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Spontaneous modification of the oxoglutarate translocator *in vivo*

Claire DUYCKAERTS, Claudine M. SLUSE-GOFFART, Francis E. SLUSE, Cécile GOSSELIN-REY, and Claude LIÉBECQ

with the technical assistance of Laurette Bertrand, Eli Dethier, Monique Cuvelier-Klimek and José Salmon

Laboratoire de Biochimie et de Physiologie Générales de l'Institut Supérieur d'Education Physique, Département de Chimie Générale et de Chimie Physique de la Faculté des Sciences; et Laboratoire des Lipides, Institut de Pathologie de la Faculté de Médecine, Université de Liège

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In studying the oxoglutarate translocator of rat-heart mitochondria over many years, we have observed an unexpected decrease in its efficiency. It has been divided by 2.48 ± 0.07 (S.E.M.) for the exchange of external oxoglutarate for internal malate at 2 °C when the internal-malate concentration is 4 mM and is accompanied by an increase in its concentration (multiplied by 1.61 ± 0.02, S.E.M.).

The affinity of the external sites of the translocator for the external oxoglutarate is unchanged as well as the binding and kinetic cooperativities of the external oxoglutarate. This shows that the external side of the translocator has not been modified and suggests that its central part has not been modified either. The apparent Michaelis constant of the internal malate is increased (multiplied by 1.74 ± 0.23, S.E.M.) suggesting that the translocator has been modified on its matricial side.

Some control experiments show that a change in the diet of the rats, despite its effect on the fatty-acid content of the mitoplasts, is probably not responsible for the observed modification. As it is nevertheless very likely that changes of the oxoglutarate translocator have occurred *in vivo*, it is proposed that the observed modification has a genetic origin.

The existence of two antagonist changes which are not directly related suggests that one of them is a response of the organism against the other; thus the oxoglutarate translocator may play a regulatory rôle in certain physiological conditions.

The oxoglutarate translocator of the inner membrane of rat-heart mitochondria performs a one-to-one exchange between external and internal dicarboxylate anions [1,2]. It is a component of the malate-aspartate shuttle which, in heart, is the main device transferring reducing equivalents produced in the cytosol to the respiratory chain in the mitochondria [3].

The influence of the external-oxoglutarate concentration on the initial rate of the external-oxoglutarate/internal-malate exchange, and the equilibrium binding of external oxoglutarate to the translocator, have been thoroughly studied from 1974 to 1977 [4,5]. Five years later, after having studied the kinetic saturation of the translocator by external malate, the binding and kinetic data of external oxoglutarate were needed to analyze the effect of external oxoglutarate on malate uptake [6]; some new control measurements of the available data [5] were desirable. It was then observed that the external-oxoglutarate binding was systematically higher than before, while the oxoglutarate-uptake rate was systematically smaller. More measurements than expected were thus required. These enabled the modification of the oxoglutarate translocator to be described precisely and are the subject of this paper.

As great care has always been taken to respect a fixed experimental protocol, unchanged over the years, the cause of the observed modification must be sought in materials rather than in methods. Among materials, rats themselves could change in spite of their common origin from the same inbred strain (for details see Materials and Methods). Of course such an event could not be directly demonstrated and could only be supported by elimination of the other possibilities. A change in the chemicals is improbable but cannot be excluded. However it will be shown that it would be difficult to account for our observations in this way, whereas an *in vivo* modification is a satisfactory hypothesis.

The food given to the rats in our laboratory during 10 days (on an average) preceding an experiment, has been changed between the two sets of experiments. As the food used before (from Hesby, see Materials) was still available, it has been compared with the new one (from U.A.R., see Materials). Rats fed on each food have also been compared.

As shown in the Discussion, the fortuitous observation of a modification of the oxoglutarate translocator *in vivo*, brings new informations and suggests a regulatory rôle for this translocator.

MATERIALS AND METHODS

Animals and diets

Male rats inbred R/A pfd f from Wistar source (supplied by the Proefdierencentrum, Katholieke Universiteit Leuven, Belgium) weighing 200 to 250 g are used for kinetic and binding studies. They are fed in our laboratory during 10 days or so, with Hesby diet (from Moulin F. Fisenne et Fils, Pepinster, Belgium) or with U.A.R. A.03 diet (from Usine d'Alimentation Rationnelle, Villemoison-sur-Orge, France).

