Quantitative Evaluation of the Activity of the Malate-Aspartate Shuttle in Ehrlich Ascites Tumor Cells

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ABSTRACT

The ability of the malate-aspartate shuttle and of the lactate dehydrogenase reaction to reoxidize glycolytic reduced nicotinamide adenine dinucleotide has been estimated quantitatively in a highly glycolyzing strain of Ehrlich ascites tumor cells. Aminoxyacetate and oxamate, which are specific inhibitors of these two systems, have been used as tools for evaluating this ability. After 30-min incubation of ascites cells with 6 mM glucose, 186 μmol of this substrate per g dry weight are consumed with a net production of 265 μmol lactate per g dry weight. Oxamate affects the glycolytic flux mainly at the levels of glucose (about 40% inhibition) and lactate (about 80% inhibition), whereas aminoxyacetate influences specifically the levels of dihydroxyacetone phosphate and fructose 1,6-diphosphate, which are markedly increased. When both inhibitors are added to the cells, a more pronounced effect on dihydroxyacetone phosphate and fructose 1,6-diphosphate is attained, but no significant differences in the rate of glucose utilization or of lactate production are observed with respect to those seen with oxamate alone. Arsenite-inhibited respiration is partly restored by 10 mM glucose by a way which is sensitive to rotenone and aminoxyacetate to the same extent. In short-term experiments, addition of glucose to arsenite-treated cells causes reduction of cytochrome b, which is partly modified by aminoxyacetate and completely abolished by the uncoupler 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole. The results indicate that in Ehrlich-Lettré ascites cells control of the nicotinamide adenine dinucleotide oxidation-reduction state is exerted mainly at the level of lactate dehydrogenase and that the operation of the malate-aspartate shuttle, although occurring per se at the maximum theoretical rate, may account for only 13% oxidation of all the reduced nicotinamide adenine dinucleotide reducing equivalents generated in the cytosol by the breakdown of glucose.

INTRODUCTION

A high rate of aerobic glycolysis is one of the main features of rapidly growing tumor cells. The reason for this metabolic behavior is, however, far from being understood. Several mechanisms have been proposed: impaired respiratory capacity, defective plasma membrane Na+-K+-ATPase, modification of the allosteric control of various glycolytic enzymes (i.e., phosphofructokinase, hexokinase, and pyruvate kinase) and deficiency of the so-called "shuttle systems" (see Refs. 17 to 19 for reviews). With respect to the last proposal, previous data collected in our laboratory have, however, provided evidence that no correlation exists between the operation of the hydrogen-translocating systems (i.e., α-glycerophosphate, malate-aspartate, and fatty acid cycles) and the enormous lactate accumulation in different strains of Ehrlich ascites tumor cells (2-5, 7, 8, 16). In particular, we have recently shown that, in the Ehrlich hyperdiploid Lettré strain, the malate-aspartate shuttle is actively functioning in the reoxidation of reduced NADH generated during physiological aerobic glucose breakdown (15). However, further investigations are required in order to assess more quantitatively the participation of such mechanisms in the metabolism of tumor cells. Indeed, the quantitative data obtained by Greenhouse and Lehninger (10), assuming on the basis of previous experiments with lactate (9) that shuttles not involving transaminations (e.g., α-glycerophosphate shuttle) do not participate in the 6 lines of ascites tested, require reviewing. The possible operation of the α-glycerophosphate shuttle has to be tested in conditions where the cytosolic level of DHAP is high, i.e., in the presence of glucose. To approach this problem, we have thought to measure the different contributions of the malate-aspartate shuttle and the lactate dehydrogenase reaction to the reoxidation of glycolytic NADH in the Ehrlich hyperdiploid Lettré strain, where it has been shown that the α-glycerophosphate shuttle is lacking (2). The efficiency of the 2 systems has been evaluated on the basis of the effect of specific inhibitors, i.e., aminoxyacetate, an aspartate transaminase (EC 2.6.1.1) inhibitor, and oxamate, a competitive inhibitor of lactate dehydrogenase (EC 1.1.1.27), on the glycolytic and respiratory activity of these cells.

The results which are the object of the present work reveal that control of the NADH oxidation-reduction state is exerted mainly at the level of lactate dehydrogenase, and the activity of the malate-aspartate shuttle, although occurring per se at the maximum theoretical rate, appears to be disproportionately low with respect to the enormous amount of NADH reducing equivalents generated during glycolysis.

