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Kinetics and Mechanism of the Exchange Reactions Catalysed by the Oxoglutarate Translocator of Rat-Heart Mitochondria.

Francis E. Sluse and Claude Liébecq.

Laboratoire de Biochimie et de Physiologie Générale,
Institut Supérieur d'Education Physique de l'Université de Liège,
1, rue des Bonnes-Villes, B-4000 Liège, Belgique.

Summary — The kinetics of the exchange reactions between 2-oxoglutarate, malate, malonate and succinate have been measured at 4°C in preparations of rat-heart mitochondria under conditions where the oxoglutarate translocator is operating exclusively. Measurements of initial rates were made at three different external and three different internal concentrations of the substrates. Double-reciprocal plots show linear relationships between v^{-1}, on the one hand, and $[S_{int}]^{-1}$ or $[S_{ext}]^{-1}$, on the other hand, with common intercept on the axis of abscissae. Therefore the Michaelis constants equals K' in all cases. The results also show that the Michaelis constants are very much higher for external substrates than for the corresponding internal substrates and that the Michaelis constant for a given substrate, either external or internal, is independent of the nature of the counter-ion.

The kinetics of the exchange reactions between internal malate and external 2-oxoglutarate or malonate have been measured at 4°C in the presence of external malate (external-product inhibitor). Measurements of initial rates were made at three concentrations of the internal substrate, of the external substrate and of the external product. The external product inhibits the exchange in a competitive way when external substrate is varied and in a non-competitive way when internal substrate is varied.

These results are in perfect agreement with only the so-called mechanism of rapid-equilibrium random bi-bi, and with a mixed dead-end and product inhibition.

It can be concluded that the translocator should possess two independent binding sites for substrates, one internal and one external, and that the transport of anions should be achieved by a simultaneous displacement of the two substrates, which would be the limiting step of the exchange reaction.

INTRODUCTION.

The inner mitochondrial membrane is impermeable to certain substances which are the substrates and the products of the intra- and extramitochondrial metabolisms. The coupling between these metabolic processes is possible because of the existence of transport systems which permit these substances to travel across the internal membrane at a high-enough rate (table I).

This coupling is determined by the properties of the translocators and the kinetic characteristics of the translocations they take part in. Extensive quantitative data could allow to elucidate the catalytic mechanism of these translocators and to understand their precise physiological rôle.

The kinetic study of a reaction is complete only if it permits to determine the mechanism of this reaction as well as the rate constants of its elementary steps.

In an enzymatic reaction it is sometimes difficult directly to study the formation and the disappearance of the intermediate enzyme-substrate complexes. The overall reaction only may then be studied by measuring the rate of disappearance of the substrate or by measuring the rate of appearance of the product when the reaction is in a steady-state, that is when the rate of disappearance of the substrate is equal to the rate of appearance of the product.

This study leads to the determination of the equation which relates the rate of the reaction to the concentration of the substrates, the products and the enzyme. This equation includes the kinetic constants which are functions of the rate constants of the elementary steps of the reaction.

Moreover, the rate equation and the interpretation of the kinetic constants depend upon the mechanism of the reaction. For every imaginable mechanisms, one can mathematically derive the rate equation and compare it with the experimental relation. This comparison permits, in general, to eliminate a certain number of mechanisms and sometimes allows to choose the mechanism which best applies to the reaction under study.

INITIAL-RATE MEASUREMENTS.

The transport of anions across the membrane, which results in an exchange between two anions, can be treated formally as an enzymatic reaction involving two substrates and two products, the translocator playing the rôle of the enzyme, and the substrates, which have travelled across the membrane, playing the rôle of the products of the reaction.

$$E + A^*_{ext} + B_{int} \leftrightarrows E + A^*_{int} + B_{ext} \quad (1)$$

In our experiments we measured the initial phase of the appearance of the external ^{14}C-labelled anion (A^*_{ext}) in the mitochondria, that is, ideally speaking, the forward reaction in absence of the products of the reaction. This simplification reduces the number of terms involved in the kinetic equation describing the exchange.

We undertook the kinetic study of the oxoglutarate translocator in the rat-heart mitochondria, by measuring the initial rates at 4°C of the twelve forward exchanges listed in table II, at different internal and external-anion concentrations. The list includes three homologous exchanges between two identical anions.