In the Hesby diet, the minimum of proteins and fats are 30% and 4%, the maximum of cellulose, inorganic matter and water are 9%, 9% and 13% respectively. The U.A.R. diet

contains at least 22% proteins and 14% fats, at the most 6% cellulose, 4.5% inorganic matter and 13% water.

The dietary effect on the lipid composition of the mitochondrial inner membrane and on the kinetics and binding of the oxoglutarate translocator has been studied by feeding 4-week-old rats (about 50 g) with Hesby or U.A.R. diets during 6 to 10 weeks. Rats then weigh 200–230 g.

Preparation of mitochondria and mitoplasts

Mitochondria from heart ventricles were prepared according to Tyler and Gonze [7]. Mitoplasts (inner membrane plus matrix) were isolated according to the method of Greenawalt [8] by disruption of the outer membrane of the mitochondria by digitonin; the mitochondrial pellet suspended in a minimal volume of isolation medium (1 ml per g of wet weight of mitochondrial pellet) was stirred 15 min at 0 °C with digitonin (12 mg per g of wet weight of mitochondrial pellet). The inner membrane in the mitoplast fraction remains structurally intact, as observed by electron microscopy (not shown) and functionally (not shown).

Kinetic and binding experiments

Special reagents, incubation medium and experimental timing and conditions have been described in [4,5].

Lipid composition of diets and mitoplasts

Extraction and fractionation. The dry crushed diets were extracted overnight by 40 volumes of $CHCl_3/CH_3OH$ (2/1, v/v); the mitoplast pellets were suspended in 5 volumes of water and the suspension extracted by 20 volumes of $CHCl_3/CH_3OH$ (2/1, v/v). The filtrates were partitioned according to Folch et al. [9]. Phospholipids from the total lipid extracts were resolved by preparative thin-layer chromatography performed on precoated silicagel 60 plates (Merck) with $CHCl_3/CH_3OH/H_2O$ (65/25/4, v/v/v) as developing system.

Fatty-acid analysis. Methyl esters of fatty acids were prepared by methanolysis in anhydrous CH_3OH/1-M HCl performed in presence of known amounts of $C_{15:0}$ as internal standard. Gas-liquid chromatography was performed isothermically at 165 °C on 20% OV-275 packed on 100/200 Chromosorb W. AW. Standard fatty acids were supplied by Supelco (Gland, Switzerland).

RESULTS

Modification of the binding curve of external oxoglutarate

It appears that the new binding values and the previous ones [5] are in a constant ratio independently of the external-oxoglutarate concentration. The mean value of this ratio, obtained from experiments made at 55 different external-oxoglutarate concentrations lower than 80 μM, is 1.61 ± 0.02 (S.E.M.), the new values being the highest ones. This is illustrated by the lower curve in Fig. 1 in which the old data [5] multiplied by 1.61 and the new data have been plotted together. The consistency of the two sets of data is clear except for external-oxoglutarate concentrations higher than 80 μM for which an additional jump was drawn in 1979 [5] whereas a constant value of 75.3 ± 1.3 (S.E.M.) pmol/mg protein is presently obtained. In fact the additional jump (dashed line in Fig. 1) was supported by four points marked with an asterisk, the one at the highest concentration having a particularly great standard error. These points have to be compared with four new ones which can be better trusted (see appendix). It is probable that in this high-concentration range the old and the new bindings are also proportional and that the binding curve of external oxoglutarate has three intermediary plateaux and not four. The straightforward conclusions are:

a) The affinity of the translocator for external oxoglutarate and the cooperativities in the external-oxoglutarate binding have not been modified. This seems to indicate that the part of the oxoglutarate translocator situated near the external surface of the membrane has not been modified.

b) The total concentration of the oxoglutarate translocator in the mitochondrial inner membrane has been multiplied by 1.61 ± 0.02 (S.E.M.). The total concentration of binding sites for the external substrate is now 75.3 ± 1.3 (S.E.M.) pmol/mg protein.

Modification of the kinetic-saturation curve

The initial rates of the external-oxoglutarate/internal-malate exchange at a fixed concentration of internal malate (4 mM) are now lower than before 1978 [5], despite the increase in the concentration of translocator revealed by the binding data. This indicates a decrease in efficiency.

