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Factors affecting the pathway of glutamate oxidation in rat kidney mitochondria

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Chimica biologica. — *Factors affecting the pathway of glutamate oxidation in rat kidney mitochondria.* Nota di ALBERTO BINDOLI, NORIS SILIPRANDI e ANTONIO TONINELLO, presentata (*) dal Corrisp. N. SILIPRANDI.

RIASSUNTO. — In presenza di dinitrofenolo ed oligomicina il glutammato non viene ossidato dai mitocondri di rene a meno che non venga aggiunto preliminarmente ADP al mezzo di incubazione. Quando l'ADP viene aggiunto dopo il disaccoppiamento non si ha alcuna ossidazione a meno che non si aggiunga ossaloacetato. La necessaria presenza di ADP viene spiegata dalla necessità di una continua disponibilità di GDP per alimentare la fosforilazione a livello di substrato tramite la reazione fosfotransferasica GTP → ADP.

La necessità di ossaloacetato è spiegata dalla rapida deplezione di ossaloacetato endogeno indotta dal disaccoppiante. Si è osservato che l'attività della glutammato deidrogenasi dei mitocondri di rene è controllata dalla quantità di GTP. Infatti il tellurito, che inibisce la α–chetoglutarato deidrogenasi, e quindi la formazione di GTP, stimola fortemente la formazione di ammoniaca dal glutammato.

Si conclude che la ossidazione del glutammato da parte dei mitocondri di rene è controllata sia dalla disponibilità di ossaloacetato che dalla concentrazione intramitocondriale di GTP.

It is generally accepted that the oxidation of glutamate in rat liver mitochondria proceeds primarily through the transamination pathway in spite of the high concentration of glutamate dehydrogenase (EC 1.4.1.3) present in these mitochondria [1]. In pigeon breast muscle and rat heart sarcosomes the transamination pathway appears to be obligatory since glutamate dehydrogenase activity is low, as evidenced by the complete inhibition of glutamate oxidation by malonate, the absence of the Krebs-Cohen dismutation and the absence of any measurable amounts of glutamate dehydrogenase by direct spectrophotometric analysis [2]. Recently Crompton and Chappel [3] found that in the presence of ADP (1) + phosphate, pig renal cortex converts added glutamate quantitatively into aspartate, confirming that also in kidney mitochondria the glutamate-oxaloacetate transamination is by far the principal route of glutamate oxidation.

However it is very probable that in isolated mitochondria the experimental imposed conditions are very important for the relative operativity of the two pathways for glutamate oxidation.

(*) Nella seduta del 20 aprile 1974.

(1) Abbreviations: ADP = adenosine-5′-diphosphate; AMP = adenosine-5′-monophosphate; ATP = adenosine-5′-triphosphate; GTP = guanosine-5′-triphosphate; NAD = oxidized nicotinamide-adenine dinucleotide; DNP = 2,4-dinitrophenol; PCP = pentachlorophenol; OLIGO = oligomycin; MAL = malonate; TELL = tellurite; GLUT = glutamate; α-OXOGLUT = α-oxoglutarate; RKM = rat kidney mitochondria.

In the present paper some characteristic features of glutamate oxidation in rat kidney mitochondria are described, which shed further light on this still unclear process. In particular it has been shown that the availability of preexisting endogenous oxaloacetate and a continuous formation of GDP are critical conditions for channelling glutamate oxidation through the transamination rather than the deamination route.

Materials and methods

Rat kidney mitochondria were prepared by the procedure of Schneider and Hogeboom [4] in 0.25 M sucrose and 1 mM EDTA. The amount of protein was determined by the biuret method, as described by Gornall [5]. Oxygen uptake was measured polarographically with a Clark electrode coupled to a Sargent model SRG recorder.

Oxaloacetate was determined by malate dehydrogenase according to Hohorst [6], aspartate by malate dehydrogenase and glutamate-oxaloacetate transaminase according to the method of Pfleiderer, Grüber and Wieland [7].

Ammonia was determined by glutamate dehydrogenase (ammonia-free) in neutralized acid axtracts according to the method of Kirsten, Gerez and Kirsten [8]. ATP was determined by the method of Steiner and Williams [9] and oxidized pyridine nucleotides ($NAD^+ + NADP^+$) by the fluorimetric procedure of Ciotti and Kaplan [10].

All enzymatic determinations were performed using a Gilford model 2400 spectrophotometer or an Aminco DW-2 UV-Vis spectrophotometer.

