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EVIDENCE FOR THE OCCURRENCE OF THE MALATE-CITRATE SHUTTLE IN INTACT EHRLICH ASCITES TUMOR CELLS

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A possible activity of the malate-citrate shuttle has been investigated in Ehrlich ascites cells by testing the effects of 1,2,3-benzenetricarboxylic acid, an inhibitor of the malate-citrate exchange, and (−)-hydroxycitrate, an inhibitor of the citrate cleavage enzyme, on the glucose-dependent oxidation-reduction rates of pyridine nucleotides and cytochrome *b* as well as on ATP levels of glycolyzing cells. Moreover, to quantitate such an activity, the effects of these two inhibitors have been compared with those induced under the same experimental conditions by aminooxyacetate, an inhibitor of the malate-aspartate shuttle which is known to operate in this strain of ascites tumor. Both benzenetricarboxylic acid and hydroxycitrate are able to increase the reduction of pyridine nucleotides, which follows glucose addition to whole cells, to about the same extent. A much more pronounced effect is elicited by aminooxyacetate under the same condition. When *n*-butylmalonate is added to slow down the flux of glycolytic reducing equivalents to the respiratory chain via the malate-aspartate shuttle, benzenetricarboxylic acid or hydroxycitrate promotes an ATP-driven reversal of electron transfer. Indeed, the glucose-induced reduction of cytochrome *b* becomes sensitive to oligomycin and the ATP level is raised significantly with respect to the value of uninhibited cells. It is concluded that the malate-citrate shuttle operates in Ehrlich ascites cells, although with a substantially lower activity with respect to the malate-aspartate shuttle.

Introduction

A high rate of aerobic glycolysis is the feature which primarily characterizes the energy metabolism of rapidly growing tumors. Among the different lines of work so far undertaken to investigate such metabolic behavior, considerable attention has been paid to the study of the extent and rate of mitochondrial oxidation of glycolytically generated NADH as well as to the identification of the shuttle system(s) involved in such oxidation (see Ref. 1 for a review). Data collected in this laboratory give strong indication for a lack of correlation between the operation of the hydrogen-translocating systems (i.e., α-glycerophosphate, malate-aspartate and fatty acid cycles) and the enormous accumulation of lactate during aerobic breakdown of glucose in Ehrlich ascites cells [2–4]. In particular, we have evaluated on a quantitative basis the different contributions of the malate-aspartate shuttle [5] and the lactate dehydrogenase reaction to the reoxidation of glycolytic NADH in the Ehrlich hyperdiploid Lettré ascites strain [6]. The data obtained have shown that control of the NAD 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.

Another approach suitable for investigating the nature of the hydrogen shuttle systems in tumor cells

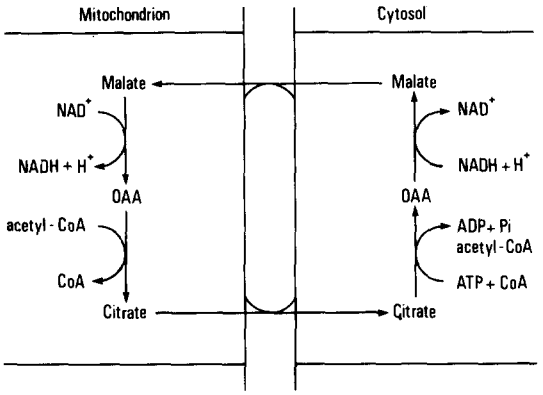

Fig. 1. Mechanism for the transport of reducing equivalents from cytosol to mitochondria by the malate-citrate shuttle. OAA, oxaloacetate.

has been that of studying the activity of the mitochondrial anion translocators [7,8]. In a previous investigation we have attempted to provide indirect evidence that certain anion translocators are actively functioning, as hydrogen carriers, in intact Ehrlich ascites tumor cells where the malate-aspartate and the malate-citrate [9] cycles may be operating [10]. In the present work experiments have been performed on Ehrlich hyperdiploid Lettré ascites cells with the aim of defining more precisely such a function, using inhibitors both of transport and metabolism. The results obtained show that the malate-asparate shuttle plays the major role in the translocation of glycolytic reducing equivalents. Moreover, they suggest that the malate-citrate shuttle (Fig. 1) is also operating although its activity appears to be substantially lower.

