that $f_A([A])$ is the same whatever the internal substrate is. In the same way $g_B([B])$ depends on B only. $V_{A:B}$ depends on the nature of both A and B. Eqn (1) means that the external-substrate concentration and the internal-substrate concentration have independent effects on the initial rate. If only a small range of substrate concentrations is explored, $f_A([A])$ and $g_B([B])$ are approximately Michaelian-type functions [4–6]. Large concentration ranges have been investigated for the exchange external oxoglutarate:internal malate [7–9], showing that the kinetic-saturation curve of the translocator by the external oxoglutarate, and thus $f_{OG}([OG])$, presents intermediary-plateau regions, like a smoothed-stair function. In contrast the kinetic-saturation curve by internal malate deviates from a Michaelian function at low concentrations only [2, 8]. The factors $f_{OG}([OG])$ and $g_{Mal}([Mal])$ are so different that they suggest an important external/internal asymmetry of the translocator. However this suggestion would be reliable only if oxoglutarate and malate situated on the same side of the membrane behaved analogously.

In order to clarify this last point, we have examined the kinetic-saturation curve of the oxoglutarate translocator by external malate – its physiological substrate – in a large range of external-malate concentrations. The Michaelian behaviour of external-malate uptake prompted us to examine the influence of external malate on the uptake rate of external oxoglutarate and the influence of external oxoglutarate on the uptake rate of external malate. Finally, Eqn (1) and its implications at the structural level will be analysed.

Part of these results have been briefly presented elsewhere [10].

## MATERIAL AND METHODS

Special reagents, preparation of the mitochondria and experimental conditions have been described in [4–8]. The time-course of the malate uptake by malate-loaded mitochondria shows that the initial phase at 2 °C extends to 3 s but for external-malate concentrations higher than 250 μM, the incubation times should not exceed 1.8 s. In the presence of mersalyl and at 2 °C, the external-malate:internal-malate exchange is exclusively catalysed by the oxoglutarate translocator [1].

## MICHAELIAN-TYPE OF THE KINETIC SATURATION BY EXTERNAL MALATE

The initial rates of the L-malate:L-malate exchange have been measured for a fixed concentration (4 mM) of internal malate and 41 concentrations of external [U-$^{14}$C]malate between 0.05 and 840 μM. Results are presented in Fig. 1 in the form of a double-reciprocal plot. A straight line may be drawn through the points, showing that the external malate is a Michaelian substrate for the translocator. The apparent maximal rate, i.e. the factor $V_{A:B}g_B([B])$ in Eqn (1), is 543 ± 23 pmol · s$^{-1}$ · (mg protein)$^{-1}$ and the Michaelis constant is 210 ± 10 μM. These two figures are derived by weighted linear regression of the double-reciprocal plot.

The observed Michaelian behaviour of external malate provides four pieces of information:

a) The oxoglutarate translocator acts differently towards external malate and external oxoglutarate despite the molecular analogy of the two substrates; the question of the asymmetry of the translocator, suggested in the introduction, remains open;

b) all active binding sites of the unloaded translocator that binds external malate have the same affinity for malate;

c) the presence of external malate bound to the translocator does not modify the affinity of the unloaded sites for malate;

d) the catalytic rate constant of the malate loaded sites does not depend on the presence of malate on other sites.

These last two points mean that either the subunits that bind external malate are not interacting or that malate binding does not induce conformational changes that modify the subunit interactions.

## INFLUENCE OF EXTERNAL MALATE ON THE UPTAKE RATE OF OXOGLUTARATE

The contrast between the complexity of the external-oxoglutarate behaviour and the simplicity of the malate behaviour may suggest the existence of regulatory sites specific for oxoglutarate only. This point will be clarified as follows.

The first step is to establish an equation that may predict a plausible malate effect and can be tested experimentally. Such an equation may be derived if: (a) each subunit of the translocator can bind either one oxoglutarate or one malate ion, not both (oxoglutarate and malate are competitive), (b) the presence of external oxoglutarate bound to the translocator does not modify the affinity of the unloaded subunits for external malate, (c) the presence of external malate bound to the translocator does not modify the catalytic rate constant of the subunits loaded with external oxoglutarate.

The initial rate of oxoglutarate uptake in absence of external malate conforms to the equation:

$$v_{OG:B} = V_{OG:B} \times \frac{\sum_{i=1}^{i=n} \alpha_i [OG]^i}{\sum_{i=0}^{i=n} \beta_i [OG]^i} \times g_B([B]) \qquad (2)$$

where B is the internal substrate, $V_{OG:B}$ the maximal rate and $n$ the number of associated subunits; $\beta_i$ is the association equilibrium constant corresponding to $E + i\,OG_{out} \rightleftharpoons E(OG)_i$ where E represents the translocator. It may be seen that $\beta_0$ equals unity. $V_{OG:B}\alpha_i g_B([B])/i\beta_i E_0$ is the mean apparent catalytic rate constant of a subunit loaded with external oxoglutarate in the complexes that contain $i$ oxoglutarate. In Eqn (2) the maximal rate appears as a separate factor so that the $\alpha_i$ coefficients differ by a constant factor from those of Eqn (3) in [7]. $E_0$ is the amount of the translocator per mg of protein. If external malate is added and binds to the unloaded subunits (assumption a) with an unique association constant, $K_{Mal}$ (assumption b), and no modification of $\alpha_i$ (assumption c), the rate equation becomes:

$$v'_{OG:B} = V_{OG:B} \times \frac{\sum_{i=1}^{i=n} \alpha_i [OG]^i \sum_{j=0}^{j=n-i} C^j_{n-i} K^j_{Mal} [Mal]^j}{\sum_{i=0}^{i=n} \beta_i [OG]^i \sum_{j=0}^{j=n-i} C^j_{n-i} K^j_{Mal} [Mal]^j} \times g_B([B]) \qquad (3)$$

which simplifies into:

$$v'_{OG:B} = V_{OG:B} \times \frac{\sum_{i=1}^{i=n} \alpha_i \{[OG]/(1 + K_{Mal}[Mal])\}^i}{\sum_{i=0}^{i=n} \beta_i \{[OG]/(1 + K_{Mal}[Mal])\}^i} \times g_B([B]). \qquad (4)$$