MATERIALS AND METHODS

Ehrlich hyperdiploid Lettré ascites cells were maintained by weekly i.p. transplantation in albino Swiss mice and harvested 6 to 8 days after the inoculation. The peritoneal fluid was washed in an isotonic medium (154 mM NaCl-8.2 mM KCl-11 mM sodium phosphate buffer, pH 7.4) and, when slightly hemorrhagic, carefully freed from hemoglobin by differential centrifugation. The packed cells were then resuspended in the same medium at the concentration of 15 to 25 mg dry weight.
whereas no appreciable differences in the levels of glucose or a more pronounced effect on DHAP and FOP is attained, aminooxyacetate. When both inhibitors are added to the cells, the level of phosphorylated intermediates much less than does the production of lactate (about 80% inhibition), but it changes etate on the utilization of glucose (about 40% inhibition) and shown). Oxamate (50 mM) is more effective than aminooxyacetate-treated cells significantly lowers the level of OHAP and aspartate [to activate the malate-aspartate cycle (3)] to glu-

**RESULTS**

Chart 1 shows the effect of different inhibitors on the level of some glycolytic intermediates after 30-min incubation of Ehrlich-Lettré ascites cells with 6 mM glucose. Control values show that 198.2 μmol glucose per g dry weight are utilized during the incubation time, with a net production of lactate of 265.3 μmol per g dry weight. DHAP and FDP accumulate in the amount of 2.5 and 10.3 μmol/g dry weight, respectively. Amino-

aminoxyacetate (0.4 mM) markedly increases the level of these 2 intermediates, whereas it scarcely affects the utilization of glucose or the production of lactate. In this respect, it should be noted that the addition of 10 mM a-oxoglutarate plus 10 mM aspartate [to activate the malate-aspartate cycle (3)] to glucose-treated cells significantly lowers the level of DHAP and FDP and influences slightly the accumulation of lactate (not shown). Oxamate (50 mM) is more effective than aminoxyacetate on the utilization of glucose (about 40% inhibition) and the production of lactate (about 80% inhibition), but it changes the level of phosphorylated intermediates much less than does aminoxyacetate. When both inhibitors are added to the cells, a more pronounced effect on DHAP and FDP is attained, whereas no appreciable differences in the levels of glucose or lactate, with respect to those seen with oxamate alone, are observed.

In order to evaluate the extent of supply of glycolytic reducing equivalents to the mitochondrial respiratory chain without interference from other substrates, we performed experiments in the presence of an inhibitor of the tricarboxylic acid cycle, i.e., arsenite. Chart 2 shows the effect of 10 mM glucose on the kinetics of O₂ consumption of Ehrlich-Lettré ascites cells treated with 5 mM arsenite. It can be seen that the endogenous respiratory rate, which is kept constantly low in the presence of the inhibitor, is stimulated by the addition of glucose. Such an effect is sensitive to rotenone and aminoxyacetate to the same extent.

Kinetic determinations of cytochrome b have been performed under experimental conditions similar to those reported in Chart 2, with the aim of elucidating further the dynamics of the respiratory events. As it is shown in Chart 3A, arsenite treatment of ascites tumor cells causes a slow oxidation of cytochrome b, which reaches a steady state after approximately 12 min. The subsequent addition of glucose induces an abrupt small oxidation followed by a large reduction. Oligomycin further reduces cytochrome b. Similar experiments (not shown) indicate that if glucose is substituted by 2-deoxyglucose or if glucose is added in the presence of iodoacetate, only an
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oxidation phase, much larger than that shown in Chart 3A, is observed. Under the latter conditions, oligomycin does not elicit a significant effect. Addition of glucose after arsenite in aminooxyacetate-treated cells (Chart 3B) causes an oxidation-reduction cycle which is much more evident than that induced in untreated cells. The evidence that the glucose-induced reduction of cytochrome \( b \), observed in arsenite-treated cells, is an energy-dependent phenomenon, comes from the experiments shown in Chart 4. In Chart 4A, reduction of cytochrome \( b \) can be observed after glucose addition, whereas, in Chart 4B, the uncoupler TTFB completely abolishes the phenomenon. \( \text{CN}^- \) is added at a concentration of 100 \( \mu \text{M} \) in both experiments, only to make the respiratory chain more sensitive to changes of electron flow and thereby to emphasize the spectroscopic effect.

DISCUSSION

The experiments presented suggest a major role of the lactate dehydrogenase reaction in the reoxidation of glycolytic NADH with respect to the malate-aspartate shuttle. The conclusion is supported by the following observations. The utilization of glucose through the glycolytic pathway is affected by oxamate to a greater extent than by aminooxyacetate; even in the presence of both inhibitors, the degree of inhibition of glucose utilization is the same as in the presence of oxamate alone. The data related to the effect of aminooxyacetate on the phosphorylated intermediates are not contradictory to such a view, because they account only for the fact that aminooxyacetate, as reported previously (15), affects the glycolytic flux at the level of glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) more specifically than does oxamate. Further evidence is, in fact, the observation that the addition of \( \alpha \)-oxoglutarate plus aspartate, which potentiates the activity of the malate-aspartate cycle, induces an effect opposite to that of aminooxyacetate on the level of DHAP and FDP but modestly inhibits the net production of lactate. This clearly signifies that the malate-aspartate shuttle, even when it is potentiated, poorly replaces the lactate dehydrogenase reaction in the reoxidation of glycolytic NADH.

