Transport of Anionic Substrates and Glutamate Metabolism in Mitochondria from Ascites Tumor Cells

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SUMMARY

A study is presented of α-oxoglutarate and glutamate transport and of glutamate oxidation in ascites tumor cell mitochondria.

Kinetics analysis of α-oxoglutarate transport in mitochondria from two strains of Ehrlich ascites tumor cells, the hyperdiploid and the hyperdiploid Lettré mutant, shows that the activity of the α-oxoglutarate carrier and its affinity for substrates are higher in the mutant than in the wild strain.

Evidence is presented showing the occurrence of carrier-mediated glutamate-OH exchange-diffusion in mitochondria from both strains. The activity of the glutamate carrier is apparently higher in the hyperdiploid Lettré mutant.

Glutamate oxidation occurs mainly through transamination to aspartate in both tumor strains. The rate of deamination in the two strains correlates directly with the level of glutamate dehydrogenase (EC 1.4.1.3.), which is higher in the wild than in the mutant strain. Thus glutamate dehydrogenase per se, and not glutamate penetration, constitutes the control step for glutamate deamination.

Data are presented on the transamination in mitochondria of glutamate with externally added oxaloacetate (arsenite present) that exclude an obligatory transport of oxaloacetate on the α-oxoglutarate carrier.

INTRODUCTION

Ascites tumor cells exhibit, like other tumors, a high rate of aerobic glycolysis (35, 40, 41). The nature of this metabolic behavior is, however, far from being understood.

The proposal that the activity of respiratory-chain oxidative phosphorylation is impaired in tumor mitochondria does not appear to have experimental support (35, 37, 40). However, a better understanding is needed of the oxidation pathways of the specific respiratory substrates in tumor mitochondria.

A crucial point in the regulation of aerobic glycolysis and energy metabolism, in general, is represented by the transport of metabolites and hydrogen between the cytosol and mitochondria. Products of the breakdown of carbohydrates, lipids, and proteins must in fact move from the cytosol to mitochondria in order to be aerobically oxidized with concomitant phosphorylation of ADP to ATP. Cytosolic NADH, produced by glycolysis, cannot be directly oxidized by mitochondria; this process appears, however, to be assured by substrate shuttles across the mitochondrial membrane. On the other hand, a number of metabolites are exported from the mitochondrial matrix to the cytosol (see Refs. 23 and 34 for review). The possibility has been entertained that inefficiency of the hydrogen shuttle mechanisms (Ref. 39; see, however, Ref. 13) might be involved in the enhanced accumulation of lactic acid in respiring tumor cells.

Many of the substances that have to permeate the mitochondrial membrane bear at physiological pH's net negative charge. The inner mitochondrial membrane is not freely permeable to these anionic substrates, but it contains specific transport systems that mediate the movement of anionic substrates between the 2 aqueous spaces separated by the membrane (7, 17, 23, 29, 34).

We have undertaken an investigation of anion-transporting systems in mitochondria of ascites tumor cells with the aims of further elucidating their regulatory functions and of examining their possible involvement in metabolic derangements of cancer (see also Refs. 19 and 37).

In this paper a study is reported of the transport of α-oxoglutarate and glutamate and of the oxidation pathways of glutamate in ascites tumor cells. Mitochondria of 2 strains of Ehrlich ascites tumor cells, the hyperdiploid and the hyperdiploid Lettré mutant, possess both the α-oxoglutarate (31) and the glutamate carrier.

The results obtained provide natural evidence in favor of a direct role of the α-oxoglutarate carrier in the malate-aspartate shuttle in ascites tumor cells. Glutamate is shown to be actively oxidized by ascites tumor cells through transamination to aspartate. The deamination pathway is, on the other hand, severely inhibited. The role of the glutamate and α-oxoglutarate carrier in the control of glutamate oxidation is examined.

MATERIALS AND METHODS

Two strains of Ehrlich ascites tumor cells, the hyperdiploid and the hyperdiploid Lettré mutant, were maintained by i.p. transplantation in albino Swiss mice. Mitochondria were prepared by the method of Kobayashi et al. (18) with slight modifications. Rat liver mitochondria were prepared...
by the method of Chance and Hagihara (5).