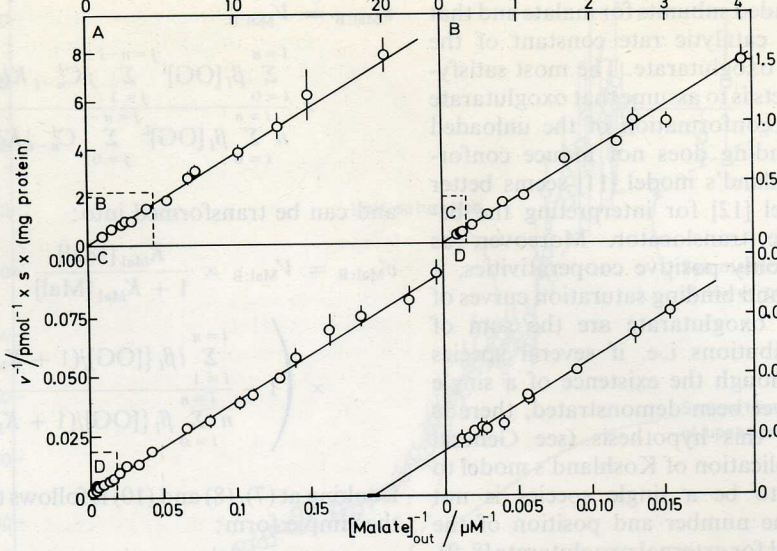


Fig. 1. *Reciprocal of the initial rate of external [$^{14}$C]malate uptake as a function of the reciprocal of its concentration.* The internal substrate is malate (4 mM). Each point is obtained by linear interpolation in the double-reciprocal plot of the rates obtained with three internal-malate concentrations around 4 mM. The standard deviation (vertical bar) is estimated by the mean of the standard deviations of the three values used to make the interpolation

Comparison of Eqns (2) and (4) shows that, for a given [B], the rate of oxoglutarate uptake in the presence of external malate, $v'_{OG:B}$, is equal to the rate of oxoglutarate uptake in the absence of external malate, $v_{OG:B}$, when oxoglutarate is at a lower concentration:

$$[OG]' = \frac{[OG]}{1 + K_{Mal}[Mal]}. \quad (5)$$

Eqn (4) can also be written:

$$v'_{OG:B}([OG]) = v_{OG:B}([OG]'). \quad (6)$$

The same reasoning applied to external-oxoglutarate binding leads to an analogous relation:

$$b'_{OG}([OG]) = b_{OG}([OG]')$$

$$= E_0 \frac{\sum_{i=1}^{i=n} i\beta_i \{[OG]/(1 + K_{Mal}[Mal])\}^i}{\sum_{i=0}^{i=n} \beta_i \{[OG]/(1 + K_{Mal}[Mal])\}^i} \quad (7)$$

where $b'_{OG}([OG])$ is the external-oxoglutarate binding in the presence of external malate and $b_{OG}([OG]')$ the external-oxoglutarate binding in the absence of external malate at a modified concentration [OG]′ related to [OG] by the same relation (5). The maximal binding value is $nE_0$.

*Results and Discussion*

$v'_{OG:B}$ has been measured for nine external-oxoglutarate concentrations between 0.32 and 308 µM and six external-malate concentrations between 0 and 1800 µM. Knowing [OG], [Mal] and $K_{Mal}$ ($K_{Mal}^{-1} = 210 \pm 10$ µM as shown above) [OG]′ is calculated according to Eqn (5) and $v_{OG:B}([OG]')$ is measured. In Fig. 2, $v_{OG:B}([OG]')$ is plotted as a function of the corresponding $v'_{OG:B}([OG])$. Linear regression gives a correlation coefficient equal to 0.996, a slope of $0.983 \pm 0.017$ and an intersection with the ordinate at $2.03 \pm 4.46$ pmol · s$^{-1}$ · (mg protein)$^{-1}$. As the slope is not significantly different from unity and the intersection with the ordinate not significantly different from zero, Eqn (6) is verified.

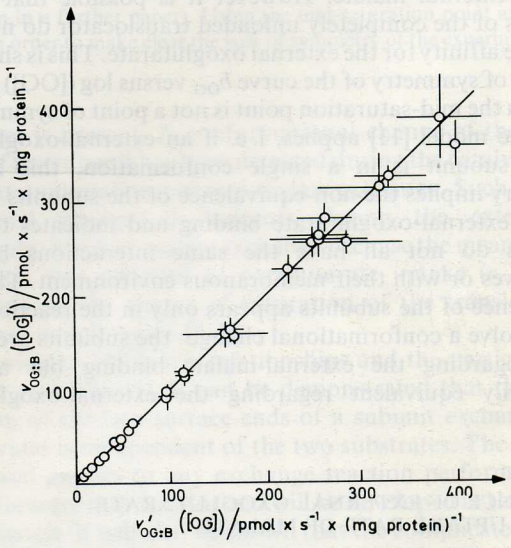


Fig. 2. *Verification of the relation (6).* $v_{OG:B}([OG]')$ is the initial rate of oxoglutarate uptake at a concentration equal to [OG]′ in the absence of external malate. $v'_{OG:B}([OG])$ is the initial rate of oxoglutarate uptake at a concentration equal to [OG] in the presence of external malate. Corresponding [OG]′ and [OG] are linked by the relation (5). [OG] is varied between 0.32 and 308 µM and the external-malate concentration is varied between 0 and 1800 µM. The internal substrate (B) is malate at a concentration of about 4 mM but the same for the two corresponding rates of oxoglutarate uptake

All the sites able to bind oxoglutarate are also able to bind malate; there are no regulatory sites specific to oxoglutarate.

It seems highly probable that each binding domain interacts with the two dicarboxylate groups common to all the substrates of the oxoglutarate translocator. However, the great difference in behaviour between oxoglutarate and malate suggests that other groups (particularly the cetonic group of oxoglutarate) may be involved.

The simple malate effect on the uptake rate of oxoglutarate implies that oxoglutarate bound to the translocator does not

modify the affinity of the unloaded subunits for malate and that malate does not modify the catalytic rate constant of the subunits loaded with external oxoglutarate. The most satisfying way to account for these facts is to assume that oxoglutarate binding does not modify the conformation of the unloaded subunits and that malate binding does not induce conformational changes. Thus Koshland's model [11] seems better adapted than Monod's model [12] for interpreting the behaviour of the oxoglutarate translocator. Moreover, as Monod's model can explain only positive cooperativities, it could apply only if the kinetic and binding saturation curves of the translocator by external oxoglutarate are the sum of independent sigmoidal contributions i.e. if several species translocate oxoglutarate. Although the existence of a single translocator species has not yet been demonstrated, there is some evidence in favour of this hypothesis (see General Discussion). However, the application of Koshland's model to the translocator considered to be a single species is not straightforward because of the number and position of the intermediary plateaux observed for external oxoglutarate [8, 9]. It is nevertheless with Koshland's model in mind, that the following work will be discussed for the sake of simplicity.