Materials: enzymes were purchased from Boehringer, Mannheim.

All other chemicals were of the highest grade available commercially.

Results

As shown in fig. 1, rat kidney mitochondria actively oxidized glutamate, both in state 3 and in the uncoupled state (trace A). As previously observed by Sallis *et al.* [11], DNP-stimulated respiration was greatly diminished in the presence of oligomycin (trace B). The successive addition of ADP had little, or no effect in restoring the oligomycin inhibited oxygen uptake (trace B). On the contrary, the inhibition of oxygen uptake produced by oligomycin + DNP was completely reversed by added oxaloacetate (trace B). The effect of added oxaloacetate was practically absent when ADP addition was omitted (trace not shown). However, as indicated by trace C of fig. 1, when ADP was added to the system prior to DNP, the uncoupler completely reversed the oligomycin inhibition of respiration and exogenous oxaloacetate was no longer required for the maximum respiration rate. Trace D shows that the effect of DNP in stimulating the respiration was much lower when ADP was added immediately prior to the uncoupler. It was generally observed that

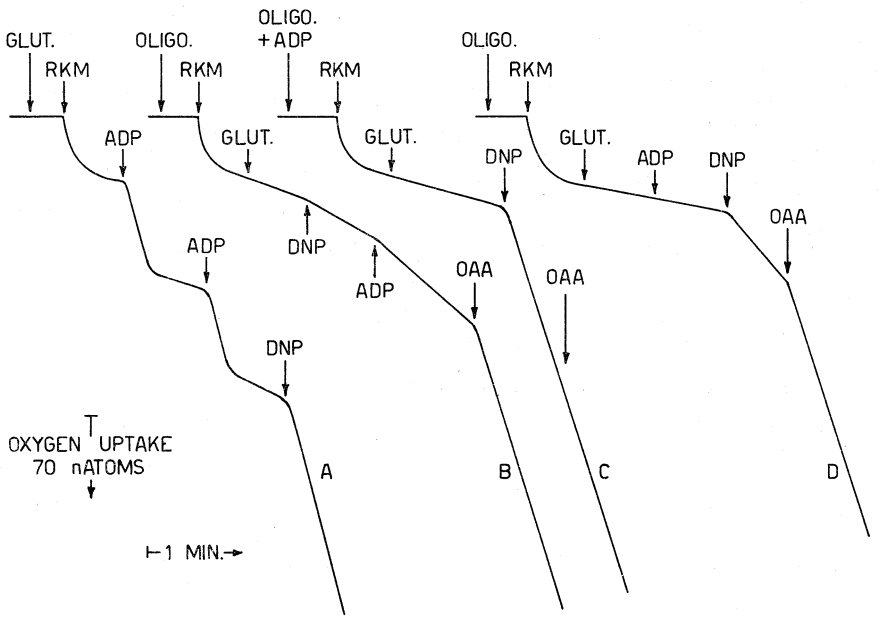

Fig. 1. – Glutamate oxidation by rat kidney mitochondria.

6 mg protein of RKM were incubated in the following medium: 11.7 mM K_2HPO_4, 2.7 mM KH_2PO_4, 10.8 mM NaF, 23.4 mM NaCl, 52.2 mM KCl, 5.4 mM $MgCl_2$ to a final volume of 2 ml at 25°. *Additions*: 10 mM glutamate, 120 μM ADP (trace A) and 1.25 mM ADP (other traces), 50 μM DNP, 17.5 mM oxaloacetate (OAA), 12 μM oligomycin.

the effectiveness of the uncoupler in restoring the oxygen uptake inhibited by oligomycin was greater, the longer was the time interval following the previous addition of ADP (compare trace C and D).

A reasonable explanation for these observations is offered by data reported in Table I. These results show that during incubation in the presence of DNP, all endogenous oxaloacetate was displaced from the mitochondria into the suspending medium.

TABLE I.

Action of DNP *on intra- and extramitochondrial oxaloacetate* [a].

FRACTION	CONDITIONS	OXALOACETATE (μ moles per mg prot.)
Supernatant	— DNP	0
	+ DNP	0.11
Pellet	— DNP	0.13
	+ DNP	0

(*a*) Incubation at 25° for 5 minutes in the medium described in the legend of fig. 1 plus 10 mM glutamate, 1.25 mM ADP, 12 μM oligomycin and 50 μM DNP were present. RKM 6 mg protein. After incubation the reaction was stopped by rapid centrifugation (at 0°, 12.000×g for 3 minutes). Supernatant and pellet fractions thus separated were deproteinized by addition of perchloric acid to a final concentration of 4 percent.

