The Pathways of Glutamate and Glutamine Oxidation by Tumor Cell Mitochondria

ROLE OF MITOCHONDRIAL NAD(P)+-DEPENDENT MALIC ENZYME*

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Little evidence has been available on the oxidative pathways of glutamine and glutamate, the major respiratory substrates of cancer cells. Glutamate formed from glutamine by phosphate-dependent glutaminase undergoes quantitative transamination by aerobic tumor mitochondria to yield aspartate. However, when malate is added there is a pronounced decrease in aspartate production and a large formation of citrate and alanine, in both state 3 and 4 conditions. In contrast, addition of malate to normal rat heart, liver, or kidney mitochondria oxidizing glutamate causes a marked increase in aspartate production. Further analysis showed that extramitochondrial malate is oxidized almost quantitatively to pyruvate + CO₂ by NAD(P)+-linked malic enzyme, present in the mitochondria of all tumors tested, but absent in heart, liver, and kidney mitochondria. On the other hand intramitochondrial malate generated from glutamate is oxidized quantitatively to oxalacetate by mitochondrial malate dehydrogenase of tumors. Acetyl-CoA derived from extramitochondrial malate via pyruvate and oxalacetate derived from glutamate via intramitochondrial malate are quantitatively converted into citrate, which is extruded. No evidence was found that malic enzyme of tumor mitochondria converts glutamate-derived malate into pyruvate as postulated in other reports. Possible mechanisms for the integration of mitochondrial malic enzyme and malate dehydrogenase activities in tumors are discussed.

A substantial body of experimental evidence indicates that glutamine is the major respiratory fuel of tumor cells. Glutamine has been shown to be an unusually good substrate for oxidation by tumor cell mitochondria (1) and intact Ehrlich cells oxidize glutamine to CO₂ at higher rates than any other amino acid present (2). Indeed, the mitochondrial glutaminase activity of several rat hepatomas was linearly correlated with the growth rates and degree of malignancy (3). It has been further concluded that glutamine, not glucose, is the major energy source for HeLa cells even in the presence of physiological levels of glucose (4). In fact, many malignant cell lines, as well as some normal cells, do not have an absolute requirement for glucose per se (5-9) and will grow in the presence of other hexoses, in which case lactate production is greatly decreased. Such studies and others (10-12) have therefore cast some doubt on the essentiality of the conversion of glucose to lactate by aerobic glycolysis for tumor cells, although hexoses are nonetheless still required for continual cell growth, apparently as anabolic precursors, particularly of ribose (9, 11-13). These considerations have focused attention on other fuels for energy production in tumor cells and have pointed specifically to glutamine as the major respiratory substrate in all malignant cell lines investigated to date. It may also be noted that glutamine is the most abundant amino acid in both blood plasma and in optimal culture media (12).

Although several previous studies have investigated the capacity of tumor mitochondria to oxidize glutamate (14) and glutamine (1), the precise route(s) of their entry into the tricarboxylic acid cycle, the fate of their carbon skeletons and amino groups, and the regulation of their oxidation have not been examined in detail. This paper describes an extensive investigation of the pathway(s) of glutamine and glutamate oxidation in mitochondria from ascites tumor cells, which are shown to differ substantially from the pathways promoted by mitochondria from normal cells.

MATERIALS AND METHODS

Animals, Tumors, and Reagents—The following ascites tumors were maintained by weekly intraperitoneal injections into the appropriate recipients: the Ehrlich ascites tumor was carried in young adult male Swiss mice (Buckberg, Tomkins Cove, NY); L1210 leukemia cells were propagated in young adult male BDF, mice (Jackson Laboratories, Bar Harbor, ME); the AS30-D hepatoma was maintained in 100-125-g female Sprague-Dawley rats (Harlan Sprague-Dawley, Walkerville, MD); the 22A/H mouse hepatoma was carried in 5-7-week-old female C3H/HeJ mice (Harlan Sprague-Dawley, Walkerville, MD); the 22A/H mouse hepatoma was carried in 5-7-week-old female C3H/HeJ mice (Harlan Sprague-Dawley, Walkerville, MD); the 22A/H mouse hepatoma was carried in 5-7-week-old female C3H/HeJ mice (Harlan Sprague-Dawley, Walkerville, MD); the 22A/H mouse hepatoma was carried in 5-7-week-old female C3H/HeJ mice (Harlan Sprague-Dawley, Walkerville, MD); the 22A/H mouse hepatoma was carried in 5-7-week-old female C3H/HeJ mice (Harlan Sprague-Dawley, Walkerville, MD); 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Glutamate Oxidation in Tumor Mitochondria