It is highly probable that the oxoglutarate translocator is made of identical subunits as indicated by their equal affinity for the external malate. However it is possible that all the subunits of the completely unloaded translocator do not have the same affinity for the external oxoglutarate. This is shown by the lack of symmetry of the curve $b_{OG}$ versus log ([OG]) (Fig. 3) in which the mid-saturation point is not a point of symmetry. If the basic model [11] applies, i.e. if an external-oxoglutarate loaded subunit is in a single conformation, this lack of symmetry implies the non-equivalence of the subunits regarding the external-oxoglutarate binding and indicates that the subunits do not all have the same interactions between themselves or with their membranous environment. The non-equivalence of the subunits appears only in the reaction steps that involve a conformational change: the subunits are equivalent regarding the external-malate binding but are not necessarily equivalent regarding the external-oxoglutarate binding.

## INFLUENCE OF EXTERNAL OXOGLUTARATE ON THE UPTAKE RATE OF MALATE

It has been seen in the previous section that external malate binding does not induce conformational changes of the translocator subunits. What happens in the next step, when the transport itself begins? Our working hypothesis will be that malate uptake is not accompanied by a conformational change so that a simple equation for the oxoglutarate effect can be established and tested experimentally. In this case the presence of external oxoglutarate does not modify the catalytic rate constant of the subunit loaded with external malate. Moreover, according to the probable identity of the subunits and to the supposed absence of conformational change during the catalytic step, all the subunits loaded with external malate and internal substrate will be considered to have the same catalytic rate constant: $V_{Mal:B}/nE_0$.

Be:

$$v_{Mal:B} = V_{Mal:B} \frac{K_{Mal}[Mal]}{1 + K_{Mal}[Mal]} g_B([B]) \quad (8)$$

the initial rate of malate uptake, in exchange with the internal substrate B, in absence of external oxoglutarate. In the presence of external oxoglutarate this equation becomes:

$$v'_{Mal:B} = V_{Mal:B} \times \frac{K_{Mal}[Mal]}{1 + K_{Mal}[Mal]} \times g_B([B]) \times \frac{\sum_{i=0}^{i=n} \beta_i [OG]^i \sum_{j=1}^{j=n-i} j C_{n-i}^j K_{Mal}^j [Mal]^j}{n \sum_{i=0}^{i=n} \beta_i [OG]^i \sum_{j=0}^{j=n-i} C_{n-i}^j K_{Mal}^j Mal]^j} \times g_B([B]) \quad (9)$$

and can be transformed into:

$$v'_{Mal:B} = V_{Mal:B} \times \frac{K_{Mal}[Mal]}{1 + K_{Mal}[Mal]} \times g_B([B]) \times \left( 1 - \frac{\sum_{i=1}^{i=n} i\beta_i \{[OG]/(1 + K_{Mal}[Mal])\}^i}{n \sum_{i=0}^{i=n} \beta_i \{[OG]/(1 + K_{Mal}[Mal])\}^i} \right). \quad (10)$$

Looking at (7), (8) and (10) it follows that (10) may be written in the simple form:

$$v'_{Mal:B} = v_{Mal:B} \left( 1 - \frac{b'_{OG}}{nE_0} \right); \quad (11)$$

$v'_{Mal:B}$, the malate uptake rate inhibited by external oxoglutarate (according to (11), $v'_{Mal:B}$ is always smaller than $v_{Mal:B}$), is equal to the uninhibited malate uptake rate, $v_{Mal:B}$, multiplied by the ratio of sites unoccupied by oxoglutarate, $(nE_0 - b'_{OG})/nE_0$.

*Results and Discussion*

In Fig. 4B, the ratio between the initial rate of malate uptake at a given external-malate concentration with external oxoglutarate ($v'_{Mal:B}$) and that obtained without external oxoglutarate ($v_{Mal:B}$) is plotted as a function of bound oxoglutarate in the presence of the same external-malate concentration ($b'_{OG}$). The external-malate concentration is 50 μM; 53 external-oxoglutarate concentrations range from 0 to 150 μM. For experimental ease, $b_{OG}$ ([OG]′) has been measured instead of $b'_{OG}$ ([OG]) [see Eqn (7)]. The rates $v'_{Mal:B}$ and the corresponding $v_{Mal:B}$ have been measured for the same mitochondrial preparation i.e. the same internal-malate concentration but it was not necessary to ensure the same internal-malate concentration from one mitochondrial preparation to another as the ratio $v'_{Mal:B} \times (v_{Mal:B})^{-1}$ does not depend on the concentration of the internal substrate B. The straight line drawn in Fig. 4 is the relation expected from (11) taken into the form:

$$\frac{v'_{Mal:B}}{v_{Mal:B}} = 1 - \frac{b'_{OG}}{nE_0}. \quad (12)$$

The intersection of this straight line with the abscissa is $nE_0 = 75.3 \pm 1.3$ (SEM) pmol × (mg protein)$^{-1}$. This maximal binding differs from the one published previously [8]; this will be discussed in a further paper.

Clearly, it appears that the simple relation (12) proposed above is not verified. The great majority of the experimental points are higher than their expected values. Moreover a slight stimulation [$v'_{Mal:B}(v_{Mal:B})^{-1} > 1$] of the malate uptake occurs for low $b'_{OG}$ while (12) predicts inhibition only.

Eqn (12) must thus be replaced by a more general one:

$$\frac{v'_{Mal:B}}{v_{Mal:B}} = \frac{\bar{k}'_{Mal:B}}{\bar{k}_{Mal:B}} \left( 1 - \frac{b'_{OG}}{nE_0} \right) \quad (13)$$

where

$$\bar{k}_{Mal:B} = V_{Mal:B}\, g_B([B])/nE_0 = \frac{v_{Mal:B}}{b_{Mal}}, \quad (14)$$