These twelve exchanges do not involve the oxoglutarate translocator exclusively. Some of these can be brought about by the dicarboxylic and/or by the tricarboxylic translocator. There may thus be a competition between translocators for the same substrates.

A rigorous analysis requires that only one translocator takes part in the exchanges studied. We were fortunate to have one such situation because the available results had showed that the tricarboxylic translocator was inactive in our experimental conditions and that the dicarboxylic translocator could be blocked by mersalyl at a concentration which did not affect the oxoglutarate translocator.

The internal anion was accumulated in the mitochondrial matrix by a long preincubation at 0°C in the presence of a high concentration of this anion. We obtained a concentration scale of the internal anion by washing the loaded mitochondria one to three times in large volumes of the ice-cold suspension medium, that is a medium which does not contain any exchangeable anions. The accumulated anion diffused very slowly out of the mitochondria.

The external radioactive anion of which we measure the uptake at 4°C was added to the incubation medium at a given time and it exchanged itself with the internal anion. After intervals of five to twenty seconds the exchange was stopped by rapid centrifugation. The radioactivity found in the mitochondrial pellet was used to plot graphs showing the appearance of the radioactive anion in the mitochondria as a function of time, from which the rate of exchange can be calculated.

In order that the measured rate be the initial rate of the forward exchange it is necessary that the concentration of the internal and external anions vary as least as possible during the measurement. For that it is necessary that the exchange remains small by a short incubation in the presence of few mitochondria.

It is also necessary that, at the beginning of the measurement, there should be no internal anion on the outside and no external anion on the inside, that is to say that it is important that there are no products of the exchange to avoid

TABLE I.

Anion translocators of mitochondria.

Translocator	Substrates
Adenine nucleotide.	ADP, ATP
Phosphate.....	phosphate, hydroxyl
Dicarboxylate.....	phosphate, malate, succinate, malonate
Oxoglutarate	oxoglutarate, malate, succinate, malonate
Tricarboxylate.....	citrate, *cis*-aconitate, isocitrate, malate, phospho*enol*pyruvate, isomalate
Pyruvate.....	pyruvate, hydroxyl
Glutamate.....	glutamate
Aspartate.....	aspartate

TABLE II.

Exchanges studied.

External radioactive anion	Internal anion
2-Oxoglutarate, or Malate, or Malonate, or Succinate	2-Oxoglutarate, or Malate, or Malonate

product inhibition. It is also important that the anions are not metabolised and that the mitochondrial matrix does not contain exchangeable anions other than those with which the mitochondria have been loaded, to avoid competition. All these conditions have been verified and followed as best as possible.

From this study Eqn 2 was derived, relating the rate of the reaction to the concentration of the substrates :

$$v = \frac{V}{1 + \frac{K_a}{[A]} + \frac{K_b}{[B]} + \frac{K_{ab}}{[A]\,[B]}} \qquad (2)$$

where v is the initial rate
V is the maximal rate
K_a and K_b are the Michaelis constants
[A] and [B] are the concentrations of the substrates, and
$K_{ab} = K_a \times K'_b = K'_a \times K_b$.

Florini and Vestling [1] developed, in the case of the enzymatic reactions which follow this simple equation, a graphic method with which K_a, K_b, K'_a and K'_b can be easily determined. This method is illustrated in fig. 1.

If the inverse of the initial rate of the reaction is plotted against the inverse of the concentration of A and B, a linear relation is obtained for each concentration of the other substrates, $[B]_1$, $[B]_2$, $[B]_3$, or $[A]_1$, $[A]_2$ and $[A]_3$. If the inverses of the apparent-maximal rates for A (*i.e.* points 1, 2, and 3) are replotted against 1/[B], another (dotted) straight line is obtained and gives $1/V$ and $-1/K_b$. If the inverses of the apparent-maximal rates for B (*i.e.* points 4, 5, and 6) are replotted against 1/[A], $1/V$ and $-1/K_a$ are similarly obtained.

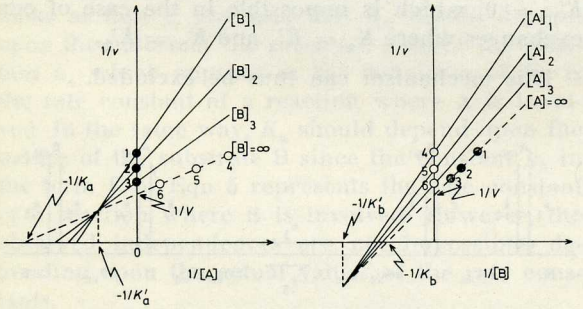

FIG. 1. — *Graphic determination of kinetic parameters, according to Florini and Vestling* [1].