The Products of Glutamate Oxidation—It is generally agreed there are two alternative mitochondrial pathways for conversion of glutamate into \( \alpha \)-ketoglutarate; dehydrogenation by glutamate dehydrogenase or transamination with oxalacetate or pyruvate. In order to investigate the relative contributions of these pathways to glutamate oxidation by tumor mitochondria, the potent transaminase inhibitor aminooxyacetic acid was employed. Appropriate control experiments using either solubilized mitochondria, matrix extracts, or purified glutamate dehydrogenase showed that up to 20 mM aminooxyacetate did not affect the activity of glutamate dehydrogenase (assayed in either direction), whereas 0.5 mM aminooxyacetate completely inhibited aspartate and alanine aminotransferase activities. Moreover, aminooxyacetate does not inhibit any of the known transport systems in mitochondria (22). This inhibitor could therefore be safely used to differentiate between the two major pathways of glutamate and glutamine oxidation.

Fig. 1 shows a series of \( O_2 \) uptake traces obtained when Ehrlich tumor mitochondria respire on various substrates in the presence of aminooxyacetate. Note that aminooxyacetate has no effect on state 3 respiration when \( \alpha \)-ketoglutarate is the respiratory substrate (Fig. 1A). Identical results were obtained with pyruvate, citrate, isocitrate, succinate, or malate as sole respiratory substrates. However, aminooxyacetate totally inhibited state 3 oxidation when glutamate was the sole substrate (Fig. 1B). Identical results were obtained with glutamine as sole respiratory substrate (data not shown). Furthermore, addition of glutamine following sequential additions of aminooxyacetate and ADP to mitochondria respiring on glutamate failed to elicit a new state 3 rate, whereas addition of \( \alpha \)-ketoglutarate resulted in a prompt resumption of oxygen consumption (Fig. 1C). Virtually identical results were obtained for the mitochondria from the L1210 leukemia and P1798 thymoma, whereas the state 3 rate of respiration in the 22H and AS30-D hepatoma mitochondria in the presence of aminooxyacetate was only 80% inhibited. Other experiments revealed that the uninhibited respiration in the 22H and AS30-D mitochondria was due to oxidation via glutamate dehydrogenase.

These observations indicated that glutamate (and glutamine) oxidation proceeds in these tumor cell lines primarily via a pathway involving an aminotransferase, presumably aspartate aminotransferase. Data in Fig. 2, which show that aspartate is formed from glutamate, substantiate this conclusion.

RESULTS

glutamine greatly exceeds the capacity of the mitochondria to oxidize glutamate under state 3 respiration by a creatine-ATP-creatine kinase system (16). In all cases, the flux through the aminotransferase pathway (as determined by aspartate efflux) remained coincident with the rates of oxygen consumption up to a 4-fold increase in oxygen uptake, with essentially no change in the glutaminase activity. No detectable oxidation through glutamate dehydrogenase occurred under any experimental conditions. Direct assay showed that the glutamate dehydrogenase activity in Ehrlich mitochondria, 750 ± 76 nmol of NADH oxidized/min/mg of mitochondrial protein (n = 10), is extremely high, approximately 4-fold higher than reported in (14). However, because glutamate dehydrogenase has numerous and complex allosteric controls (26), it is possible that its activity may vary greatly depending upon the metabolic state and the conditions of measurement.