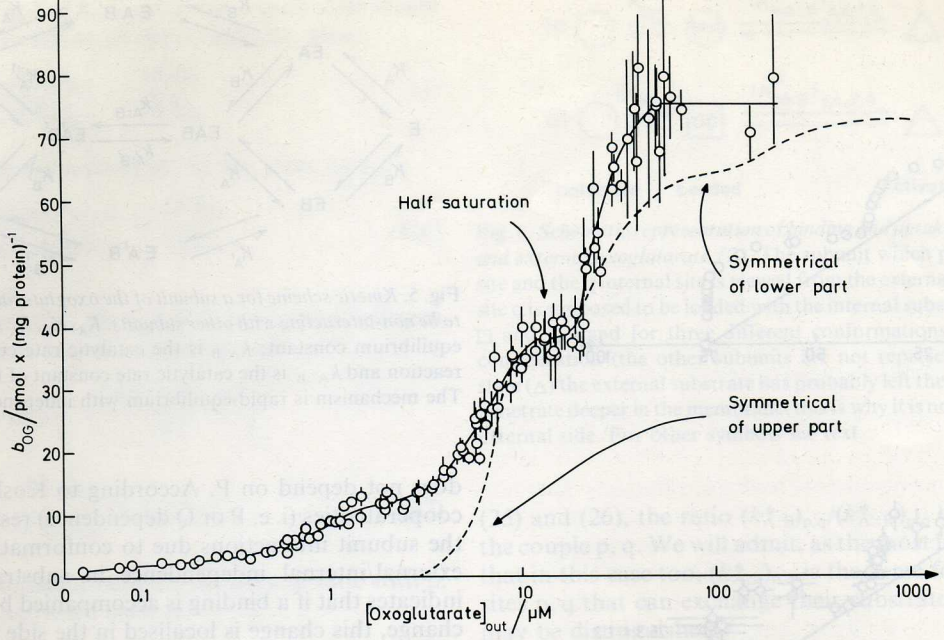


Fig. 3. *Bound external oxoglutarate as a function of the logarithm of the free external-oxoglutarate concentration.* Each point is the mean of five measurements made with the same mitochondrial preparation and is represented with its SEM (vertical bar). The method of measurement is described in [8]. The data of this figure are not those published in [8] (this will be commented on in a further paper). Using the mid-saturation point as a point of symmetry, the symmetrical of the lower part and of the upper part have been drawn in order to make clear the lack of symmetry of the experimental curve

i.e. the mean apparent catalytic rate constant of subunits loaded with external malate in the absence of external oxoglutarate. It does not depend on the external-malate concentration; $b_{Mal}$ is the bound external malate in the absence of external oxoglutarate.

$$\bar{k}'_{Mal:B} = \frac{v'_{Mal:B}}{b'_{Mal:B}} = \frac{v'_{Mal:B}}{b_{Mal}\left(1 - \frac{b'_{OG}}{nE_0}\right)} \qquad (15)$$

is the mean catalytic rate constant of subunits loaded with external malate in the presence of external oxoglutarate. It depends on both the external-malate and the external-oxoglutarate concentrations. Fig. 4B shows that

$$\frac{v'_{Mal:B}}{v_{Mal:B}} \geq 1 - \frac{b'_{OG}}{nE_0} \qquad (16)$$

so that

$$\bar{k}'_{Mal:B} \geq \bar{k}_{Mal:B}. \qquad (17)$$

The external oxoglutarate increases the mean apparent catalytic rate constant of malate uptake. This effect indicates that malate uptake is accompanied by a conformational change, contrary to our working hypothesis.

## GENERAL DISCUSSION

A translocator subunit may be seen as a channel that has a binding site accessible to the external substrate and a binding site accessible to the internal substrate. When the exchange reaction occurs the two substrates must leave the sites situated near the surfaces of the membrane to penetrate into the channel. This transfer is accompanied by a conformational change that modifies the subunit interactions and leads to cooperative phenomena at the level of the catalytic rate constant when a ligand whose binding induces conformational changes is present. A conformational change of the external side of the subunit has been detected during the catalytic step of the external-malate:internal-malate exchange. Such a conformational change also occurs during the external-oxoglutarate:internal-malate exchange because the mean apparent catalytic rate constant of oxoglutarate uptake ($v_{OG:Mal}/b_{OG}$) depends on the degree of saturation of the translocator by external oxoglutarate [8, 9].

Owing to Haldane's relationships and the particular form of the rate Eqn (1), it can be demonstrated that the conformation of the two surface ends of a subunit exchanging two substrates is independent of the two substrates. The reasoning followed applies to any exchange reaction performed by the oxoglutarate translocator and will be developed in this General Discussion. It will also be shown that the complicated effect of the external oxoglutarate on the malate uptake (Fig. 4B) was in fact predictable and can be characterized by a simple equation. Two possible structures of the translocator will be proposed; they are suggested by the theoretical analysis and allow a straightforward explanation of the conclusions reached. Finally, the possibility of the presence of an isozymic mixture of oxoglutarate translocators in our mitochondrial preparations will be briefly discussed.

### Part 1. *Haldane's Relationships and the Particular Form of the Rate Equation*

The net rate of exchange between an external substrate A and an internal substrate B when the external product A′ and the internal product B′ are present is equal to the difference between the two opposed fluxes. As the net rate is zero at equilibrium, relations exist between kinetic coefficients of the two unidirectional rates and the overall equilibrium constant. These relations are known as Haldane's relationships. This will be shown first in a simplified case where the oxoglutarate translocator is not oligomeric but made of a single subunit with

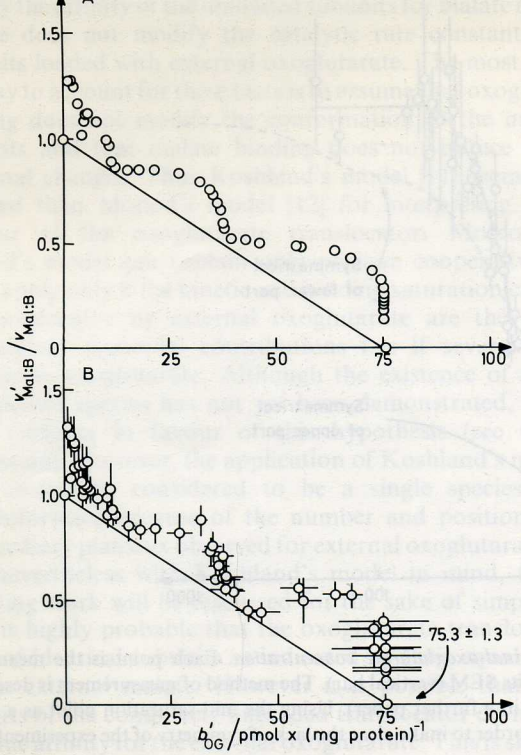


Fig. 4. *Ratio between the initial rate of malate uptake in the presence of external oxoglutarate* ($v'_{Mal:B}$) *and the initial rate of malate uptake in the absence of external oxoglutarate* ($v_{Mal:B}$) *as a function of the bound external-oxoglutarate in the presence of external malate* ($b'_{OG}$). Bound oxoglutarate is measured as described in [8]. The external-malate concentration is 50 μM and the external-oxoglutarate concentration is varied between 0 and 150 μM. The internal substrate (B) is malate. The straight line has been calculated according to the relation (12). A: Calculated ratios according to equation (40). B: Experimental data