Determination of the Exchange-Diffusion of Mitochondrial [α-¹⁴C]Oxoglutarate with External Malate. The initial rate of [α-¹⁴C]oxoglutarate efflux from mitochondria was measured by the multilayer centrifugation-filtration technique as described by Pfaff (Ref. 36; see also Ref. 29). Mitochondria of both strains of Ehrlich ascites tumor cells (2.5 to 3.5 mg proteins) were preincubated in a reaction medium containing the following standard components: 150 mM sucrose, 30 mM Tris-HCl, 1 mM MgCl₂, and 0.5 mM EDTA. In addition 1 mM arsenite, 1 mM ADP, 1 mM potassium phosphate, and carrier-free [α-¹⁴C]oxoglutarate were present.

After 1 min, 10 mM glutamate was added, followed 3 min later by simultaneous addition of 1 μg rotenone, 0.5 μg antimycin, and 10 μg oligomycin. Final volume was 1 ml; final pH was 7.2; temperature was 20°. Two min after the addition of the inhibitors, mitochondria were layered on the top of a 2nd incubation layer at 4° and then spun down through this layer into HCIO₄ by rapid centrifugation.

The 2nd incubation layer was of the same composition as the preincubation mixture (except the labeled substrate), plus different concentrations of malate. A discontinuous density gradient increasing toward the bottom of the centrifuge tube was made by addition of dextran to the 2nd layer. This was separated from the HCIO₄ at the bottom of the tube by a layer of silicon oil (29, 36). The exposure time of the mitochondria to the 2nd incubation layer was estimated to be about 15 sec by measuring the oxidation of 3-hydroxybutyrate to acetoacetate. [α-¹⁴C]Oxoglutarate was measured both in HCIO₄ extracts of the mitochondrial pellet and in the supernatant. The substrate content of the matrix space was calculated by correcting the amount in the mitochondrial extract with that in the sucrose-permeable space plus adherent supernatant. This was determined with [¹⁴C]sucrose. The total H₂O of the mitochondrial pellet was determined with H₂¹⁴O. The mitochondrial content of α-oxoglutarate was also determined enzymatically (see assays below).

Determination of the Exchange-Diffusion of Mitochondrial [¹⁴C]Malate with External α-Oxoglutarate. The efflux of mitochondrial [¹⁴C]malate induced by external α-oxoglutarate was measured essentially as described for the [α-¹⁴C]oxoglutarate-malate exchange. Mitochondria (about 2.5 mg protein for both strains, Ehrlich hyperdiploid and Ehrlich hyperdiploid Lettré mutant) were preincubated for 1 min in a reaction medium containing 150 mM sucrose, 50 mM Tris-HCl, 1 mM MgCl₂, 0.5 mM EDTA, 1 μg rotenone, 0.5 μg antimycin, and 10 μg oligomycin. After 1 min, 1 mM [¹⁴C]malate was added. Final volume was 1 ml; final pH was 7.2; temperature was 20°. Two min after the addition of the labeled substrate, the mitochondria were centrifuged at 4° through a 2nd incubation layer containing the standard components as the preincubation mixture (except the labeled substrate), plus different concentrations of α-oxoglutarate. [¹⁴C]Malate was measured as described for [α-¹⁴C]oxoglutarate.

Passive Uptake of Glutamate into Mitochondria. Swelling of mitochondria due to passive influx of glutamate was followed at 546 nm in an Eppendorf filter photometer at 20° in 2 ml medium containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.8 μg rotenone, 3 μg antimycin, and 100 mM ammonium lactate. Enough mitochondria were added to give an initial A₄₅₆ of 0.8 to 0.9.