These results demonstrate that glutamine is hydrolyzed to ammonia and glutamate via a mitochondrial glutaminase, which has been shown to be localized exclusively in the mitochondrial matrix of Ehrlich ascites tumor cells (18) and is activated by inorganic phosphate. We have verified those observations and found that the glutaminase activity in Ehrlich mitochondria is activated >10-fold by high (50 mM) phosphate concentrations.

Effect of Other Substrates on Glutamine Oxidation—It is highly unlikely that tumor mitochondria in vivo are presented with only a single respiratory substrate as in these in vitro experiments. Therefore, it seemed appropriate to examine the effects of other likely substrates on glutamine oxidation by both normal and tumor mitochondria. Malate and pyruvate were of special interest, the former because it readily passes into mitochondria from the cytosol (as in the malate-aspartate shuttle (22)) and the latter because it is formed in large amounts during aerobic glycolysis. Fig. 4, A and B, shows that malate added to normal rat liver and rat kidney mitochondria increases the rate of aspartate production from glutamate, presumably because the added malate is oxidized by mitochondrial malate dehydrogenase to oxalacetate, which can undergo transamination with glutamate. A parallel increase in oxygen consumption was also observed.

However, when malate was added to Ehrlich and AS30-D mitochondria respiring on glutamate, a strikingly different relative fluxes through the respective pathways remained unaltered even when the mitochondria were shifted into increasing rates of state 3 respiration by a creatine-ATP-creatine kinase system (16). In all cases, the flux through the aminotransferase pathway (as determined by aspartate efflux) remained coincident with the rates of oxygen consumption up to a 4-fold increase in oxygen uptake, with essentially no change in the glutaminase activity. No detectable oxidation through glutamate dehydrogenase occurred under any experimental conditions. Direct assay showed that the glutamate dehydrogenase activity in Ehrlich mitochondria, 750 ± 76 nmol of NADH oxidized/min/mg of mitochondrial protein (n = 10), is extremely high, approximately 4-fold higher than reported in (14). However, because glutamate dehydrogenase has numerous and complex allosteric controls (26), it is possible that its activity may vary greatly depending upon the metabolic state and the conditions of measurement.

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result was observed (Fig. 5, A and B). The addition of malate resulted in a prompt and extensive inhibition of aspartate formation from glutamate, by as much as 80%; this effect was not due to inhibition of glutamate disappearance, as will be shown. The inhibitory effect of added malate on aspartate formation was also observed when glutamine replaced glutamate. The inhibition of aspartate formation persisted throughout a 4-fold increase in oxygen consumption (i.e. in state 3 respiration), and could be produced by concentrations of malate as low as 0.2 mM. The inhibitory effect of added malate on aspartate formation was also observed in mitochondria isolated from other tumor cell lines examined (L1210 leukemia, P1798 thymoma, and 22aH hepatoma). Thus, inhibition of aspartate production from glutamate by added malate was a rather unexpected characteristic of five different tumor cell lines and contrasted sharply with the stimulatory effect of added malate on aspartate formation in the normal mitochondria tested.

How Does Added Malate Inhibit Aspartate Production from Glutamate?—One possible explanation for the observed decrease in aspartate formation from glutamate would be direct inhibition of mitochondrial aspartate aminotransferase by malate, since dicarboxylic acids are known to inhibit aminotransferases, but only at very high concentrations (27). However, this explanation is unlikely since added malate does not inhibit aspartate production in normal rat liver and rat kidney mitochondria. Moreover, examination of the effect of malate on aspartate aminotransferase activity in extracts of tumor mitochondria or with the purified enzyme revealed insignificant (<15%) inhibition by very high malate concentrations (50 mM). Alternatively, there was considered that added malate might activate the glutamate dehydrogenase pathway in tumor mitochondria. However, this explanation was ruled out by the finding that no ammonia production from glutamate could be detected. Since no effects of malate on glutamate transport have been described (22), malate inhibition of the glutamate:aspartate exchange translocase did not seem likely. Furthermore, glutamate utilization by tumor mitochondria took place at nearly the same rate in the presence or absence of malate (see data below). Further insight into the mechanism came from examination of the products of oxidation of added malate by tumor mitochondria.