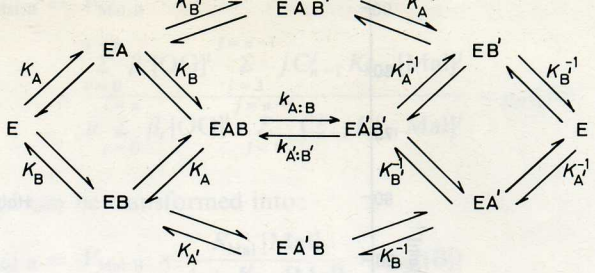


Fig. 5. *Kinetic scheme for a subunit of the oxoglutarate translocator supposed to be non-interacting with other subunits.* $K_A$, $K_B$, $K_{A'}$ and $K_{B'}$ are association equilibrium constant; $k_{A:B}$ is the catalytic rate constant of the forward reaction and $k_{A':B'}$ is the catalytic rate constant of the backward reaction. The mechanism is rapid-equilibrium with independent binding [4–9]

an internal and an external sites. In accordance with the kinetic scheme (rapid-equilibrium random bi-bi mechanism with independent binding [4–9]) of Fig. 5 one has:

$$v = E_0 \frac{k_{A:B}K_AK_B[A][B] - k_{A':B'}K_{A'}K_{B'}[A'][B']}{(1 + K_A[A] + K_{A'}[A'])(1 + K_B[B] + K_{B'}[B'])} . \quad (18)$$

At equilibrium:

$$\frac{[A']_e[B']_e}{[A]_e[B]_e} = K_e = 1 \quad (19)$$

so that,

$$k_{A:B}K_AK_B = k_{A':B'}K_{A'}K_{B'} . \quad (20)$$

This Haldane relation (20) may be generalized to the oligomeric translocator case and this gives a set of relations:

$$(k_{A:B})_{p,q,P,Q}(K_A)_{p,P}(K_B)_{q,Q} = (k_{A':B'})_{p,q,P,Q}(K_{A'})_{p,P}(K_{B'})_{q,Q} \quad (21)$$

where p and q designate the external loaded site and the internal loaded site under consideration. The occupancy of the other sites is designated by P (for the external sites) and Q (for the internal sites). Here P and Q do not have exactly the same meaning as in [7] where they represented the occupancy of all sites including the sites p and q. Moreover the situation analysed in [7] was that in which the products A′ and B′ are absent. P and Q being given, the external site p has affinities $(K_A)_{p,P}$ for A and $(K_{A'})_{p,P}$ for A′ which does not depend on Q, and the site q has affinities $(K_B)_{q,Q}$ for B and $(K_{B'})_{q,Q}$ for B′ that does not depend on P. According to Koshland's model [11], cooperativities (i. e. P or Q dependence) result from changes in the subunit interactions due to conformational changes. The external/internal independence in substrate binding [4–9] indicates that if a binding is accompanied by a conformational change, this change is localised in the side of the translocator where the substrate binds. When p is loaded with A and q is loaded with B, they exchange their substrates with the rate constant $(k_{A:B})_{p,q,P,Q}$. The same couple of sites exchange A′ and B′ with the rate constant $(k_{A':B'})_{p,q,P,Q}$. These rate constants are zero if the sites p and q cannot exchange their substrates.

As already shown [7], the particular form of the initial-rate Eqn (1) imposes a particular form to each catalytic rate constant:

$$(k_{A:B})_{p,q,P,Q} = (k^*_{A:B})_{p,q}(\chi_A)_{p,P}(\chi_B)_{q,Q} . \quad (22)$$

This is a particular form of a more general relation:

$$(k_{A:B})_{p,q,P,Q} = (k^{\ddagger}_{A:B})_{p,q} \exp\left(-\frac{(\Delta G^{\ddagger}_{A:B})_{p,q,P,Q}}{RT}\right) \quad (23)$$

where $(k^{\ddagger}_{A:B})_{p,q}$ is the rate constant of the activited state, and $(\Delta G^{\ddagger}_{A:B})_{p,q,P,Q}$ is the free energy of activation. The factor $(k^*_{A:B})_{p,q}$ in (22) contains the factor $(k^{\ddagger}_{A:B})_{p,q}$ of (23) and that part of exp $[-(\Delta G^{\ddagger}_{A:B})_{p,q,P,Q}/RT]$ which depends of both A and B in a non-dissociable way. This last factor cannot depend on the occupancy (P and Q) of the other sites. The factor $(\chi_A)_{p,P}$ is the part of exp $[-(\Delta G^{\ddagger}_{A:B})_{p,q,P,Q}/RT]$ which depends of the external substrate only. It may depend on p and P but not on q and Q. The corresponding factor for the internal substrate is $(\chi_B)_{q,Q}$. Eqn (22) indicates that the activation step may be accompanied by independent conformational changes of the external side (leading to a P dependence) and of the internal side (leading to a Q dependence). Combination of Eqns (21) and (22) gives:

$$1 = \frac{(k^*_{A:B})_{p,q}}{(k^*_{A':B'})_{p,q}} \times \frac{(\chi_A)_{p,P}(K_A)_{p,P}}{(\chi_{A'})_{p,P}(K_{A'})_{p,P}} \times \frac{(\chi_B)_{q,Q}(K_B)_{q,Q}}{(\chi_{B'})_{q,Q}(K_{B'})_{q,Q}} . \quad (24)$$

As P may be different for a given Q it may be seen that the ratio

$$\frac{(\chi_A)_{p,P}(K_A)_{p,P}}{(\chi_{A'})_{p,P}(K_{A'})_{p,P}}$$

does not depend on P, i. e. it is not influenced by the changes of interactions between the external part of the p subunit and the external parts of the other subunits during the binding and the activation steps of p. Let $(K^*_A)_p$ and $(K^*_{A'})_p$ be the intrinsic values

of $(\chi_A)_{p,P}(K_A)_{p,P}$ and $(\chi_{A'})_{p,P}(K_{A'})_{p,P}$, i.e. the values they would have in absence of interaction changes, one can write:

$$\frac{(\chi_A)_{p,P}(K_A)_{p,P}}{(\chi_{A'})_{p,P}(K_{A'})_{p,P}} = \frac{(K_A^*)_p}{(K_A^*)_p} \tag{25}$$

for any couple of external substrates. In the same way,

$$\frac{(\chi_B)_{q,Q}(K_B)_{q,Q}}{(\chi_{B'})_{q,Q}(K_{B'})_{q,Q}} = \frac{(K_B^*)_q}{(K_B^*)_q} \tag{26}$$

whatever B and B′ are.