Assays. α-Oxoglutarate and ammonia were determined in the neutralized perchloric acid extracts by enzymatic methods as described elsewhere (33), using an Eppendorf spectrophotometer or the split-double beam Amino-Chance spectrophotometer at 340 to 375 nm. Aspartate was determined in the neutralized acid extracts with α-oxoglutarate, NADH, malate dehydrogenase (EC 1.1.1.37), and aspartaseaminotransferase (EC 2.6.1.1.), using an Eppendorf photometer. The reaction mixture (2 ml) contained 40 mM triethanolamine hydrochloride, 8 mM MgSO₄, 4 mM EDTA, 5 mM α-oxoglutarate, 70 μM NADH, 10 μM malate dehydrogenase, and an aliquot of the neutralized extract; the reaction was started by the addition of 0.1 mg aspartate aminotransferase. Proteins were determined by the usual biuret method.

Chemicals. All radiochemicals were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. N-Ethylmaleimide was obtained from British Drug Houses Ltd., Poole, Dorset, England. Enzymes, coenzymes, and substrates for metabolite determination were obtained from Boehringer und Sohne, Mannheim, West Germany. All other chemicals were from Sigma Chemical Company, St. Louis, Mo. or from E. Merck, Darmstadt, West Germany.

RESULTS

Anion Transport. The transport of α-oxoglutarate across the mitochondrial membrane is carrier mediated and takes place through a tightly coupled exchange-diffusion with malate and other dicarboxylates (8, 27, 29). The activity and the saturation kinetics of the α-oxoglutarate translocator in ascites tumor cell mitochondria were studied following either the exchange of intramitochondrial α-oxoglutarate with externally added malate or the exchange of intramitochondrial malate with external α-oxoglutarate (see Ref. 29). In the 1st case α-oxoglutarate was produced in the mitochondrial matrix by deamination of added glutamate. Carrier-free [α-¹⁴C]oxoglutarate was added to label the α-oxoglutarate produced. The system also contained arsenite to prevent further oxidation of α-oxoglutarate. α-Oxoglutarate-loaded mitochondria were then exposed to a 2nd incubation medium containing, in separate samples, varying concentrations of malate.

Controls showed that, in the conditions used, the decline in the rate of α-oxoglutarate efflux was negligible in the 1st 15 sec following malate addition.

In Chart 1 the results of a typical experiment of this series, carried out on mitochondria from Ehrlich hyperdiploid ascites tumor cells, are presented in the form of a Line-weaver-Burk plot. The efflux of intramitochondrial [α-¹⁴C]oxoglutarate driven by external malate follows saturation kinetics. The plot gives a V₉₅ of 31 nmoles of α-oxoglutarate driven out per min per mg protein and a Kₘ for malate of 100 μM.

To follow the kinetics of the reverse reaction, i.e., the efflux of mitochondrial malate in exchange with externally
added α-oxoglutarate, mitochondria were preloaded with [14C]malate in the presence of inhibitors of metabolism and then exposed to a 2nd medium containing varying concentrations of α-oxoglutarate. This procedure turned out to be more practical than the previous one and to give more reproducible results; it was therefore adopted for systematic study of the α-oxoglutarate transport in mitochondria from the 2 strains of ascites tumor cells.

Chart 2 illustrates the saturation kinetics of the malate, α-oxoglutarate exchange-diffusion in the 2 strains. The reaction followed in both strains saturation kinetics. The V_max obtained in the Ehrlich hyperdiploid strain is practically equal to that measured by following the α-oxoglutarate, malate exchange (see Chart 1). This confirms the reliability of the methods used. Furthermore in the Ehrlich hyperdiploid strain the K_M for external α-oxoglutarate (79 μM) is very close to that for external malate (100 μM; see Chart 1).

Comparison of the kinetics of the malate, α-oxoglutarate exchange-diffusion in the 2 strains shows that the V_max of the malate, α-oxoglutarate exchange in the Ehrlich hyperdiploid Lettré mutant strain is higher and the K_M is lower than in the Ehrlich hyperdiploid strain. The significance of these differences was documented by statistical analysis (31). It is therefore apparent that both the content of the carrier molecules in the membrane and the affinity of the carrier for α-oxoglutarate are, in mitochondria of the mutant, higher than in those of the wild strain.