Fig. 6 shows that oxidation of added malate by tumor mitochondria (in the absence of glutamate) was accompanied by a high rate of pyruvate formation. Production of pyruvate from malate by Ehrlich and other tumor mitochondria was first shown in earlier work from this laboratory (28) to be due to the presence of a mitochondrial form of malic enzyme (28, 29). Subsequent reports have shown that NAD(P)⁺-linked malic enzyme is present in many tumor cell lines (29, 30). In addition to pyruvate, the oxidation of added malate resulted in formation of citrate. This indicated not only that pyruvate was oxidized to acetyl-CoA but also that some of the added malate was oxidized by malate dehydrogenase, to provide the oxalacetate required for the formation of citrate from acetyl-CoA. Thus, oxidation of added malate produces two other oxidizable substrates, pyruvate and citrate. These observations therefore suggested that the formation of either or both of these substrates might be responsible for the inhibition of aspartate production from glutamate by added malate.

Fig. 7 shows the effect of added pyruvate on aspartate production from glutamate. Pyruvate caused a large decrease in the aspartate formed, but as soon as the added pyruvate was essentially consumed (<20 μM extramitochondrial concentration), a high rate of aspartate production began. 

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**Fig. 5.** Mitochondrial efflux of aspartate in Ehrlich (A) and AS30-D (B) mitochondria: effect of malate. The Ehrlich and AS30-D mitochondria were resuspended at 3 mg/ml in the medium described in Fig. 1. The respiratory substrate(a) was 5 mM glutamate ± 2 mM malate. Aspartate was measured on the normalized supernatants as described. A, Ehrlich mitochondria: □, aspartate efflux from glutamate alone; A, aspartate efflux from glutamate plus malate. B, AS30-D tumor mitochondria: A, aspartate efflux from glutamate alone; A, aspartate efflux from glutamate plus malate.

**Fig. 6.** Mitochondrial efflux of pyruvate and citrate during malate oxidation. Ehrlich and AS30-D mitochondria were resuspended at 2 mg/ml in the medium described in Fig. 1. The final concentration of malate was 5 mM. Pyruvate and citrate were determined on the neutralized extracts of the supernatant. A, Ehrlich tumor mitochondria: □, pyruvate; △, citrate. B, AS30-D tumor mitochondria: □, pyruvate; ◇, citrate.

**Fig. 7.** Mitochondrial efflux of aspartate during glutamate oxidation: effect of pyruvate. Ehrlich tumor mitochondria were resuspended at 2 mg/ml in the medium described in Fig. 1. The final concentration of glutamate was 5 mM. The effect of pyruvate was tested at two concentrations indicated. O, aspartate efflux (glutamate alone); □, aspartate efflux, and △, pyruvate consumption (0.20 mM pyruvate initially); A, aspartate efflux, and ◇, pyruvate consumption (0.50 mM pyruvate initially).
paralleled the rate in the absence of pyruvate. Further experiments showed that the disappearance of added pyruvate during glutamate oxidation was accompanied by the appearance of large amounts of alanine. As much as 60-80% of the added pyruvate consumed by the mitochondria appeared in the suspending medium as alanine during glutamine oxidation (data not shown). These findings indicated that the formation of alanine from pyruvate was brought about by the presence of alanine aminotransferase in the tumor mitochondria. This was confirmed by direct assay in extracts of Ehrlich mitochondria (specific activity of 120 nmol/min/mg of mitochondrial protein) and is the first demonstration that Ehrlich mitochondria contain a mitochondrial form of alanine aminotransferase (however, see Ref. 1). However, we have not ruled out the possibility that alanine could also arise by the action of glutamine transaminase-ω-amidase (31), but this enzyme has not been reported in tumor cells or tumor mitochondria (32). Furthermore, the specific activity of alanine aminotransferase (which is specific for glutamate) was more than adequate to account for the appearance of alanine.

### Balance Sheets for Oxidation of Glutamate by Tumor Mitochondria

Fig. 8 contrasts the products of glutamate oxidation by tumor mitochondria in the presence and absence of malate. The data were obtained from measurements of the relevant metabolites made in 3-min intervals over a 30-min period. Oxygen uptake measurements were also made and were fully consistent with the metabolite transformations shown.