From Eqns (22) and (23), the equilibrium constant between the normal loaded state and the activated state is:

$$(K^{\ddagger}_{A:B})_{p,q,P,Q} = \exp\left(-\frac{(\Delta G^{\ddagger}_{A:B})_{p,q,P,Q}}{RT}\right)$$

$$= \frac{(k^*_{A:B})_{p,q}}{(k^{\ddagger}_{A:B})_{p,q}}(\chi_A)_{p,P}(\chi_B)_{q,Q}\,. \tag{27}$$

The equilibrium constant between the normal state with p, q unloaded and the activated state is:

$$(K^{\varnothing}_{A:B})_{p,q,P,Q} = (K^{\ddagger}_{A:B})_{p,q,P,Q}(K_A)_{p,P}(K_B)_{q,Q} \tag{28}$$

or

$$(K^{\varnothing}_{A:B})_{p,q,P,Q} = \frac{(k^*_{A:B})_{p,q}}{(k^{\ddagger}_{A:B})_{p,q}}(\chi_A)_{p,P}(K_A)_{p,P}(\chi_B)_{q,Q}(K_B)_{q,Q}\,. \tag{29}$$

Applying Eqn (29) to any two couples of substrates $A_1:B_1$ and $A_2:B_2$ and taking into account Eqns (25) and (26) one may derive that:

$$\frac{(K^{\varnothing}_{A_1:B_1})_{p,q,P,Q}}{(K^{\varnothing}_{A_2:B_2})_{p,q,P,Q}} = \frac{(k^*_{A_1:B_1})_{p,q}(k^{\ddagger}_{A_2:B_2})_{p,q}(K^*_{A_1})_p(K^*_{B_1})_q}{(k^{\ddagger}_{A_1:B_1})_{p,q}(k^*_{A_2:B_2})_{p,q}(K^*_{A_2})_p(K^*_{B_2})_q} \tag{30}$$

so this ratio does not depend on P and Q. This means that an activated subunit has the same conformation of its two surface ends whatever the couple of substrates with which it is loaded. This suggests — and this is common sense — that in the activated state the two substrates that are exchanging are no more bound to the surface sites. It is also probable that the conformation of the two surface sides of the subunit changes during a first step in which the two substrates leave the surface sites to penetrate deeper in the subunit (the two-substrate transfer step) and that the next steps occuring in the middle of the subunit are not accompanied by conformational changes of the two surface sides of the subunit. The two-substrate transfer step is in rapid-equilibrium and has a very small equilibrium constant.

A schematical representation of the external side of a subunit is shown in Fig. 6 in the case where $A_1$ is the external malate and $A_2$ the external oxoglutarate.

The form of Eqn (1) imposes also that all factors that depend of both A and B must be contained in the $V_{A:B}$ factor (this aspect of the problem has not been stressed in [7]). It means either that $(k^*_{A:B})_{p,q}$ is the same for all the couples of sites p, q that can exchange their substrates or that the $V_{A:B}$ factor contains the $\sum_{p,q}(k^*_{A:B})_{p,q}$. As shown in [7] the second possibility occurs if all the external sites are equivalent (in [7] equivalent sites were called identical sites), and if all the internal sites are equivalent. This implies that $(K_A^*)_p$ does not depend on p and that $(K_B^*)_q$ does not depend on q. Then, according to Eqns (24),

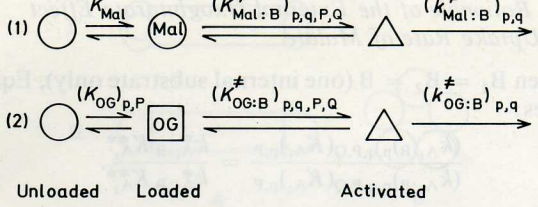


Fig. 6. *Schematic representation of binding and uptake of external malate (1) and external oxoglutarate (2)*. The subunit which possesses the p external site and the q internal site is viewed from the external medium. The internal site q is supposed to be loaded with the internal substrate B. The symbols ○, □ and △ stand for three different conformations of the subunit under consideration (the other subunits are not represented). In the activated state (△) the external substrate has probably left the external binding site to penetrate deeper in the membrane, this is why it is no longer visible from the external side. For other symbols see text

(25) and (26), the ratio $(k^*_{A:B})_{p,q}/(k^*_{A':B'})_{p,q}$ does not depend on the couple p, q. We will admit, as the most likely interpretation, that in this case too, $(k^*_{A:B})_{p,q}$ is the same for all the couples of sites p, q that can exchange their substrates. Two possibilities may be distinguished.

*First Possibility*. Any loaded external site can exchange its substrate with any loaded internal site so that $(k^*_{A:B})_{p,q}$ has the same value, $k^*_{A:B}$, whatever p and q (this first possibility has not been considered in [7]). An interpretation of this situation will be proposed in part 3 of this General Discussion (Model I). With this assumption Haldane's relationships become:

$$k^*_{A:B}(K^*_A)_p(K^*_B)_q = k^*_{A':B'}(K^*_{A'})_p(K^*_{B'})_q\,. \tag{31}$$

As p and q can be any external and internal sites, it follows that $(K_A^*)_p/(K_{A'}^*)_p$ does not depend on p and that $(K_B^*)_q/(K_{B'}^*)_q$ does not depend on q. This may be written

$$(K_A^*)_p/(K_{A'}^*)_p = K_A^{**}/K_{A'}^{**} \tag{32}$$

and

$$(K_B^*)_q/(K_{B'}^*)_q = K_B^{**}/K_{B'}^{**} \tag{33}$$

*Second Possibility*. A given loaded external site can exchange its substrate with only one loaded internal site. These two corresponding sites will receive the same label so that $(k^*_{A:B})_{p,q}$ is either equal to zero (if $p \neq q$) or to $k^*_{A:B}$ (if $p = q$). This corresponds to Model II proposed in part 3 of this General Discussion. In this case one has:

$$k^*_{A:B}(K^*_A)_p(K^*_B)_p = k^*_{A':B'}(K^*_{A'})_p(K^*_B)_p\,. \tag{34}$$

It has been shown in [7] that if $(k^*_{A:B})_{p,q}$ depends on the couple p, q, as is the case here, all the subunits must be equivalent for the substrates on one side of the membrane at least. It follows that at least one of the relations (32) and (33) is satisfied. Eqn (34) shows then that both Eqns (32) and (33) must be satisfied.