The four straight lines, on the left part, intersect at a common point, the abscissa of which is $-1/K'_a$ and the other four lines, on the right part, intersect at another common point, the abscissa of which is $-1/K'_b$. These common points of intersection can be above, below, or on the axis of the abscissae. In the latter case, $-1/K_a = -1/K'_a$, and $-1/K_b = -1/K'_b$.

We applied this graphic method to the results of the exchanges measured. Fig. 2 illustrates the results of the exchange internal malonate against external oxoglutarate for three concentrations of malonate and oxoglutarate. The common point of the four straight lines coincides with the point of intersection of the dotted line with the abscissae. This means that $-1/K'_a = -1/K_a$ and that $-1/K'_b = -1/K_b$. These two relations have been observed for all the twelve exchanges studied [2, 3].

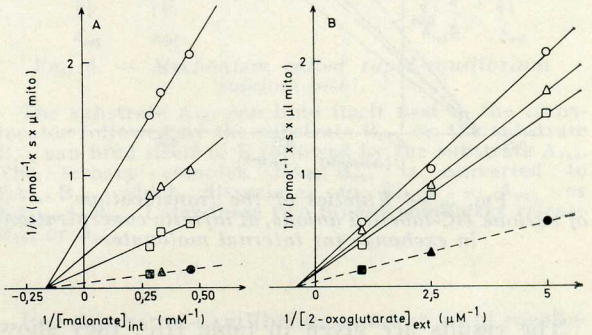

FIG. 2. — *Kinetics of 2-oxo [5-14C] glutarate influx in exchange for internal malonate.*

Heart mitochondria (final concentration = 0.58 mg protein/ml). (A) $1/v$ against 1/[internal substrate] ; external 2-oxo [5-14C] glutarate = 0.2 µM (O), 0.4 µM (△) or 1 µM (□) ; the filled symbols (●, ▲, ■) correspond to the three values of $1/V_b$ (apparent-maximum rates) determined in Fig. 2 B and give $1/V$ by extrapolation (— — — —). (B) $1/v$ against 1/[external substrate] ; internal malonate = 2.17 mM (O), 2.98 mM (△) or 3.51 mM (□) ; the filled symbols (●, ▲, ■) correspond to the three values of $1/V_a$ (apparent-maximum rates determined in Fig. 2 A and give $1/V$ by extrapolation (— — — —). v is expressed in pmol/s per µl mitochondria. (From Sluse *et al.* [3]).

Moreover the graphic comparison reveals that the abscissa of this common point for a given anion is the same if this anion is exchanged against any four possible counter-anions. This means that the abscissa of this common point does not depend upon the nature of the counter-anion of the exchange (fig. 3). From then on, the values of each constant have been calculated by extrapolation of twelve best-fitting straight lines obtai-

ned from four different exchanges (see Appendix in [4]).

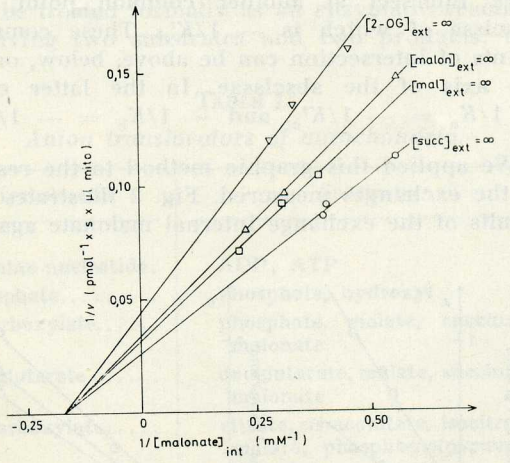

FIG. 3. — *Kinetics of the translocation of various ^{14}C-labelled anions, at infinite concentration, in exchange for internal malonate.*

The results are given in table III ; they show that the Michaelis constants of the external substrates are different from one another and very much smaller than the constants of the corresponding internal substrates.

TABLE III.

Michaelis constants for the translocated substrates.