It becomes now clear that the inhibition of oxygen uptake in the presence of DNP and oligmycin is secondary to the unavailability of oxaloacetate induced by the uncoupler and to the consequent inhibition of the transamination pathway for glutamate oxidation.

Furthermore, as is shown in Table II, when glutamate oxidation was prevented by DNP plus oligomycin (compare trace B in fig. 1), very small amounts of aspartate and of ammonia were formed, indicating that both the transamination and the deamination routes for glutamate oxidation are inhibited.

TABLE II.

Aspartate and ammonia formed from glutamate oxidation [a].

CONDITIONS	ASPARTATE (μ moles per min.)	AMMONIA (per mg prot.)
ADP	14.00	2.24
OLIGO + DNP	1.40	2.30
OLIGO + DNP + ADP	4.75	2.69
OLIGO + DNP + MAL	traces	1.54
OLIGO + DNP + TELL + NAD^+	traces	17.35

(*a*) Incubation for 10 minutes at 25° in the medium described in the legend of fig. 1. RKM 6 mg proteim. *Additions*: 10 μM glutamate, 1.25 mM ADP, 12 μM oligomycin, 50 μM DNP, 5 mM malonate, 1.5 mM tellurite, 1.5 mM NAD^+. Reactions were stopped by adding 4 percent perchloric acid.

When ADP was added before DNP, that is, under the conditions of trace C (fig. 1), more aspartate was formed, while ammonia formation remained unchanged. From Table II it can also be observed that with the addition of malonate, which makes oxaloacetate unavailable by preventing its formation from α-oxoglutarate, aspartate formation was blocked, but ammonia formation did not increase over the values found in the absence of malonate. Therefore when endogenous oxaloacetate is unavailable, either because it was displaced out of the mitochondria by the uncoupler, or because its formation through the Krebs cycle was prevented by malonate, oxygen uptake, aspartate and ammonia formation were inhibited.

In the light of these results it would appear that glutamate dehydrogenase activity is almost silent under the described conditions.

Incidentally, Table II shows that the amount of aspartate formed in the presence of malonate was lower than that in the presence of DNP. This would mean that the loss of oxaloacetate produced by the uncoupler was partially compensated by the neoformation of oxaloacetate from its precursors in the Krebs cycle.

We have previously found that 1 mM tellurite [12] inhibits the activity of all mitochondria NAD linked dehydrogenases and that this inhibition can be relieved, with the exception of α-oxoglutarate and pyruvate dehydrogenases, by addition of NAD^+ [13].

Indeed, as shown in fig. 2 (traces B and C), tellurite inhibited both glutamate and α-oxoglutarate oxidation by rat kidney mitochondria. However, as expected, addition of NAD^+ restored oxygen uptake in the presence of glutamate, but not in the presence of α-oxoglutarate (trace C).

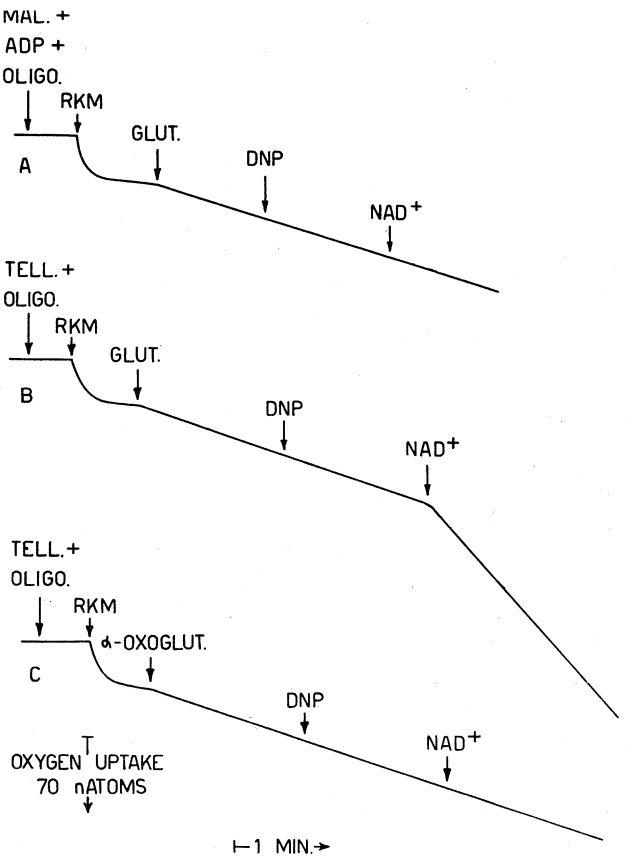

Fig. 2. – Glutamate and α-oxoglutarate oxidation by rat kidney mitochondria.