In liver mitochondria glutamate transport across the inner membrane has been shown to take place either through exchange-diffusion with hydroxyl ions (that is formally equivalent to glutamate-H+ symport) (22, 24) or exchange-diffusion with aspartate (1). The former reaction can be easily studied by following swelling of mitochondria suspended, in the presence of respiratory inhibitors, in isoosmotic ammonium glutamate.

Chart 3 shows that both Ehrlich hyperdiploid and Ehrlich hyperdiploid Lettré mutant strain mitochondria undergo large-amplitude swelling when suspended in 100 mM ammonium L-glutamate. For comparison the behavior of rat liver mitochondria is also shown. Both the rate and the extent of swelling of ascites cell mitochondria are smaller than those observed for rat liver mitochondria.

Furthermore, at a concentration that completely blocks the swelling of rat liver mitochondria, N-ethylmaleimide, an inhibitor of the glutamate-OH− exchange (22, 24), causes only partial inhibition of swelling of ascites cell mitochondria. Thus the glutamate-OH− exchange system is less active in mitochondria of tumor cells than in rat liver mitochondria. Refs. 22 and 24). Judging from the extent of the N-ethylmaleimide-sensitive swelling, it might be inferred that the glutamate-OH− exchange is more active in the Ehrlich hyperdiploid Lettré mutant strain than in the Ehrlich hyperdiploid strain. The residual N-ethylmaleimide-insensitive swelling of ascites tumor mitochondria is probably due to partial damage of the osmotic barrier in ascites cell mitochondria.

Glutamate Oxidation. Glutamate can be oxidized in mitochondria by 2 pathways: the deamination pathway, the activity of which is measured by ammonia production, and the transamination pathway, which leads to aspartate formation (2, 21, 33).

Chart 4 illustrates the time course of aspartate and NH₃ formation during glutamate oxidation by ascites cell mitochondria in the presence of ADP and Pₐ (State 3 (6)). It can be seen that the activity of the deamination pathway, which at the beginning of the incubation (1 min) is as high as that of the transaminase pathway, sharply declines with the incubation time, whereas the latter remains fairly constant.

The rate of glutamate deamination, measured at 1 min incubation is 10 nmoles/min/mg protein in the Ehrlich hyperdiploid strain mitochondria and 5 nmoles in the Ehrlich hyperdiploid Lettré mutant strain as compared to a rate of 20 nmoles in rat liver mitochondria (32, 33). This correlates with the estimated levels of glutamate dehydrogenase which amount to about 900 nmoles NADH oxidized per min per mg protein in rat liver mitochondria, 183 in Ehrlich hyperdiploid strain. Ehrlich strain mitochondria indicated 150 μM N-ethylmaleimide was present. Experimental details are given under "Materials and Methods."

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hyperdiploid strain mitochondria, and 100 in Ehrlich hyperdiploid Lettré mutant strain mitochondria (O. Dionisi, unpublished data).

Chart 5 shows the effect of malonate on deamination of glutamate in the presence of arsenite, which suppresses further oxidation of $\alpha$-oxoglutarate. Malonate, which exchanges with $\alpha$-oxoglutarate (8, 27) and thus prevents accumulation of the oxo acid in the mitochondrial matrix, causes a marked stimulation of glutamate deamination (8). However, the rate of deamination remains higher in the Ehrlich hyperdiploid strain (higher level of glutamate dehydrogenase) than in the Ehrlich hyperdiploid Lettré mutant strain (lower level of glutamate dehydrogenase). The activity of the glutamate carrier is apparently higher in the Ehrlich hyperdiploid Lettré mutant strain than in the Ehrlich hyperdiploid strain mitochondria.

Chart 6 shows the concentration dependence curve for the stimulatory effect of malonate on glutamate deamination in the presence of arsenite. Half-maximal stimulation is obtained at 300 $\mu$M malonate in the Ehrlich hyperdiploid strain and at 150 $\mu$M in the Ehrlich hyperdiploid Lettré mutant strain mitochondria. This confirms that the $\alpha$-oxoglutarate carrier has in the Ehrlich hyperdiploid Lettré mutant strain a higher affinity for its substrates than in the Ehrlich hyperdiploid strain.