*In Both Cases*. The translocator has only one value of $(k^*_{A:B})_{p,q} \neq 0$ for a given A:B couple, one value of the ratio $(K_A^*)_p/(K_{A'}^*)_p$ and one value of $(K_B^*)_q/(K_{B'}^*)_q$. At the molecular level, it means that all the subunits of the oxoglutarate translocator are identical in their central part. It may be deduced that:

$$\frac{(k_{A_1:B_1})_{p,q,P,Q}(K_{A_1})_{p,P}(K_{B_1})_{q,Q}}{(k_{A_2:B_2})_{p,q,P,Q}(K_{A_2})_{p,P}(K_{B_2})_{q,Q}} = \frac{k^*_{A_1:B_1}K^{**}_{A_1}K^{**}_{B_1}}{k^*_{A_2:B_2}K^{**}_{A_2}K^{**}_{B_2}}\,. \tag{35}$$

This relation (35) will allow to rationalize the experimental results relative to the external-oxoglutarate effect on the malate-uptake rate.

Part 2. *Rationale of the External-Oxoglutarate Effect on the Uptake Rate of Malate*

When $B_1 = B_2 = B$ (one internal substrate only), Eqn (35) becomes:

$$\frac{(k_{A_1:B})_{p,q,P,Q}(K_{A_1})_{p,P}}{(k_{A_2:B})_{p,q,P,Q}(K_{A_2})_{p,P}} = \frac{k^*_{A_1:B}\,K^{**}_{A_1}}{k^*_{A_2:B}\,K^{**}_{A_2}}. \quad (36)$$

The initial rate of the exchange between $A_1$ and B is given by:

$$v_{A_1:B} = \sum_P \sum_Q \sum_p \sum_q (k_{A_1:B})_{p,q,P,Q}(K_{A_1})_{p,P}(K_B)_{q,Q}[C_{P,Q}]\,[A_1]\,[B] \quad (37)$$

and the initial rate of the $A_2$:B exchange is:

$$v_{A_2:B} = \sum_P \sum_Q \sum_p \sum_q (k_{A_2:B})_{p,q,P,Q}(K_{A_2})_{p,P}(K_B)_{q,Q}[C_{P,Q}]\,[A_2]\,[B] \quad (38)$$

where $[C_{P,Q}]$ is the concentration of a complex that has the p and q sites unloaded and is characterized by a P occupancy of the other external sites and a Q occupancy of the other internal sites. The same $[C_{P,Q}]$ will be involved in (37) and (38) if $A_1$ and $A_2$ are simultaneously present in the external medium. As previously $v'$ is the uptake rate in the presence of both external substrates. Then, examination of Eqns (36), (37) and (38) shows that:

$$\frac{v'_{A_1:B}}{v'_{A_2:B}} = \frac{k^*_{A_1:B}\,K^{**}_{A_1}[A_1]}{k^*_{A_2:B}\,K^{**}_{A_2}[A_2]}. \quad (39)$$

When two external substrates are present the ratio of their initial rates of uptake in exchange with a same internal substrate is proportional to the ratio of their concentrations. This equation will be further modified in order to use the measured ratio $v'_{Mal:B}/v_{Mal:B}$

Taking Eqns (8), (39) and (5) into account one has:

$$\frac{v'_{Mal:B}}{v_{Mal:B}} = \frac{k^*_{Mal:B}\,K^{**}_{Mal}}{k^*_{OG:B}\,K^{**}_{OG}\,V_{Mal:B}K_{Mal}\,g_B([B])}\,\frac{v'_{OG:B}}{[OG]'} \quad (40)$$

[B] being fixed, the ratio between the uptake rate of malate with external oxoglutarate ($v'_{Mal:B}$) and without external oxoglutarate ($v_{Mal:B}$) varies as the ratio between the uptake rate of oxoglutarate in the presence of external malate ($v'_{OG:B}$) and the modified external-oxoglutarate concentration ([OG]′) that can be calculated according to (5).

Fig. 7 illustrates this relation with the experimental data obtained when B is the internal malate at a concentration of 4 mM. A straight line has been drawn passing through the origin and with a slope equal to the mean value of the $y/x$ ratio that is $0.045 \pm 0.001\ \mu l^{-1} \cdot s \cdot mg$ protein. As this value must be equal to $(\lim_{[OG]'\to 0} v'_{OG:B}/[OG]')^{-1}$, i.e. $(V_{OG:B} \times \alpha_1 \times g_B([B]))^{-1}$, it can be determined, in principle at least, from the kinetic data of the external-oxoglutarate uptake as only external substrate. The straight line satisfactorily accounts for the experimental results. Using its slope and the values of $v'_{OG:B}/[OG]'$, the ratios $v'_{Mal:B}/v_{Mal:B}$ have been calculated and plotted as a function of $b'_{OG}$ in Fig. 4A also showing good agreement with experimental results.

The calculated curve and the straight line of Fig. 4A seem to rejoin each other at three intermediate values of oxoglutarate binding. These three binding values are those of the end of the three intermediary plateaux of the external-oxoglutarate binding curve. This surprising feature as well as the exact causes of the structures in the oxoglutarate-saturation curves are still unexplained.

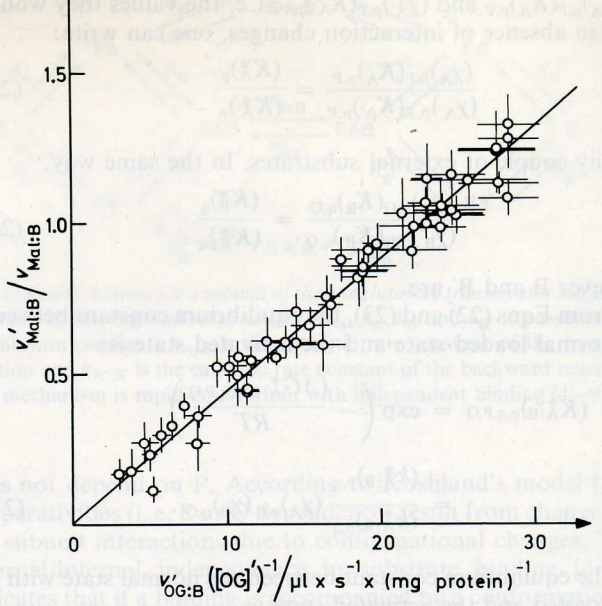


Fig. 7. *Verification of the relation (40)*. Ratio between the initial rate of malate uptake in the presence of external oxoglutarate ($v'_{Mal:B}$) and the initial rate of malate uptake in the absence of external oxoglutarate ($v_{Mal:B}$) as a function of the initial rate of oxoglutarate uptake in the presence of external malate ($v'_{OG:B}$) divided by [OG]′ calculated according to Eqn (5). The internal substrate is malate. Its concentration is 4 mM when $v'_{OG:B}$ is measured. The straight line drawn through the points passes through the origin with a slope equal to the mean $y/x$ ratio ($0.045 \pm 0.001\ \mu l^{-1} \cdot s \cdot mg$ prot). The $y$ values are the same as in Fig. 4B

From the experiments described in the first section of this paper it is known that $V_{Mal:B}K_{Mal}g_B([B]) = 543/210\ \mu l \cdot s^{-1} \cdot (mg\ protein)^{-1}$ so that the proportionality factor of Eqn (39) can be calculated:

$$\frac{k^*_{Mal:B}\,K^{**}_{Mal}}{k^*_{OG:B}\,K^{**}_{OG}} = 0.116 \pm 0.003.$$

This ratio is determined when B is the internal malate and does not necessarily have the same value with another internal substrate. The obtained figure means that each subunit of the oxoglutarate translocator is about 8.6 times more efficient in exchanging oxoglutarate with malate (whatever the direction of the exchange) than in exchanging malate with malate.