Material and Methods

Ehrlich hyperdiploid Lettré ascites cells were maintained by weekly intraperitoneal transplantation in albino Swiss mice and harvested 6–8 days after inoculation. The peritoneal fluid was washed in an isotonic medium (154 mM NaCl, 6.2 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 resuspended in the same medium at different concentrations, depending on the experimental conditions. All the experiments were carried out in the presence of arsenite to avoid interference from substrates of the Krebs cycle (see Ref. 6). For ATP measurements the cells (15–20 mg dry wt./ml) were incubated at 38°C with 20 mM glucose plus 5 mM arsenite in Warburg manometric flasks gassed with O_2. After 50 min, the reaction was stopped at −15°C (ice/salt mixture) by adding to the cells an equal volume of a mixture containing 8% (w/v) $HClO_4$, 40% ethanol and 5 mM EDTA. The extracts, neutralized with 0.5 M triethanolamine/3 M K_2CO_3 [11], were used for the assay [12]. Spectrophotometric recordings of pyridine nucleotides and cytochrome *b* were performed at room temperature in a dual-wavelength split-beam Aminco-Chance spectrophotometer at 340 minus 375 nm and at 430 minus 410 nm, respectively. Enzymes, coenzymes and substrates were purchased from Boehringer und Sohne. Aminooxyacetic acid and rotenone were obtained from K and K Laboratories, Inc. Oxamic acid was obtained from British Drug Houses Ltd., 1,2,3-benzenetricarboxylic acid from Merck-Schuchardt and *n*-butylmalonic acid from Aldrich Chemical Co. (−)-Hydroxycitrate was a generous gift from Dr. Y.S. Lewis [13]. All other chemicals were products of Sigma Chemical Co. or E. Merck.

Results

Fig. 2 shows the oxidation-reduction rates of pyridine nucleotides in Ehrlich hyperdiploid Lettré ascites cells treated with arsenite and supplemented with glucose (oxamate was employed in order to eliminate interference from the lactate dehydrogenase reaction). It can be seen that arsenite causes a slow oxidation of the nucleotides. The subsequent addition of glucose induces an abrupt reduction (Fig. 2A) which is greatly enhanced by the transaminase inhibitor aminooxyacetate (Fig. 2B). A significant effect on the nucleotide reduction is also produced by 1,2,3-benzenetricarboxylic acid (Fig. 2C), an inhibitor of the malate-citrate exchange [14] or by (−)-hydroxycitrate (Fig. 2D), an inhibitor of the cytosolic citrate cleavage enzyme (ATP citrate (*pro*-3*S*)-lyase) [15].

Fig. 3 shows kinetic determinations of the cytochrome *b* redox state performed under experimental conditions similar to those reported in Fig. 2. A reduction of the pigment occurs following addition of glucose to arsenite-treated cells with no further effect elicited by oligomycin (Fig. 3A). *n*-Butylmalonate,

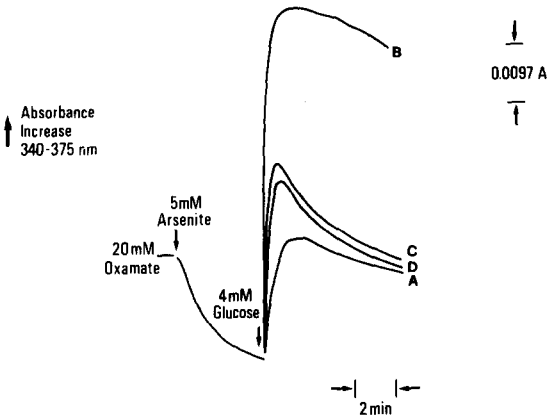

Fig. 2. Spectrophotometric recordings of pyridine nucleotides in Ehrlich hyperdiploid Lettré ascites cells inhibited with oxamate. In B, C and D, the cells were pretreated with 0.4 mM aminooxyacetate, 10 mM benzenetricarboxylic acid and 2 mM hydroxycitrate, respectively. An upward deflection of the trace corresponds to reduction of the nucleotides. The cell concentration was 11.5 mg dry wt./ml. For further details see Material and Methods.