Experiments of O\(_2\) consumption in arsenite-treated cells may be utilized to make a quantitative estimate of the shuttle activity.
(see below). Indeed, the finding that under such conditions the glucose-dependent enhancement of respiration is affected by aminooxyacetate and rotenone to the same extent allows us to attribute this phenomenon almost entirely to the transfer of NADH reducing equivalents into the respiratory chain via the malate-aspartate shuttle. It should be pointed out, as reported previously (2), that in the strain of ascites cells used in the present work the α-glycerophosphate shuttle contributes very poorly to the mitochondrial supply of glycolytic reducing equivalents.

The spectroscopic measurements of cytochrome b were performed in the attempt to validate the data obtained by the experiments shown in Chart 2. Indeed, addition of glucose to arsenite-treated cells induces a marked reduction of cytochrome b, preceded by a slight oxidation (Chart 3A). There are data which allow us to attribute such an effect to the entry of glycolytic reducing equivalents into the respiratory chain, e.g., the fact that glucose is able only to oxidize cytochrome b when the cells are poisoned with iodoacetate, and a similar response is observed when glucose is substituted by 2-deoxyglucose, i.e., in the absence of NADH equivalents. The oxidation of cytochrome b, which is larger with 2-deoxyglucose or glucose plus iodoacetate than with glucose, may be explained in all the 3 cases in terms of ADP supply to the respiratory chain. The difference in the extent of such oxidation probably lies in the fact that only with glucose is the ADP effect immediately counteracted by the supply of NADH equivalents, which tend to reduce the respiratory chain. On the other hand, the further reduction of cytochrome b induced by oligomycin excludes the possibility that glucose acts on the cytochrome through an ATP-driven reversed electron transport (2, 6). As shown in Chart 3B, pretreatment of the cells with aminooxyacetate causes a substantial modification of the glucose-dependent oxidation-reduction kinetics of cytochrome b; the oxidation is more pronounced (a condition which resembles that with glucose plus iodoacetate or 2-deoxyglucose), and the half-time of the reduction is somewhat increased. Thus, even if the inhibitor is unable to abolish the reduction of cytochrome b in short-term experiments, it already influences the kinetics of hydrogen flux into the respiratory chain. The view that mitochondrial oxidation of glycolytic NADH proceeds through the malate-aspartate shuttle is strengthened by the data presented in Chart 4. Indeed, the sensitivity of cytochrome b reduction to the uncoupler TTFB is consistent with the energy-dependent process of aspartate efflux from the mitochondria (13, 14) essential for the activity of such a cycle.

From the data reported in Table 1, the activity of the malate-aspartate shuttle can be calculated as net respiratory increases induced by glucose in arsenite-treated cells. Such a value (0.91 μl oxygen consumed per hr per mg dry weight) represents about 13% of the oxygen consumption of ascites tumor cells respiring in the presence of glucose, and it is quite close to the theoretical NADH shuttle activity, which should be one-sixth of the oxygen consumption due to glucose oxidation alone. However, this activity, which corresponds to a net transport of 0.082 μmol NADH per hr per mg dry weight, accounts for only 13% oxidation of all the NADH-reducing equivalents (0.610 μmol/hr/mg dry weight) generated by ascites tumor cells during aerobic glucose breakdown in our experimental conditions (see Chart 1).

In summary, the ability of 2 systems, i.e., the lactate dehydrogenase reaction and the malate-aspartate shuttle, to reoxidize glycolytic NADH has been explored in the Ehrlich hyperdiploid Leittré strain of ascites tumor cells. As judged from the effect exerted by oxamate and aminooxyacetate (which are specific inhibitors of such systems) on the utilization of glucose through the glycolytic pathway, the lactate dehydrogenase reaction seems to be the most important system utilized by the cells for this function. However, it has also been demonstrated that such an unbalanced contribution does not arise from deficiency of the shuttle system, which has been shown to operate at the maximum theoretical rate, but from the fact that the amount of cytosolic reducing equivalents generated during pyruvate formation from glucose by far exceeds the normal mitochondrial oxidizing capacity. As a consequence, much more lactate is obligatorily produced to provide an extra oxidation of the residual NADH. Thus, the present data are in agreement with previous results obtained using lactate (4, 9) or glucose (10, 15) as sources of cytosolic reducing equivalents. By contrast, they disprove the conclusion of Kováčevec (12), that the malate-aspartate shuttle cannot function at a sufficiently high rate because of the low concentration of aspartate in glycolyzing ascites cells.

In light of the above conclusions, we are inclined to believe that the reasons for the high glycolytic activity of tumor cells have to be searched for mainly in perturbations of the system(s) which control the rate of glucose utilization (e.g., the phosphate potential), rather than in an inadequacy of the shuttle mechanisms.

REFERENCES

6. Galeotti, T., Azzi, A., and Chance, B. The redoxation of cytoplasmic reducing...
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