Anions	External (µM)	Internal (mM)
2-Oxoglutarate	2.34	1.48
Malate.....	11.6	3.6
Malonate.....	424	5.9
Succinate	141	

Interpretation.

Eqn 2 is well known in enzyme kinetics and the theory of the mechanisms to which it corresponds has been substantiated and discussed by Alberty [5], Frieden [6] and Cleland [7-9]. Among the many mechanisms for the two substrate-two product reactions described by Cleland, only three are compatible with the results of our experiments.

There is *first* the mechanism called steady-state ping-pong bi-bi illustrated in fig. 4. One observes that in this mechanism the ternary complex (translocator-internal substrate-external substrate) is absent and the translocator exists in two distinct states, that is E_{int} and E_{ext}. The initial rate, in the case of such a mechanism, is given by Eqn 3 :

$$v = \frac{V}{1 + \frac{K_a}{[A]} + \frac{K_b}{[B]}} \qquad (3)$$

where K_a and K_b are the Michaelis constants for the substrates A and B and where $K_{ab} = 0$. Given that $K_{ab} = K'_a \times K_b = K'_b \times K_a$, it is necessary,

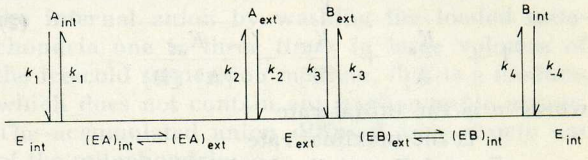

FIG. 4. — *Mechanism called steady-state ping-pong bi-bi.*
The reaction sequence is written from left to right, the translocator is represented by E, the addition of substrates and the dissociation of the complexes are represented by the vertical arrows. The rate constants are written on the side of these arrows. Those on the left for the forward reaction and those on the right for the backward reaction. In this mechanism the substrate on the internal side of the membrane, for example, A_{int} fixes itself on the translocator E_{int}, which forms the binary complex EA_{int}. This displaces itself in the membrane and reaches the other side where it becomes EA_{ext} and where A_{ext} is liberated. The substrate B_{ext} then fixes itself on the translocator E_{ext}, forms EB_{ext}, is transported in the other direction and is liberated in its turn on the other side of the membrane.

in order that $K_{ab} = 0$, that the constants $K'_a = K'_b = 0$, which is impossible in the case of our exchanges where $K_a = K'_a$ and $K_b = K'_b$.

This mechanism can thus be excluded.

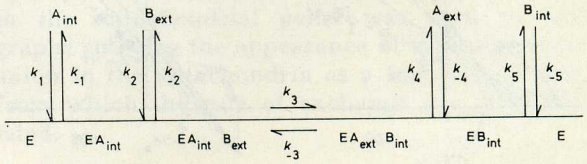

FIG. 5. — *Mechanism called steady-state ordered bi-bi.*
In this mechanism, one of the substrate, A_{int} for example, binds first to the translocator E and forms the binary complex EA_{int} to which the substrate B_{ext} binds later to give the ternary complex $EA_{int}B_{ext}$. This complex is converted to EA_{ext} B_{int} and dissociates first to give A_{ext} + EB_{int} and later to E + B_{int}.

The *second mechanism* compatible with our results is called steady-state ordered bi-bi (fig. 5). The constants k_1, k_2, k_3, k_4, k_5 and k_{-1}, k_{-2}, k_{-3}, k_{-4} and k_{-5} are the rate constants of the various steps of the reaction.

Alberty showed that the initial rate of the reaction is described by Eqn 2 where V is given by Eqn 4, K_a by Eqn 5, K'_a by Eqn 6 and K_b by Eqn 7.

$$V = [E]_t/(\frac{1}{k_3} + \frac{1}{k_4} + \frac{1}{k_5} + \frac{k_{-3}}{k_3 k_4}) \quad (4)$$

where $[E]_t$ is the total concentration of the translocator,

$$K_a = V/[E]_t k_1 \quad (5)$$

$$K'_a = k_{-1}/k_1 \quad (6)$$

$$K_b = \frac{V}{[E]_t k_2}(1 + \frac{k_{-2}}{k_3} + \frac{k_{-2}k_{-3}}{k_3 k_4}) \quad (7)$$

In order that K_a be equal to K'_a, as we observed for every exchange, it would be necessary that k_{-1}, be equal to the expression 8 derived from the Eqns 4, 5 and 6 by putting $K_a = K'_a$.

$$k_{-1} = 1/(\frac{1}{k_3} + \frac{1}{k_4} + \frac{1}{k_5} + \frac{k_{-3}}{k_3 k_4}) \quad (8)$$

If this equality repeats in every exchange for every substrate studied, one can explain it only by a series of coincidences. It is necessary in effect that every time, k_{-1} be equal to this expression which involves four rate constants of different reactions. One cannot however, exclude such a series of coincidences, perhaps not so improbable.