The conditions of incubation and the medium are described in the legend of fig. 1. *Additions*: 10 mM glutamate, 12 μM oligomycin, 50 μM DNP, 1.5 mM NAD^+. When present: 1.25 mM ADP, 5 mM malate, 1.5 mM tellurite, 10 μM α-oxglutarate.

Consequently, since the oxidation of α-oxoglutarate is blocked by tellurite, the oxygen uptake in the presence of glutamate is exclusively due to glutamate dehydrogenase activity. This is confirmed by the formation of a consistent amount of ammonia observed in the presence of tellurite (see Table II). Therefore, addition of tellurite plus NAD^+ to mitochondria offers a convenient experimental condition for preventing glutamate transamination and for unmasking glutamate dehydrogenase activity.

De Haan *et al.* [1] have correlated the percentage contribution of glutamate dehydrogenase to the metabolism of glutamate by rat liver mitochondria with the availability of $NAD(P)^+$. Since tellurite maintains the mitochondrial NAD(P) in the oxidized state, it was thought that the increased ammonia production in the presence of tellurite could be the consequence of a higher availability of $NAD(P)^+$ for glutamate dehydrogenase. However, as shown in Table III, no significant difference in the amount of mitochondrial $NAD(P)^+$ has been observed in the presence and in the absence of tellurite. In both cases, a very high amount of $NAD(P)^+$ was observed. Evidently the action of the uncoupler masked that of tellurite in keeping the NAD(P) pool in a highly oxidized state.

An alternative explanation is that mitochondrial glutamate dehydrogenase *in situ*, as well as at the molecular level, is inhibited by GTP [14] produced by the substrate level phosphorylation. That this could be the case is indicated by data of Table III, which show that in the presence of tellurite plus malonate a much lower amount of ATP than in the presence of malonate alone was produced.

TABLE III.

ATP *formation and* $NAD(P)^+$ *concentration during glutamate oxidation.*

CONDITIONS	$NAD(P)^+$ (a)	ATP (b)
	(μ moles per mg prot.)	
NONE	2.20	—
ADP	2.50	—
MAL. + PCP	5.24	26.9
TELL. + PCP	5.15	8.7

(a) Incubation at 25° for 10 minutes in the medium described in the legend of fig. 16. RKM 6 mg protein. *Additions*: 10 mM glutamate, 12 μM oligomycin in all samples and when present 5 mM malonate, 50 μM PCP, 1.5 mM tellurite, 1.25 mM ADP.

(b) Incubation at 25° for 30 minutes in the medium described in the legend of fig. 1. RKM 6 mg protein. *Additions*: 10 mM glutamate, 12 μM oligomycin, 50 μM PCP, 3 mM NAD^+, 1.25 mM AMP, 25 mM glucose, 10 I.U. hexokinase and when present 5 mM malonate, 3 mM tellurite.

(a–b) Reactions were halted by adding 4 percent perchloric acid.

Undoubtedly ATP production is an expression of substrate level phosphorylation: in fact the relative experiments have been carried out in the presence of DNA and oligomycin, that is, under those conditions in which phosphorylation linked to the respiratory chain is suppressed.

These results would indicate that tellurite stimulates glutamate dehydrogenase activity by preventing the formation of GTP, which is inhibitory, rather than by maintaining the mitochondrial NAD(P) pool in an oxidized state.

DISCUSSION

The dominance of the transamination pathway for glutamate oxidation, already observed by previous Authors [15–16] in mitochondria of other tissues, appears very evident also in kidney mitochondria in which, unlike liver mitochondria, glutamate oxidation is strongly inhibited by addition of DNP plus oligomycin. The cause of this inhibition seems to be the lack of phosphate acceptor for substrate level phosphorylation. This explanation is strongly supported by the observation that addition of ADP before the uncoupler prevents the inhibition of glutamate oxidation. It is very likely that exogenous ADP, by supplying the substrate for GTP–ADP phosphotransferase, ensures a continuous formation of GDP, necessary for the oxidation of α–oxoglutarate deriving from glutamate.