In the presence of arsenite, which blocks the transamination pathway of glutamate oxidation at the level of $\alpha$-oxoglutarate dehydrogenase, glutamate transamination depends upon the external supply of oxaloacetate. Chart 7 shows the oxaloacetate concentration dependence curve for transamination of glutamate with externally added oxaloacetate.

It can be seen that the transamination reaction follows saturation kinetics in both strains. The $V_{max}$ values obtained in these experiments are much lower than those estimated for the transamination reaction in disrupted mitochondria (13). Glutamate-oxaloacetate transaminase is located within the osmotic barrier of mitochondria (matrix and/or internal side of the inner mitochondrial membrane) (28). Evidently, the rate of transamination of externally added glutamate and oxaloacetate is limited by penetration of oxaloacetate and glutamate into the mitochondria.

It is disputed whether oxaloacetate is transported in mitochondria by the $\alpha$-oxoglutarate (25) and/or the dicarboxylate carrier (14). The observation (Chart 7) that the apparent affinity of oxaloacetate for its entry into mitochondria is slightly higher in the Ehrlich hyperdiploid strain ($K_m = 475 \mu$M) than in the Ehrlich hyperdiploid Lettré mutant strain ($K_m = 550 \mu$M), whereas the affinity of the $\alpha$-oxoglutarate
carrier for its substrates is higher in the Ehrlich hyperdiploid Lettré mutant strain than in the Ehrlich hyperdiploid strain (Chart 2; see also Ref. 31), indicates that oxaloacetate is not exclusively transported by the α-oxoglutarate carrier. Additional support for this conclusion is provided by the experiment of Chart 8. In this experiment the effect of malonate on the transamination of externally added glutamate and oxaloacetate in mitochondria of both strains was examined. If oxaloacetate entered the mitochondria through an obligatory exchange with α-oxoglutarate generated in the matrix, the transamination should be inhibited by malonate which would compete with oxaloacetate for exchange with mitochondrial α-oxoglutarate. However, the experiment shows that malonate has no significant effect on the transamination of glutamate with oxaloacetate. Possible inhibition by malonate of oxaloacetate entry on the dicarboxylate carrier (in exchange with P₄) might be compensated by promotion of α-oxoglutarate efflux on the α-oxoglutarate carrier.

DISCUSSION

The mechanisms proposed for hydrogen transfer between the mitochondrial and cytosolic pools of NAD and NADP include the malate-oxaloacetate (9, 15, 20), the α-glycerophosphate-dihydroxyacetone phosphate (42), and the malate-aspartate shuttle (3) for intramitochondrial oxidation of cytosolic NADH and the α-oxoglutarate-(iso)citrate shuttle (26) for the transfer of NADP reducing equivalents between cytosol and mitochondria. α-Oxoglutarate-malate exchange-diffusion on the α-oxoglutarate carrier would be involved in both the malate-aspartate and α-oxoglutarate-(iso)citrate shuttles.

The 2 strains of ascites tumor cells used in this study exhibit equal rates of aerobic glycolysis; however, the activity of the α-glycerophosphate cycle is strongly depressed in the mutant strain compared to the wild strain (10, 13).

Very recently, Eboli et al. (12) have obtained data that would exclude any major role of the malate-oxaloacetate shuttle for the mitochondrial oxidation of cytosolic NADH in ascites tumor cells.

The malate-aspartate shuttle is based on the existence in the cell of cytosolic and mitochondrial malate dehydrogenase and glutamate-oxaloacetate transaminase (3). Cytosolic malate dehydrogenase and glutamate-oxaloacetate transaminase have in the Ehrlich hyperdiploid Lettré mutant strain a higher activity than in the Ehrlich hyperdiploid strain (13). This, together with the observation (11, 12) that aminooxyacetate, an inhibitor of glutamate-oxaloacetate transaminase, causes a strong inhibition of the oxidation of lactate to pyruvate in the Ehrlich hyperdiploid Lettré mutant strain but not in the Ehrlich hyperdiploid strain, indicates that the malate-aspartate shuttle is highly active for the oxidation of cytosolic NADH in the mutant but not in the wild strain. The observation that the activity of the α-oxoglutarate carrier in both strains correlates directly with the activity of the malate-aspartate shuttle (see also Ref. 31) provides natural evidence in favor of a direct involvement of the α-oxoglutarate carrier in the malate-aspartate shuttle.