On the other hand, we observe that the Michaelis constant for a given substrate in a given phase does not depend upon the nature of the other substrate against which it exchanges. However, if one looks at Eqn 7, one sees that K_b should depend upon the nature of the substrate A since the constant k_4 which appears in the definition of K_b is the rate constant of a reaction where A is involved. In the same way, K_a should depend upon the nature of the substrate B since the constant k_5 in the term V of Eqn 5 represents the rate constant of a reaction where B is involved. However, the observed independences are not impossible, depending upon the actual values of the rate constants.

It appears that these considerations do not exclude the second mechanism although it does not provide a particularly satisfying interpretation.

The *third mechanism* compatible with the kinetic equation deduced from our experiments is called rapid-equilibrium random bi-bi (fig. 6). In this mechanism, there is no determined order for the binding of the substrates to the translocator and for the release of the products. Moreover, the conversion step of the ternary complex $EA_{int}\ B_{ext}$ to $EA_{ext}\ B_{int}$, that is the transport itself, is the rate-limiting step of the reaction.

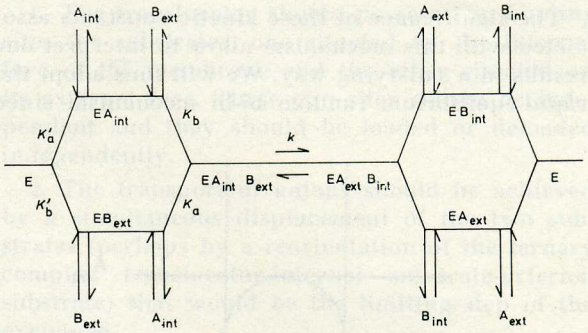

FIG. 6. — *Mechanism called rapid-equilibrium random bi-bi.*

The substrate A_{int} can bind itself first to the translocator followed by the substrate B_{ext} or, the substrate B_{ext} can bind itself to E followed by the substrate A_{int}. The ternary complex $EA_{int}\ B_{ext}$ is converted to $EA_{ext}\ B_{int}$ which dissociates to $EB_{int} + A_{ext}$ or $EA_{ext} + B_{int}$. Later these two binary complexes release B_{int} or A_{ext}.

For this rapid-equilibrium random bi-bi mechanism, the significance of the constants K_a, K_b, K'_a K'_b is the following :

K'_a is the dissociation constant of the complex EA_{int} to $E + A_{int}$;

K'_b is the dissociation constant of the complex EB_{ext} to $E + B_{ext}$;

K_a is the dissociation constant of the complex $EA_{int}\ B_{ext}$ to $EB_{ext} + A_{int}$;

K_b is the dissociation constant of the complex $EA_{int}\ B_{ext}$ to $EA_{int} + B_{ext}$.

K'_a and K'_b which are the dissociation constants of the binary complexes EA_{int} and EB_{ext} characterise the random binding of the first substrate to the translocator ; they are independent of the later fixation of the second substrate and of course of its nature. This is what we observe in our results where the constant K' for one given substrate is independent of the nature of the counter-substrate.

K_a and K_b which are the dissociation constants of the ternary complex $EA_{int}\ B_{ext}$ characterise the fixation of the second substrate to the binary complex and are not necessarily independent of the presence and the nature of the first substrate already bound to the translocator. But if the binding of the first substrate does not modify the affinity of the translocator for the second substrate, then the constants K_a and K_b are necessarily equal to K'_a and K'_b. It is what we observed

in our results where the Michaelis constants for a given substrate are equal to the corresponding constant K'.

The significance of these kinetic constants associated with this mechanism allow to interpret our results in a satisfying way. We will thus adopt the rapid-equilibrium random bi-bi mechanism since this is the mechanism which fits best with our results.