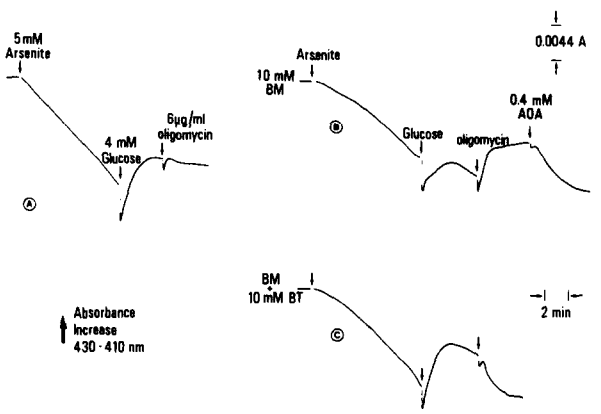

Fig. 3. Redox changes of cytochrome *b* induced by glucose in arsenite-inhibited cells. In B and C the cells were pretreated with butylmalonate (BM) and butylmalonate plus benzenetricarboxylic acid (BT), respectively. An upward deflection of the trace corresponds to reduction of cytochrome *b*. The rapid decrease in absorbance upon glucose addition is due to dilution of the cell suspension. The slow oxidation before oligomycin addition, especially evident in traces B and C, can be attributed to the persistence of the arsenite-induced oxidation of the pigment. The cell concentration was the same as in Fig. 2. For further details see Material and Methods. AOA, aminooxyacetate.

an inhibitor of the dicarboxylate carrier [14], greatly diminishes the glucose-dependent reduction of cytochrome *b* (Fig. 3B). In this case oligomycin increases the residual reduction of the pigment, insensitive to butylmalonate, and aminooxyacetate completely abolishes such an effect. It should be noted that in the absence of glucose no significant effect is elicited by aminooxyacetate using the same sequence of additions (not shown). When benzenetricarboxylic acid (or hydroxycitrate, not shown) is added together with butylmalonate, a reversal of electron transfer seems to take place, as judged by the effect of oligomycin on the glucose-dependent reduction of cytochrome *b* (Fig. 3C).

Table I shows the results of long-term experiments where ATP levels have been measured after 50 min incubation of the cells treated with arsenite and supplemented with glucose. Control values are significantly decreased by the addition of aminooxyacetate. Moreover, in the presence of α-oxoglutarate plus aspartate, which are specific substrates of the malate-aspartate shuttle and increase per se the ATP content with respect to the control (from 5.8 to 6.9 nmol/mg protein), aminooxyacetate still displays its inhibitory effect. It should be noted that aminooxyacetate inhibits the glucose-stimulated O_2 uptake of arsenite-

TABLE I

EFFECT OF DIFFERENT SUBSTRATES AND INHIBITORS ON THE STEADY-STATE LEVEL OF ATP IN GLYCOLYZING EHRLICH-LETTRÉ ASCITES CELLS

Ehrlich hyperdiploid ascites tumor cells were analyzed for their content in ATP after 50 min aerobic incubation at 38°C in the presence of the substrates and inhibitors indicated below. Under all conditions the cells were supplemented with 20 mM glucose and 5 mM arsenite. Other experimental details are given in Material and Methods. Values are expressed as means ± S.E. (number of observations).

Conditions	ATP (nmol/mg dry wt.)
Control	5.8 ± 0.52 (6)
Aminooxyacetate (0.4 mM)	3.6 ± 0.38 (6)
α-Oxoglutarate (10 mM) + aspartate (10 mM)	6.9 (2)
α-Oxoglutarate + aspartate + aminooxyacetate	4.6 (2)
(−)-Hydroxycitrate (2 mM)	7.5 ± 0.76 (4)
(−)-Hydroxycitrate + aminooxyacetate	5.0 ± 0.45 (4)
Rotenone (10 μM)	1.3 (2)

treated cells, whilst α-oxoglutarate plus aspartate enhance it significantly (unpublished results). Conversely, hydroxycitrate promotes an increase of ATP from 5.8 to 7.5 nmol/mg protein. This effect is still present when aminooxyacetate is added to the system together with hydroxycitrate. The last value in the table gives the level of ATP in the presence of rotenone, i.e., under conditions where the mitochondrial energy generation is completely inhibited. It should be noted that the accumulation of lactate does not significantly change under all the conditions reported in Table I (not shown).