However, when ADP was added after the uncoupler, in spite of the induced availability of phosphate acceptor, glutamate was not oxidized unless exogenous oxaloacetate was added. This indicates that an initial transamination of glutamate with endogenous oxaloacetate is a necessary preliminary condition for glutamate oxidation.

In fact, under the described conditions, no endogenous oxaloacetate is available for the reason that DNP causes the efflux of all mitochondrial oxaloacetate into the external medium. In the absence of sparking amounts of oxaloacetate, glutamate cannot transaminate and in turn the formation of new oxaloacetate, necessary to ensure the continuation of the process is prevented. When ADP is added to the system before the uncoupler, preliminary transamination occurs due to the oxaloacetate originally present. The newly formed α-oxoglutarate, owing to the availability of the phosphate acceptor, is oxidized producing a continuous supply of oxaloacetate.

De Haan *et al.* [1] have previously observed that uncouplers inhibit the transamination pathway of glutamate oxidation. However they suggested that in the absence of energy, oxaloacetate is not removed with sufficient rapidity from the malate dehydrogenase compartment to aspartate transaminase.

On the contrary, as can be deduced from the increased ammonia production, tellurite stimulates the activity of mitochondrial glutamate dehydrogenase and inhibits the substrate linked phosphorylation. Therefore it can be assumed that tellurite does not stimulate glutamate dehydrogenase « per se », but by preventing GTP accumulation through the block of α-ketoglutarate dehydrogenase activity.

A further demonstration that the deamination pathway of glutamate oxidation is silent in isolated kidney mitochondria is provided by the experiments with malonate. Malonate blocks aspartate production as well as oxygen uptake, but does not affect ammonia formation over values found in the absence of malonate. This means that no competition between the two routes of glutamate utilization exists and that also when the transamination reaction is inhibited, the dehydrogenation of glutamate is silent.

Tellurite, as can be deduced from the stimulation of ammonia production, stimulates the activity of mitochondrial glutamate dehydrogenase. The strong decrease in the formation of ATP produced by tellurite under conditions in which only the substrate linked phosphorylation was operative, strongly indicates that glutamate dehydrogenase is inhibited by accumulated GTP [4].

The action of tellurite on glutamate oxidation is summarized in the scheme of fig. 3.

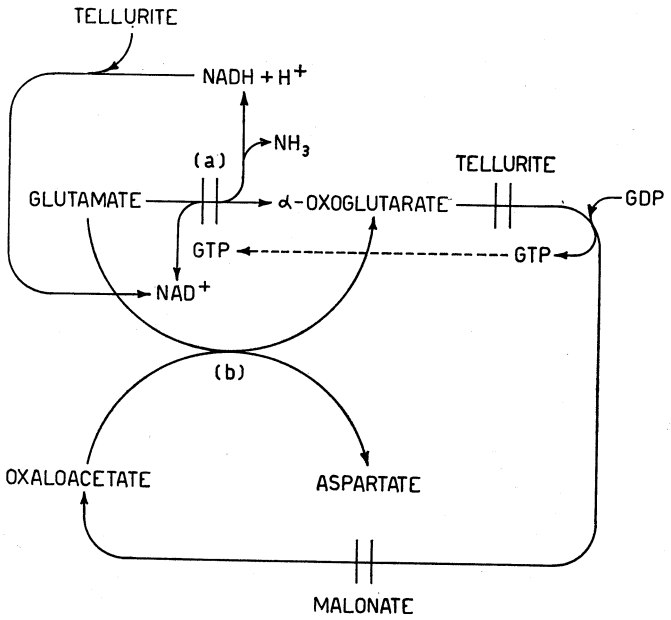

Fig. 3. – Model of action of tellurite and malonate on glutamate oxidation in rat kidney mitochondria.
(a) GLUTAMATE DEHYDROGENASE.
(b) GLUTAMATE OXALOACETATE TRANSAMINASE.

The present results confirm from a general point of view the recent findings of La Noue *et al.* [16] and in particular demonstrate that in kidney mitochondria glutamate utilization through the dehydrogenation and the transamination routes is controlled by the availability of mitochondrial oxaloacetate and by the GTP level. Oxaloacetate availability and a high level of GTP favour the transamination route, while oxaloacetate unavailability and a low level of GTP favour the dehydrogenation route.

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