The ratio between the data published previously [5] and the present ones seems to be independent of the external-oxoglutarate concentration and is 1.54 ± 0.04 (S.E.M.). This value has been obtained from experiments made at 22 different external-oxoglutarate concentrations lower than 150 μM. The new data and the old ones divided by 1.54 are shown together in the upper curve in Fig. 1. The consistency of the two sets of data is good except at oxoglutarate concentrations higher than 150 μM: the decrease of the oxoglutarate-uptake rate reported in 1979 [5] (dashed line in Fig. 1) is no longer observed. The inhibition was supported by two points only while the saturation plateau is supported by three points of greater statistical significance (see appendix). The local discrepancy between the two sets of data is more likely to be due to two erratic points (those marked with an asterisk) than to a failure of the proportionality observed elsewhere.

It can be concluded that: (a) the cooperativities induced by external oxoglutarate at the level of the catalytic rate constant have not been modified; (b) the efficiency of the studied exchange, i.e. the ratio between the initial rate of oxoglutarate uptake and the total concentration of the translocator, has been divided by a factor of 2.48 ± 0.07 (S.E.M.) at 2 °C when the internal-malate concentration is 4 mM.

These conclusions together with those derived from the binding data could be hardly explained by an *in vitro* effect. Indeed an unmasking of some translocators, due to the chemicals, is not expected to lead to a decrease of the uptake rate. In contrast, the observation of two antagonist and not directly related changes (decrease in efficiency and increase in concentration) is quite consistent with an *in vivo* modification of the translocator (see Discussion).

It is conceivable that the efficiency of the physiological exchange (external malate/internal oxoglutarate) *in vivo* has also decreased and that the increase in the translocator concentration almost compensates the decrease in efficiency under physiological conditions.

Modification of the apparent Michaelis constant for internal malate

An apparent Michaelis constant for internal malate equal to 1.30 ± 0.14 mM has been published in 1979 [5]; it had been