Part 3. *Structure of the Oxoglutarate Translocator*

The results obtained so far seem to indicate that the conformational changes of the oxoglutarate translocator giving rise to cooperativities, either at the level of the binding constant or at the level of the catalytic rate constant, are those of the two surface ends of the translocator. The substrate binding does not modify the central part but can modify the surface ends, contrary to the exchange step occuring in the central part of the subunit; the latter can modify the central part but not the two ends which are then in a conformation which does not depend on the two exchanged substrates. This strongly suggests that a functional subunit of the oxoglutarate translocator is composed of different specialized parts: binding and catalytic subunits.

*Binding subunits* are located near the surfaces of the membrane. Each of them has a binding site directly accessible

to the substrates in solution. The conformation of the loaded subunit depends on the substrate to which they are bound and may depend on the presence of effectors. This explains the cooperative phenomena and may play a rôle in regulation. The loaded subunits can transfer their substrates to a catalytic subunit in a step which is the first of the catalytic process itself; this transfer is accompanied by a conformational change. The conformation of a binding subunit which has transferred its substrate to a catalytic subunit is independent of the substrate that has 'passed through it'.

*Catalytic subunits* are situated deeper in the membrane. They receive an external substrate from an external binding subunit and an internal substrate from an internal binding subunit. It is through a catalytic subunit that the two substrates are exchanged. The possible conformational change of a catalytic subunit during the transfer step and the next steps of the exchange are not perceived in our experiments. Information on the events that occur at the level of a catalytic subunit is not yet available. This subunit may itself be composite and have mobile parts. We will however designate it as a channel. It is interesting to note that a catalytic subunit need not be equipped with a special device to ensure a one-to-one exchange: it only has to allow migration of the ions it contains and let an ion exit on one side only if it has received an ion coming from this side. Such a mechanism is plausible and only requires that a binding subunit that has transferred its substrate to a catalytic subunit is in a conformation such that its site is accessible only from the catalytic subunit whereas, before the transfer step, a binding subunit is in a different conformation in which it can only bind the substrates present in the aqueous solution. Two models can be proposed.

*Model I: the Single-Channel Model* (Fig. 8.I). The translocator is composed of a central catalytic subunit the ends of which are surrounded by several binding subunits. This model corresponds to the first possibility considered in part 1 of this General Discussion.

*Model II: the Multi-Channel Model* (Fig. 8.II). The translocator is the association of several groups of subunits, each group containing a catalytic subunit, an external binding subunit and an internal binding subunit. Each group of three subunits forms a functional subunit of the translocator (Fig. 8.II.A). This model corresponds to the second possibility considered in part 1 of this General Discussion. The theoretical analysis leads to the conclusions that: (a) all the catalytic subunits are identical as indicated by their identical $k^*_{A:B}$ value and by the relations (32) and (39); (b) on one side of the membrane at least, the binding subunits are equivalent [7]. Their arrangement is thus probably annular as shown in Fig. 8.II.B.

In both models, external binding subunits may differ from the internal binding subunits but both probably operate according to a similar mechanism. The number of binding subunits may differ inside and outside in the first model but must be the same in the second model. It has been observed in this paper that all the external binding subunits are equivalent for malate binding and this suggests that the external binding subunits are identical. The schematic representation of a catalytic subunit in Fig. 8 and the applied term, channel, does not portend what its actual structure and working could be.

It has already been proposed from completely different observations on various translocases (see [13] for a review) that a membranous carrier could be composed of peripheral receptor proteins (the binding subunits) and of integral proteins (the catalytic subunits). Whereas the catalytic subunits are necessarily integral proteins, the binding subunits of the oxoglutarate translocator may be either peripheral or partly embedded in the membrane.

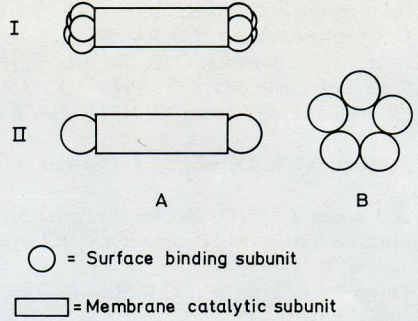


Fig. 8. *Two possible structures for the oxoglutarate translocator of rat-heart mitochondria.* I. The oxoglutarate-translocator according to the single-channel model. II.A. A functional subunit of the oxoglutarate translocator according to the multi-channel model. II.B. Probable arrangement in the plane of the membrane

Part 4. *Is the Oxoglutarate Translocator a Single Species?*

If the oxoglutarate translocator is a single species the number of associated external binding subunits (in Model I as well as in Model II) must be high (18 at least) in order to account for the binding saturation curve of the external oxoglutarate [8, 9]. Moreover it is difficult to develop a simple model of subunit interaction that could account for the observed structures. It is why the possibility of enzyme heterogeneity has been considered [7–9]. It would explain the lack of symmetry of the curve in Fig. 3. However the different kinds of oxoglutarate translocators considered to coexist have many characteristics in common. Indeed if these different species are distinguished by the external oxoglutarate they must fulfil the following conditions: (a) they must have the same $g_B([B])$ function; this is required by the fact that the measured rate of oxoglutarate uptake must obey an equation of type (1) even if it is the sum of independent contributions; (b) the affinity of their external binding subunits must be the same for external malate; (c) Eqn (1) requires identical $k^*_{A:B}$; moreover, Eqn (37) requires the same $k^*_{Mal:B} K^{**}_{Mal}/k^*_{OG:B} K^{**}_{OG}$ ratio.

These three conditions seem to indicate that all the hypothetical species have the same binding subunits and the same catalytic subunits and this drastically limits the possibilities of heterogeneity. It cannot be ruled out that species could coexist which differ in the way their subunits are associated, in the number of associated subunits or in their membranous environment. In any case, the first of these three conditions remains a particularly worrying constraint. In the absence of any direct evidence, we prefer the simple view well supported by our results, that the mitochondrial preparation contains a single species of oxoglutarate translocator and not an isozymic mixture.

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