When glutamate is oxidized in the absence of malate it is quantitatively converted into aspartate (+ CO₂), as already shown in Fig. 2. This conversion occurs via transamination of glutamate with oxalacetate, formed via malate dehydrogenase, as shown in Fig. 9. This situation, which we have observed in mitochondria of Ehrlich tumor mitochondria, may occur via two different pathways, depending upon the origin of the malate, whether from external or cytosolic sources or from an internal source, i.e. from a-ketoglutarate. External malate appears to be oxidized almost exclusively via malic enzyme of the tumor mitochondria, with formation of pyruvate + CO₂, whereas internally generated malate is oxidized almost exclusively via matrix malate dehydrogenase, as shown schematically in Fig. 9. This situation, which we have observed in mitochondria of liver and kidney, may be also the case in tumor mitochondria. When glutamate oxidation in the presence of external malate, both were oxidized at rates about equal to the rates observed when they were added separately. However, the yield of aspartate from glutamate was now very greatly reduced in favor of a large formation of citrate + isocitrate. The sum of aspartate and citrate + isocitrate (together with CO₂) accounted almost quantitatively for the glutamase carbon disappearing. However, the yield of pyruvate from added malate was very greatly reduced when glutamate was oxidized simultaneously (see Fig. 6); in this case, the formation of alanine and the acetyl groups of the accumulated tricarboxylates accounted almost quantitatively for the malate that was utilized.
five different animal tumors, is entirely different from the behavior of normal liver or kidney mitochondria, which lack malic enzyme and consequently oxidize both external and internal malate via malate dehydrogenase to yield oxalacetate.

**DISCUSSION**

Three major consequences follow from the observations reported here. First, our data fully confirm and extend the evidence that mitochondrial malic enzyme plays a unique role in tumor mitochondria. That this enzyme is characteristically present in tumor mitochondria was first shown in early work from this laboratory (28) and verified in other studies (29, 30). A particularly significant finding is that mitochondrial malic enzyme is one of the few progression-linked enzymes in the Morris hepatoma series (30), as is the phosphate-dependent glutaminase (3), which strongly suggests an important role of malic enzyme in glutamine metabolism in tumors.

The second major point established by these experiments concerns current concepts of glutamine oxidation by tumors. Recent studies in other laboratories have led to the hypothesis that malic enzyme catalyzes a key step in the oxidation of glutamine and glutamate in a simple linear pathway (30), referred to as glutaminolysis, by analogy with glycolysis (32). This intramitochondrial pathway is represented by the sequence

\[
\text{Glu} \rightarrow \text{Glu} \rightarrow \alpha\text{-ketoglutarate} \rightarrow \text{succinyl-CoA} \rightarrow \text{succinate} \rightarrow \text{fumarate} \rightarrow \text{maleate} \rightarrow \text{pyruvate} + \text{CO}_2
\]

The data presented here clearly do not support this simple linear scheme. Rather, they indicate not only a very different role for malic enzyme but also the active cooperation of malate and/or pyruvate from the medium (or cytosol) in the oxidation of glutamine, leading to the nearly quantitative formation and extrusion into the medium of citrate and alanine, as shown in Fig. 9. Thus, the oxidation of glutamate by tumor mitochondria is accompanied by metabolic interactions with cytosolic malate and/or pyruvate, both of which are readily available in the cytosol through known metabolic pathways.

The two major products of the pathway described here, citrate and alanine, have important roles in tumor metabolism. Citrate is required as the major source of cytosolic acetyl-CoA for fatty acid and cholesterol biosynthesis, the latter believed to be characteristically enhanced in tumor cells (33, 34). Alanine is required in cytosolic amino acid transformations and in protein synthesis. Glutamine is a neutral amino acid and can enter mitochondria readily by simple uniport (22). Malate may enter on the dicarboxylate carrier in exchange with phosphate or as dicarboxylate, or it may enter by exchange with a tricarboxylate on the tricarboxylate carrier (22). Indeed, the near-agreement (Fig. 8) between the rates of malate uptake and citrate extrusion suggests these transport events are coupled through the action of the tricarboxylate carrier.