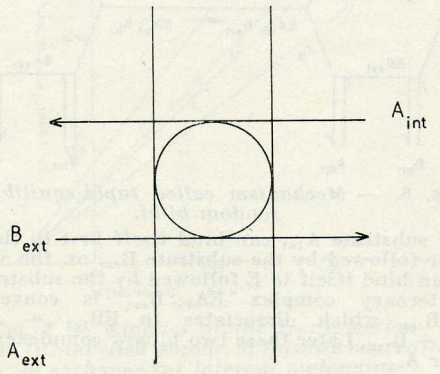

FIG. 7. — *External-product inhibition.*
The exchange A_{int}/B_{ext} is measured at many concentrations of A_{ext}.

We conclude thus that the anions present in the extra- or intramitochondrial compartments bind to the oxoglutarate translocator in a completely independent manner and that the Michaelis constants and the constants K' are dissociation constants.

External-product inhibition.

In order to confirm the fitness of this mechanism we studied the inhibition by the product of the reaction. This study plays an important rôle in the elucidation of the mechanisms of reaction because two mechanisms of which the initial rates follow the same kinetics can have different types of inhibition by the products of the reaction.

We studied the kinetics of the inhibition by the external products of the following two exchanges : the exchange internal malate/external oxoglutarate and the exchange internal malate/external malonate at many concentrations of external malate which is the external product of the reaction.

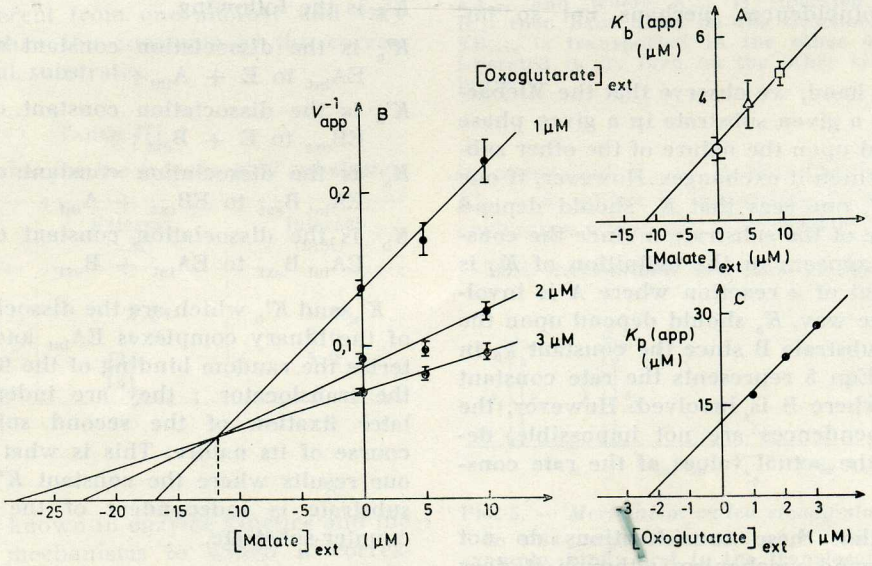

FIG. 8. — *Determination of the inhibition constant* (K_p) *for L-malate in the external-product inhibition of the influx of 2-oxo*[5-^{14}C]*glutarate in exchange for internal L-malate.*

(A) Apparent Michaelis constant for external oxoglutarate, K_b (app), as a function of the concentration of external product malate ; the vertical bars represent the mean ± standard deviation ; n = 9 (O), 3 (△), and 5 (□). (B) V^{-1}_{app} at infinite [malate]$_{int}$, internal substrate, as a function of [malate]$_{ext}$, external product. The vertical bars represent mean ± standard deviation of V^{-1}_{app}. The three lines have a common intercept corresponding to K_p, whereas they intercept with the axis of the abscissae at three values of K_p (app), one for each concentration of external oxoglutarate. V_{app} is expressed in pmol/s per µl mitochondria. (C) K_p (app) as a function of the external substrate (oxoglutarate), taken from (B). (Modified from Sluse *et al.* [4]).

If one studies the exchange of A_{int} against B_{ext} in the presence of the product A_{ext} (fig. 7) and if one supposes that the mechanism of the reaction is the rapid-equilibrium random bi-bi with a dead-end complex $EA_{int}A_{ext}$, the inhibition by the product A_{ext} will be competitive if one varies the concentration of the external substrate B and will be non-competitive if one varies the concentration of the internal substrate A.