Discussion

In a previous work we have already demonstrated that the malate-aspartate shuttle is operating in intact glycolyzing Ehrlich hyperdiploid Lettré ascites cells [4]. The major evidence has been provided by the fact that the transaminase inhibitor aminooxyacetate strongly affects the free cytosolic NADH/NAD ratio which in turn is responsible for the changes in the glycolytic intermediates at the level of both glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase. Moreover, further studies have quantitated such an activity in terms of O_2 consumption coupled to the net transport of glycolytic NADH to the respiratory chain [6]. In this respect, it has been shown that the activity of the malate-aspartate shuttle closely corresponds in these cells to the theoretical NADH shuttle activity, i.e., one-sixth of the oxygen consumption due to glucose oxidation alone. On the other hand, preliminary data, obtained by using a different experimental approach, have suggested that the malate-citrate shuttle may be also operating in Ehrlich hyperdiploid Lettré ascites cells [10]. In the present work we have reinvestigated both cycles, with the aim of comparing and defining more precisely their relative activity, by studying the in vivo function of certain anion translocators which are known to operate inside them. The use of arsenite eliminates interference from substrates of the Krebs cycle, thus demonstrating the effects of glucose specifically dependent on the transport of glycolytic reducing equivalents to the respiratory chain [6].

The major effect on the glucose-dependent reduction of pyridine nucleotides is promoted by aminooxyacetate. Similarly, in cells pretreated with *n*-butylmalonate (to block the entry of malate generated from the cytosolic endogenous pool of oxaloacetate) aminooxyacetate abolishes completely the reduction of cytochrome *b* (Fig. 3B). In such an experiment oligomycin was also used, as an inhibitor of the coupled mitochondrial electron flux, with the purpose of raising the reduced steady state of the pigment for better demonstration of the aminooxyacetate effect. These observations and the fact that in the absence of glucose no significant effect on cytochrome *b* is elicited by aminooxyacetate make the redox changes of the pigment highly specific for NADH entry into the respiratory chain via the malate-aspartate shuttle.

1,2,3-Benzenetricarboxylic acid and (–)-hydroxycitrate are able to affect the reduction of pyridine nucleotides (Fig. 2). Moreover, when one of these inhibitors is added together with butylmalonate a reversal of electron transfer seems to occur (Fig. 3C). This is probably because (i) benzenetricarboxylic acid or hydroxycitrate increases the glycolytic ATP/ADP ratio by reducing significantly ATP utilization in the citrate lyase reaction and (ii) butylmalonate limits the forward electron flux linked to malate oxidation via the malate-aspartate cycle. The view that benzenetricarboxylic acid or hydroxycitrate interferes with an ATP-consuming reaction is further supported by the results shown in Table I. Indeed, hydroxycitrate induces an increase in ATP which is still evident under conditions where the activity of the malate-aspartate shuttle is also inhibited. On the other hand, the effect of α-oxoglutarate plus aspartate as well as that of aminooxyacetate on the level of ATP confirms the data on pyridine nucleotides and cytochrome *b* with respect to the operation of the malate-aspartate shuttle.

It can be concluded that: (i) the malate-aspartate shuttle plays the major role in the translocation of glycolytic reducing equivalents in Ehrlich hyperdiploid Lettré ascites cells; (ii) the effect of benzenetricarboxylic acid or hydroxycitrate on the kinetics of pyridine nucleotides and cytochrome *b* as well as on the ATP level in glycolyzing cells suggests that the malate-citrate cycle is also functioning. Such a system (Fig. 1) could in fact bring about a reoxidation of glycolytic reducing equivalents by a way which involves cytosolic ATP-dependent cleavage of citrate and entry of malate in exchange with mitochondrial

citrate via the malate-citrate carrier. This shuttle would operate in addition to the malate-aspartate shuttle, although its activity is probably substantially lower.

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