The third point raised by these observations concerns the mechanism of regulation of glutamine and malate metabolism via the overall pathway described here. The first question, which is also applicable to behavior of mitochondria from normal tissues, may be stated as follows. Why does glutamate oxidation usually proceed entirely via transamination, either with oxalacetate or pyruvate, rather than by direct dehydrogenation via glutamate dehydrogenase, which is present in very large amounts in both normal liver and tumor mitochondria? The results presented here imply that glutamate dehydrogenase activity of tumor mitochondria, under the conditions tested, is very strongly inhibited, possibly by a negative allosteric effector such as GTP (26). This well recognized problem requires further analysis, particularly in tumor mitochondria. The second question is why is external malate oxidized almost exclusively by malic enzyme, whereas internally generated malate is oxidized by malate dehydrogenase? Although this difference remains to be confirmed by direct isotopic experiments, which are under way, two types of explanation seem possible. First, it is possible that the malate entering the mitochondrial matrix via a membrane carrier has “preferred access” to mitochondrial malic enzyme, which could conceivably be located at or near the inner face of the malate transport system so that it can act exclusively on transported malate but not on malate generated in the matrix. This type of explanation has a counterpart in the preferred access of ATP generated by heart mitochondria to the mitochondrial creatine kinase isozyme (35). Such an explanation would also require that internally generated malate is unable to gain access to malic enzyme. An alternative possibility is that the activities of malic enzyme and malate dehydrogenase are both regulated in an integrated manner, so that acetyl-CoA and oxalacetate are formed at equal rates, in order to yield citrate as the sole end product. Studies on the regulation of tumor malic enzyme are described in a following communication (36).

The data reported here also raise further questions about the overall scheme of tumor cell metabolism. Cytosolic malate is readily available in tumor cells and presumably can be diverted away from participation in the malate-aspartate shuttle for cytosolic NADH oxidation, which most tumors tested are potentially capable of promoting (37). Since our data show that incoming malate is oxidized primarily by mitochondrial malic enzyme to pyruvate, which is extruded, two immediate and important consequences could follow. Pyruvate formed by malic enzyme from cytosolic malate could not only undergo reduction to lactate via cytosolic lactate dehydrogenase but might also compete with glycolytic pyruvate for entry into the mitochondrial matrix and oxidation via the pyruvate dehydrogenase complex. Furthermore, mitochondrial malic enzyme might be viewed as an essential link in an oxidative malate → citrate conversion. Since citrate extruded from mitochondria is the primary acetyl-CoA precursor for cholesterol and fatty acid biosynthesis in the cytosol, and since tumor cells generally exhibit a greatly enhanced and unregulated rate of cholesterologenesis (33, 34), mitochondrial malic enzyme activity would provide pyruvate for subsequent conversion to citrate. Citrate efflux would occur via the tricarboxylate exchange carrier, and malate carbon formed from citrate in the cytosol would eventually reenter the mitochondria due to the sequential action of ATP-dependent citrate lyase, in part a mitochondrially bound enzyme (38), and cytosolic malate dehydrogenase. Indeed, some evidence for such a shuttle was recently presented (39).

Finally, the special metabolic pathways observed in tumor mitochondria in this study may be related to or be the product of reversible multienzyme complex formation between sequential enzymes in the tricarboxylic acid cycle. Several studies have demonstrated that mitochondrial malate dehydrogenase can form reversible binary complexes with fumarase (40), citrate synthase (40–42), aspartate aminotransferase (43, 44), and glutamate dehydrogenase (45). In mitochondria, where the matrix volume is very small and the protein concentration very high (46), such binary or higher order multienzyme complexes may be subject to specific regulatory mechanisms and could thus be responsible for the pathways observed in this study. Preliminary studies in this laboratory strongly suggest that some of the matrix enzymes involved in glutamate metabolism in tumor mitochondria may be associated in a multienzyme cluster.
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