We observed what the theory predicted. The apparent Michaelis constants K_b for the external substrate, here oxoglutarate, increases with the concentration of the external product, here external malate (fig. 8A). The straight line which joins these three points cuts the abscissa at $-1/K_p$ (Mal_{ext}). The inhibition constant is approximatively 12 µM and is about the same as the Michaelis constant of external malate determined by initial rate studies and which was 11.6 µM.

On the other hand, the inverses of the apparent maximal rates (V_{app}^{-1}) for each concentration of the external substrate, here oxoglutarate, increase with the concentration of the external product, here external malate (fig. 8B). The three lines intersect at a point of which the abscissa is $-1/K_p(Mal_{ext})$: the constant is also 12 µM. Each line cuts the abscissa at a point which corresponds to an apparent negative inhibition constant for each concentration of the external substrate. If we plot these apparent inhibition constants, K_p(app), against the concentration of the external substrate, we obtain a line which cut the abscissa at $-K_m$ ($Oxogl._{ext}$). This is about 2.3 µM as that determined in the initial rate study (fig. 8C).

These results are thus in accordance with the mechanism rapid-equilibrium random bi-bi with a dead-end complex $EA_{int}A_{ext}$.

It should be realised however that the inhibition pattern observed could also be explained by a steady-state ordered bi-bi mechanism, which we could not exclude earlier, where the only binary complexes would be those formed by the translocator and the external ions and where the external product would not form the dead-end ternary complex $EA_{int}A_{ext}$. This last restriction however is in flagrant contradiction with the fact that the complex $EA_{int}A_{ext}$ must exist since a substrate can exchange against itself as shown in table II.

The external-product-inhibition pattern thus favours the rapid-equilibrium random bi-bi mechanism. This confirms the fitness of this mechanism which we chose after the initial-rate studies.

Conclusion.

The translocator thus should have the following characteristics :

1. The translocator should possess two binding sites for substrates, one situated on the internal face of the membrane and the other situated on its external face. These two sites should be independent and they should be loaded or deloaded independently.

2. The transport of anions should be achieved by a simultaneous displacement of the two substrates (perhaps by a reorientation of the ternary complex translocator-internal substrate-external substrate) that would be the limiting step of the exchange.

3. The translocator must remain unchanged after the exchange.

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RÉSUMÉ.

Les réactions d'échange entre le 2-oxoglutarate, le malate, le malonate et le succinate ont été étudiées à 4°C dans des préparations de mitochondries de cœur de rat dans des conditions où le transporteur oxoglutarate était le seul transporteur capable de les effectuer. Les mesures de vitesses initiales ont été faites pour trois concentrations du substrat interne et du substrat externe. Les graphiques des inverses montrent que l'on obtient des relations linéaires entre v^{-1}, d'une part, et $[S_{int}]^{-1}$ ou $[S_{ext}]^{-1}$, d'autre part, et que ces lignes droites coupent l'axe des abscisses en un même point. Dans chaque cas la constante de Michaelis est donc égale à la constante K'. Les résultats obtenus montrent aussi que les constantes de Michaelis sont beaucoup plus petites pour les substrats externes que pour les substrats internes correspondants et que la constante de Michaelis pour un substrat donné, qu'il soit interne ou externe, est indépendante de la nature de l'ion partenaire de l'échange.

Les réactions d'échange entre le malate interne et le 2-oxoglutarate ou le malonate externes ont été étudiés à 4°C en présence de malate externe (inhibition par le produit externe de la réaction). Les vitesses initiales ont été mesurées pour trois concentrations du substrat interne, du substrat externe et du produit externe. Le produit externe inhibe l'échange d'une façon compétitive quand on fait varier le substrat externe et d'une façon non compétitive quand on fait varier le substrat interne.

Ces résultats sont exclusivement en accord avec le mécanisme de réaction appelé *rapid-equilibrium random bi-bi* et avec une inhibition mixte par le produit et le complexe *dead-end.*

On peut conclure que le transporteur possède deux sites indépendants sur lesquels se fixent les substrats (un site interne et un site externe) et que le transport d'anions s'effectue par déplacement simultané des deux substrats, ce qui est l'étape limitante de la réaction.

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