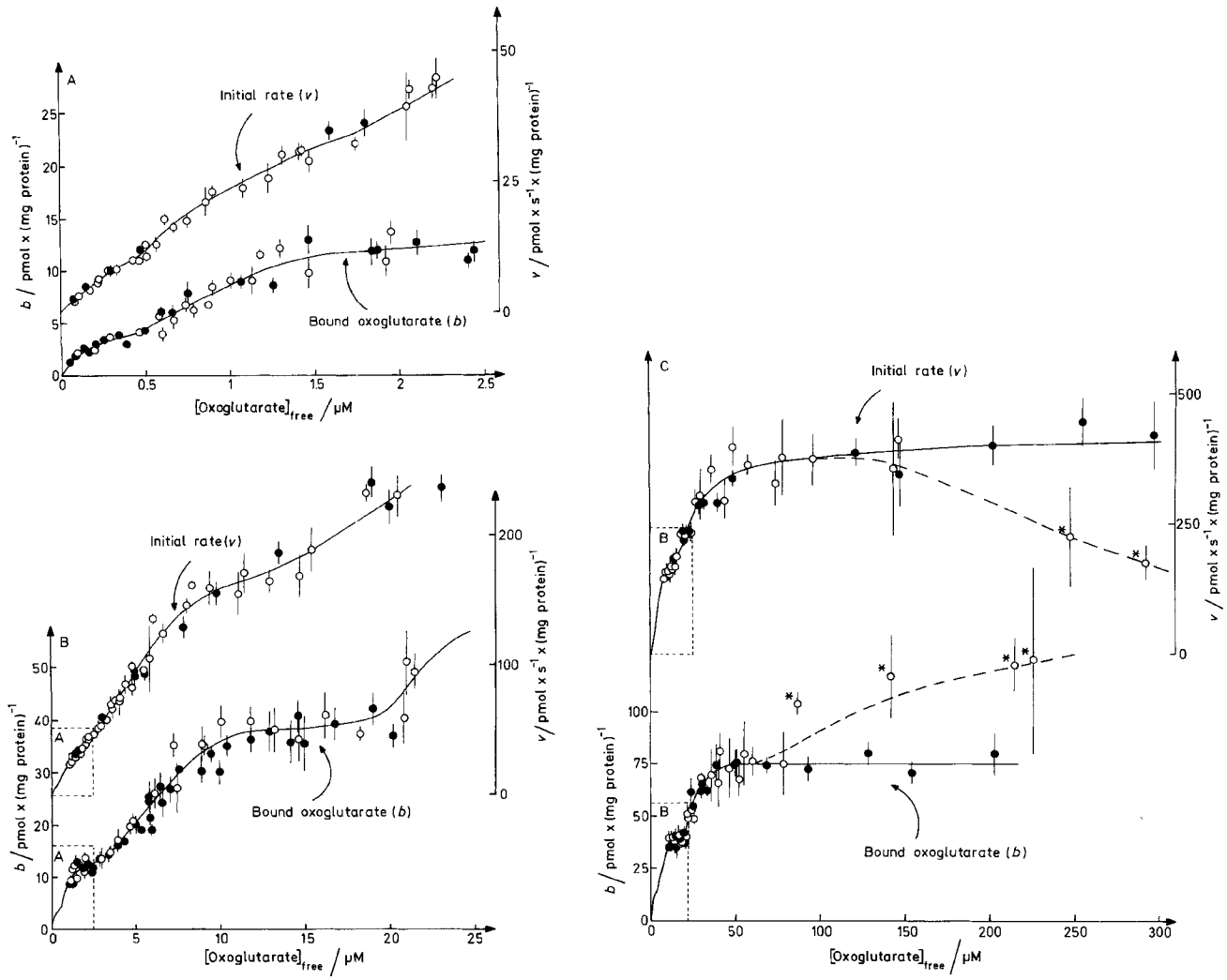

Fig. 1. *Bound oxoglutarate (lower curve) and rate of oxoglutarate uptake (upper curve) as a function of free external-oxoglutarate concentration.* (Lower curve): malate-depleted mitochondria; vertical bars represent S.E.M. (○): values published in [5] multiplied by 1.61; (●): new values. (Upper curve): malate-loaded mitochondria; each point corresponding to the rate at 4 mM-internal malate is obtained by linear interpolation of the double-reciprocal plots of the rates measured with three internal-malate concentrations around 4 mM; the vertical bars represent the standard deviations. (○): values published in [5] divided by 1.54; (●): new values. Both lines pass through the adjusted points (not shown) calculated by the smoothing method [5]. Asterisks mark old data diverging from present ones (see text)

calculated from the initial rates at two internal-malate concentrations (2 mM and 6 mM) and several external-substrate concentrations.

The apparent Michaelis constant obtained now by the same method is 2.26 ± 0.18 mM. This suggests that the translocator has been modified on its matricial side.

Modification of the diet

Results published in 1979 [5] have been obtained with rats fed during an average of 10 days with Hesby diet. The new results described in this paper are from rats fed during the same number of days with U.A.R. diet.

Fatty-acid content of both foods has been determined and is shown in Table 1. The Hesby diet contains a greater proportion of unsaturated fatty acids than the U.A.R. diet and has a greater double-bond index. The difference is due to a greater proportion of linoleic acid (18:2) in the Hesby diet. The food received by the rats before their arrival in our laboratory is very similar to the U.A.R. diet (not shown).

Fatty-acid content of the mitoplasts. Rats weighing around 50 g have been fed with Hesby diet during 70 days or with U.A.R. diet during 42 days. After these periods, the rats of both groups have reached the same weight, between 200 and 230 g, as for usual experiments. Heart mitochondria have been isolated from which mitoplasts (mitochondria without their external membrane) have been prepared. In the mitoplasts, the amount of fatty acids in the free form or in the triacylglycerols and in sphingomyelin is negligible compared with the amount present in the glycerophospholipids. The proportion between the saturated and the unsaturated fatty acids as well as the double-bond index are analogous for the membranes of the two groups of rats (Table 1). However, the arachidonic acid (20:4), synthesized in the organism from the linoleic acid (18:2), is in greater proportion in the mitoplasts of rats fed with the Hesby diet. This is mostly due to the contribution of the

Table 1. *Comparison of the fatty-acid contents.*
Values are molar percentage. Commercial origin of the diets in Materials and Methods. Preparation of heart mitoplasts from rats fed with the two diets as described in Materials and Methods