Furthermore, the above data would suggest the existence in the cell of a self-regulatory mechanism that allows the mutative depression of 1 of the 2 pathways of intramitochondrial oxidation of cytosolic NADH to be compensated for by an increased activity of the other.

It would be interesting to extend these studies to the tricarboxylate carrier to elucidate its role and that of the α-oxoglutarate carrier in the transfer of NADP-reducing equivalent across the mitochondrial membrane.

The present study shows that, as in mitochondria isolated from mammalian tissues (2, 21, 33), transamination to aspartate represents the main route for glutamate oxidation in mitochondria from ascites tumor cells. In fact, in tumor cell mitochondria, as in rat liver mitochondria (32, 33), the deamination pathway is almost as active as the transamination pathway at the beginning of the incubation; however, net deamination of glutamate slows down and comes to a halt with time while the rate of transamination remains fairly constant. Papa et al. (30, 33, 38) have presented evidence showing that this inhibition of glutamate deamination in isolated rat liver mitochondria is due to accumulation of NADPH. The inhibition might result from a preferential reaction of glutamate dehydrogenase with NADP (38) (the isolated enzyme reacts with both NAD and NADP) and/or a mass action of NADPH (16).

Comparison of the activity of the deamination pathway in mitochondria of the 2 tumor strains and rat liver shows that the initial rate of deamination, although extremely low when referred to the content of glutamate dehydrogenase, is directly related to the enzyme level in mitochondria. On the other hand, the time-dependent decline of net glutamate deamination is larger and faster in ascites cell mitochondria (Chart 9), especially in the Ehrlich hyperdiploid Lettré mutant strain, which has the lower content of glutamate dehydrogenase, than in rat liver mitochondria. These observations lend support to the proposal that the inhibition of the glutamate dehydrogenase pathway is due to enzyme inhibition (or deactivation) by accumulation of NADPH (30, 33, 38) and not simply to equilibrium effects.

Net uptake of glutamate apparently takes place in mitochondria through an exchange-diffusion reaction with hydroxyl ions (or H⁺ symport) mediated by a N-ethylmaleim-
ide-sensitive carrier. In rat liver mitochondria the activity of this system and its affinity for glutamate are considerably lower (4, 24) than those reported for other mitochondrial anion translocators. Recently, Kovacevic and Morris (19) reported that mitochondria isolated from ascites cells and rapidly growing hepatomas are relatively impermeable to glutamate. The experiment of Chart 3 shows the occurrence of an N-ethylmaleimide-sensitive glutamate-\( \text{OH} \) exchange across the membrane of mitochondria from ascites tumor cells. However, the activity of the system in these mitochondria is lower than in rat liver mitochondria. Furthermore the exchange is apparently more active in the Ehrlich hyperdiploid Lečtre mutant strain than in the Ehrlich hyperdiploid strain.

The finding that the rate of glutamate deamination is, under the various conditions tested (see Charts 4 and 5), lower in the Ehrlich hyperdiploid Lečtre mutant strain than in the Ehrlich hyperdiploid strain, which has a higher content of glutamate dehydrogenase but which exhibits a lower activity of the glutamate translocator than does the Ehrlich hyperdiploid Lečtre mutant strain, indicates that glutamate penetration into mitochondria does not constitute the rate-limiting step for glutamate deamination.

In conclusion, this investigation shows the occurrence of alterations in the activity and kinetic parameters of anion translocators in tumor mitochondria with respect to normal tissues (cf. Refs. 19, 31, and 37).

These alterations, in conjunction with modifications of metabolic pathways for substrate utilization in mitochondria, might lead to definite derangements in energy metabolism in cancer. In this respect further elucidation of the characteristics of metabolite-transporting systems and of respiratory substrate utilization in tumor mitochondria should contribute to a better understanding of the metabolic behavior of cancer cells.

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