Fatty acid	Diet		Composition of heart mitoplasts after feeding on	
	Hesby	U.A.R.	Hesby diet	U.A.R. diet
16:0	17	22	11	12
18:0	2	4	19	20
16:1	–	7	–	–
18:1	20	27	8	8
18:2	57	35	33	35
18:3	4	5	–	–
20:4	–	–	21	15
22:6	–	–	8	10
Saturated (total)	19	26	30	32
Unsaturated (total)	81	74	70	68
Double-bond index	146	119	206	198

Table 2. *Comparison of the oxoglutarate translocator of rat-heart mitochondria from rats fed with Hesby or U.A.R. diet.*
External-oxoglutarate bound (±S.E.M.) by malate-depleted mitochondria. Initial rates of oxoglutarate uptake (±S.D.) by malate-loaded mitochondria (4 mM)

External oxoglutarate concentration	External oxoglutarate binding		Initial rate of oxoglutarate uptake	
	Hesby diet	U.A.R. diet	Hesby diet	U.A.R. diet
μM	pmol × (mg protein)$^{-1}$		pmol × s^{-1} × (mg protein)$^{-1}$	
0.4	–	–	10.12 ± 0.93	8.61 ± 0.88
2	11.1 ± 1.4	12.6 ± 1.4	46.3 ± 4.4	34.3 ± 3.5
5	–	–	98.9 ± 13.4	94.6 ± 4.9
12	38.6 ± 2.7	36.1 ± 2.6	184.6 ± 9.7	170.7 ± 11.5
20	39.2 ± 2.7	36.9 ± 2.3	–	–
30	–	–	275.3 ± 19.4	286.6 ± 16.1

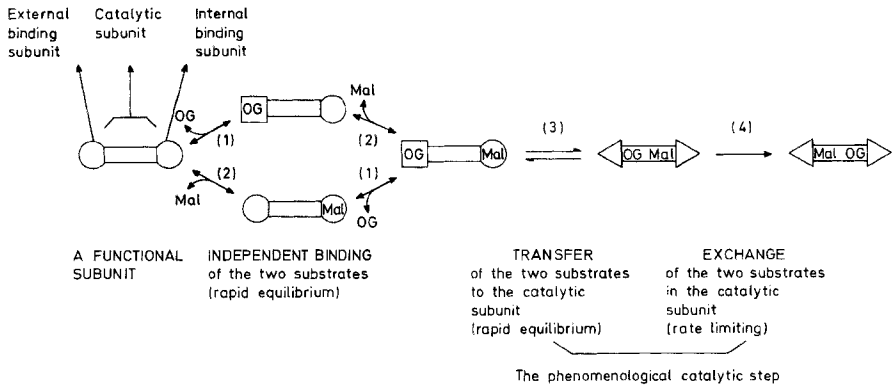

Fig. 2. *Schematic representation of the steps involved in the initial rate of the exchange of external oxoglutarate (OG) with internal malate (Mal).* A functional subunit only is represented. The whole translocator (not shown) may be either the association of several such functional subunits or a single catalytic subunit surrounded by several binding subunits [6]. The symbols ○, □ and △ stand for three conformations of binding subunits

arachidonic acid present in the phosphatidylcholines (14% of the total fatty acids in the heart mitoplasts of rats fed with the Hesby diet and 9% of the total fatty acids in the heart mitoplasts of rats fed with the U.A.R. diet). The distribution of the different fatty acids in the other phospholipids has been determined but is not reported since a similar phospholipid composition of mitoplasts was observed in both groups of rats.

External-oxoglutarate binding and its initial rate of exchange with internal malate (4 mM) have been determined at 2 °C, for different external-oxoglutarate concentrations, with isolated heart mitochondria. The comparison of the two groups of rats is made in Table 2. There is no systematic difference, showing that the modification of the oxoglutarate-translocator properties described in this paper is probably not related to diet. This finding does not exclude that other diets with more important differences in the lipid composition may influence the oxoglutarate translocator.

DISCUSSION

Location of the observed modification

The theoretical analysis of the available data on the oxoglutarate translocator of rat-heart mitochondria has led us to propose two structural models [6]. In both, the oxoglutarate translocator is an association of different kinds of subunits: binding subunits situated near both inner membrane surfaces and catalytic subunits embedded in the membrane between the external binding subunits and the internal ones.

A functional subunit composed of an external binding subunit, a catalytic subunit, and an internal binding subunit is shown in Fig. 2. Binding of external oxoglutarate induces a conformational change. Internal-malate binding is represented in Fig. 2 without conformational change. This is quite possible, given the behaviour of external malate [6], but has not yet been demonstrated for internal malate [5,2]. As shown in [6], the catalytic step can be decomposed in a substrates' transfer step (3) accompanied by a conformational change, and an exchange step (4) which is the rate-limiting step.

The scheme of Fig. 2 provides a likely interpretation of the experimental data [6] among which is the particular form of the initial-rate equation:

$$v_{OG/Mal} = V_{OG/Mal} \times f_{OG}([OG]) \times g_{Mal}([Mal])$$

where the concentrations of the two substrates [OG] and [Mal] appear in two separate and specific functions, f_{OG} and g_{Mal}. Only the maximal rate $V_{OG/Mal}$ depends on both sub-

strates. The modification described in this paper deals with the product $V_{OG/Mal} \times g_{Mal}([Mal])$ but not with the factor $f_{OG}([OG])$.

The preservation of $f_{OG}([OG])$ and of the ratio between the bound oxoglutarate and the total concentration of binding sites means that the external binding subunits have not been modified. Moreover it suggests that the catalytic subunits have not been modified since this would probably affect the interaction between the external binding subunits and the modified catalytic subunits, thus modifying the affinity of the external subunit for external oxoglutarate. The increase in the apparent Michaelis constant of the internal malate supports the view that the internal binding subunits have been modified. This modification affects the binding of internal malate and probably the transfer step. It is the combination of the two effects that leads to a decrease by a 2.5 factor of the efficiency of the oxoglutarate uptake at 2 °C in exchange with 4-mM internal malate.

The observed modification and the interpretation of the saturation curves of external oxoglutarate

The intermediary plateaux present in the binding and kinetic saturation curves of the external oxoglutarate may be due either to the sum of independent sigmoidal contributions from isozymic translocators, or to changeovers of negative and positive cooperativities in a single oligomer [4–6].

As shown in [6], the different isozymic species that could coexist, must be made of the same subunits which could be associated in different ways, in various numbers or to various membraneous environments. Moreover the different species must be equivalent for the internal substrate so that it is probable that the proposed differences apply only to the external binding subunits.

The spontaneous modification described in this paper was the same for all the species that could coexist and this confirms that they are made of the same building blocks. It is particularly interesting that an increase of the total concentration of the translocator has not modified the ratio of the different putative contributions. Unless a large constant reserve of unassociated external binding subunits (unable to bind the substrates) is available, this strongly suggests that the different species would also have the same number of external binding subunits.

A new argument in favour of the view that a single oligomeric species is involved can thus be added to those developed in [6].

Origin of the observed modification

As shown, a slight modification in the lipid composition of the inner membrane, due to diet, is not responsible for the observed modification of the oxoglutarate translocator. Fortunately for the organism, it is to be expected that a regulatory catalyst (see below) be insensible to such commonplace and small fluctuations in diet.

An explanation that can be proposed is that the observed modification has a genetic origin, either direct (affecting the translocator itself) or indirect (affecting an effector of the translocator). It must be stressed that the inbred and inline strain of the rats (page 30 in [10]) (at a given generation, all breeding pairs of rats have a common ancestor, four generations previously), leads to an homozygote population. This homozygotism may be disturbed by a mutation but tends to be restored in course of time. Though the mutation has a greater probability of being eliminated than to be selected this last possibility exists.

We have examined all the experimental results obtained between 1977 and 1982 and believe that the modification occurred in 1978.

Possible regulatory rôle of the oxoglutarate translocator

It is quite possible that the increase in the concentration of the oxoglutarate translocator is an adaptation of the organism against the decrease in efficiency of the catalyst. Such an adaptation would be required only if the oxoglutarate translocator has a significant control strength in certain physiological situations and if the concerned reactions are of vital importance for the organism.

The last condition can be considered as verified. The oxoglutarate translocator is part of the malate-aspartate shuttle. This shuttle is 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].

Concerning the possible regulatory rôle of the oxoglutarate translocator, strong evidence is not yet available. Such a rôle is suggested by the great complexity of its kinetics which, like those of known regulatory enzymes, presents cooperative phenomena [5,6]. Moreover the translocator seems to have a physiological effector: aspartate [2]. The increase in the concentration of the translocator observed together with a decrease in its efficiency may be considered as additional indication.

Concluding comments

Biological materials are not stable over many years despite whatever precautions may be taken. However, large changes are probably not to be expected in organisms such as rats. In the example developed in this paper, quantitative details have been modified but the underlying mechanism remained unchanged.

On the one hand, thorough studies are necessary to describe complex systems and, possibly, their spontaneous modifications. On the other hand, such studies are so long that the likelihood of undesirable modifications cannot be ignored. Frequent control measurements are thus indispensable despite their time and money requirements. By chance, instead of resulting in disaster, the modification of the oxoglutarate translocator has been turned to advantage by simply making a small set of measurements.

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C. Duyckaerts, F. E. Sluse, and C. Liébecq,
Laboratoire de Biochimie et de Physiologie Générales, Institut Supérieur d'Education Physique de l'Université de Liège,
Institut de Chimie, Université de Liège au Sart-Tilman,
B-4000 Liège, Belgium

C. M. Sluse-Goffart,
Département de Chimie Générale et de Chimie Physique, Faculté des Sciences de l'Université de Liège,
Institut de Chimie, Université de Liège au Sart-Tilman,
B-4000 Liège, Belgium

C. Gosselin-Rey,
Laboratoire de Recherche sur les Lipides, Faculté de Médecine de l'Université de Liège,
Institut de Pathologie, Centre Hospitalier Universitaire, Université de Liège au Sart-Tilman,
B-4000 Liège, Belgium

Supplementary material to

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by

C. Duyckaerts, C.M. Sluse-Goffart, F.E. Sluse, C. Gosselin-Rey and C. Liébecq

Comparison of the statistical weight of old [5] and present data obtained at high external-oxoglutarate concentrations

It must be stressed that the strict requirements described in details for initial-rate measurements (page 3 in [4]) and for binding measurements (page 9 in [5]) are difficult to reach at oxoglutarate concentrations higher than 100 μM.

Binding values of oxoglutarate at concentrations higher than 80 μM are given in Table 3. Old values [5] are multiplied by 1.51 as in Fig.1 and are the mean of 4 to 5 determinations (*n*). Their S.E.M. vary from 5 to 43% of the mean. Present bindings are the mean of 4 to 14 determinations and their S.E.M. vary from 7 to 13% only.

Table 3. *Comparison of binding data at high external-oxoglutarate concentrations*

Oxoglutarate	Old [5] binding values (adjusted)	Present binding values
μM	pmol × (mg protein)$^{-1}$	
96	104 ± 6 (*n* = 5)	–
92	–	73 ± 6 (*n* = 12)
108	–	80 ± 9 (*n* = 14)
161	117 ± 19 (*n* = 4)	–
184	–	71 ± 5 (*n* = 4)
202	–	79 ± 10 (*n* = 4)
214	122 ± 13 (*n* = 3)	–
256	125 ± 54 (*n* = 5)	–

Initial rates of oxoglutarate translocation at concentrations higher than 150 μM are given in Table 4. Old values [5] are divided by 1.54 as in Fig.1 ; they are the slopes (± S.D.) of the straight lines calculated from 5 incubation times (*n*). Their S.D. are 4% and 10% of the slope. Present initial rates are the slopes of the straight lines calculated from 12 or 13 incubation times (*n*) over the same period of time (1 s) and their S.D. vary from 9 to 16% of the slope.

Table 4. *Comparison of initial-rate data at high external-oxoglutarate concentrations*

Oxoglutarate	Old [5] initial rates (adjusted)	Present initial rates
μM	pmol × s^{-1} × (mg protein)$^{-1}$	
203	–	402 ± 37 (*n* = 13)
242	228 ± 91 (*n* = 5)	–
254	–	447 ± 49 (*n* = 12)
293	174 ± 32 (*n* = 5)	–
297	–	424 ± 70 (*n* = 12)