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KINETICS OF REDOX CHANGES OF NICOTINAMIDE-ADENINE DINUCLEOTIDES IN EHRLICH ASCITES TUMOUR CELLS

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1. Introduction

In a previous paper [1] we have reported a method for distinguishing cytosolic and mitochondrial nicotinamide-adenine dinucleotides in intact Ehrlich–Lettré ascites tumour cells. This was allowed by simultaneous measurements of fluorescence and absorbance of the adenine nucleotides and calculation of the F/A ratios. Indeed, such ratios appear to be different for the two species of the coenzyme, i.e. they are higher for mitochondrial than cytosolic nucleotides. By means of this method it has been also possible to show that oxygenation of an anaerobic suspension of ascites cells, supplemented with glucose, causes a biphasic response of nicotinamide nucleotides. A fast phase is followed by a slow phase and by the values of the F/A ratios one may establish that the former is mitochondrial and the latter cytosolic. Thus, a kinetic demonstration has been given of aerobic oxidation of cytosolic NADH in glycolyzing ascites cells. It has also been shown that the slow oxidation of nicotinamide nucleotides, which is not accompanied by a parallel slow oxidation of flavoproteins, is abolished by iodoacetate and by the uncoupler TTFB.

In the present work we have extended the absorption measurements of nicotinamide nucleotides to the wild strain of Ehrlich hyperdiploid ascites tumour cells and we have estimated the level of two glycolytic intermediates, glucose-6-phosphate (G-6-P) and fructose-1,6-diphosphate (FDP), under steady-state conditions, in both the strains during anaerobic and aerobic glycolysis.

Since the two tumour strains have different shuttle mechanisms for hydrogen transport across the mitochondrial membrane [2–5], namely the α-glycerophosphate shuttle [6, 7] is operating in the wild cells and the malate–aspartate shuttle [8] in the mutant cells, they afford a unique system where the relationship between shuttle mechanism and the redox state of cytosolic nicotinamide nucleotides and/or glycolytic intermediates can be investigated. The results obtained in the present study show that the behaviour of the two ascites tumour cell strains is different with respect to nicotinamide nucleotides reduction and the level of glycolytic intermediates.

2. Experimental

Ehrlich hyperdiploid (H) ascites tumour cells and the Lettré mutant (H–L) were grown in albino Swiss mice by weekly transfer of 0.2 to 0.3 ml of ascites fluid. The cells were harvested after 6–8 days from the inoculation, washed in an isotonic saline medium (154 mM NaCl, 6.2 mM KCl, 11 mM sodium

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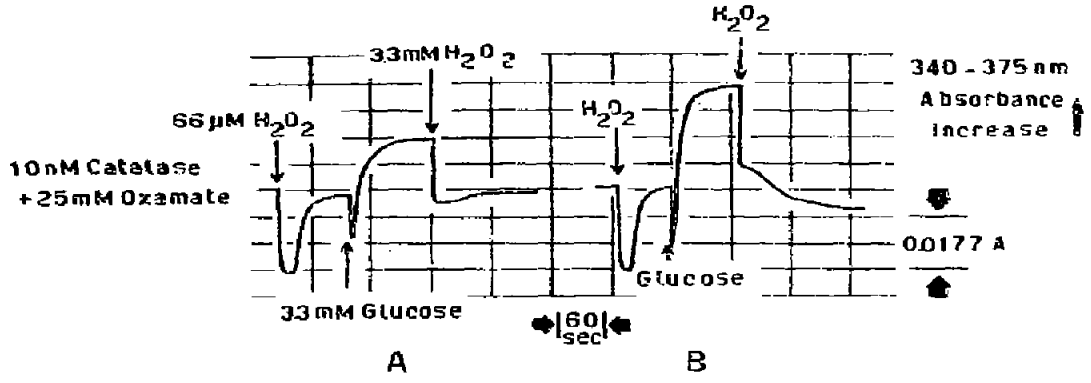

Fig. 1. Spectrophotometric recordings of redox changes of nicotinamide nucleotides in intact Ehrlich hyperdiploid (A) and Lettré mutant (B) ascites tumour cells. The cell suspension concentrations were 5.9 and 6.5 mg dry wt./ml for H (A) and H–L (B) cells, respectively. The cells, pretreated with catalase and oxamate, were allowed to become anaerobic by endogenous substrates. Addition of a small amount of H_2O_2 caused an oxidation–reduction cycle. Glucose reduced the cytosolic pool of nicotinamide nucleotides. An aerobic steady-state is finally obtained by addition of excess H_2O_2 to the anaerobically glycolyzing cells.

phosphate buffer, pH 7.4) [9] and resuspended in the same medium at the concentration of 50–60 mg dry weight per ml.

Spectrophotometric measurements of nicotinamide nucleotides were performed at room temp. in the dual wavelength/split-beam Aminco–Chance spectrophotometer at 340–375 nm. For the estimation of metabolites 200 μl of cell suspension were harvested with an Eppendorf pipette from the spectrophotometric cuvette and poured in a centrifuge tube containing an equal amount of ice-cold 10% (v/v) $HClO_4$. The deproteinized material was then centrifuged and the supernatant neutralized with a 0.5 M triethanolamine–3 M K_2CO_3 mixture. G-6-P and FDP were assayed fluorimetrically by enzymic methods [10].

Rotenone was purchased by K and K Lab. Inc., Plainview, New York. The 4, 5, 6, 7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) was kindly supplied by Dr. R.B. Beechey of Shell Research Ltd., Milstead laboratory of Chemical Enzymology, Sittingbourne, Kent (Great Britain). Enzymes, coenzymes and substrates for metabolites determination were obtained from Boehringer und Soehne (Mannheim) and Sigma (St. Louis).

3. Results and discussion

3.1. *Kinetics of redox changes of nicotinamide nucleotides in H and H–L cells*

Fig. 1 shows the oxidation–reduction changes of nicotinamide nucleotides in the wild (A) and mutant (B) strain of Ehrlich ascites tumour cells. The cells have been pretreated with catalase (to allow oxygen evolution from added H_2O_2) and oxamate (to inhibit reoxidation of cytosolic NADH at the lactate dehydrogenase level) [11]. The kinetics of nicotinamide nucleotides in the H–L cells, shown in trace B, are comparable to those described previously [1]. Addition of a small amount of H_2O_2 (66 μM) causes in both the strains an oxidation of reduced nicotinamide nucleotides of 0.029 A units, which is followed by a reduction when oxygen is exhausted. The further addition of glucose to the anaerobic cells brings about a reduction of cytosolic nicotinamide nucleotides corresponding to 0.02 A units in the H cells (A) and 0.035 A units in the H–L cells (B). It should be noted that the dry weight of the H cells was only slightly lower (about 10%) than that of H–L cells. The half-time of glucose induced nucleotides reduction was 25 sec and 12 sec in the H and H–L cells, respectively. Another difference between the two strains is observed when excess of oxygen (3.3 mM H_2O_2) is added to the anaerobically glycolyzing cells. While H–L cells show, as described previously [1], a bi-

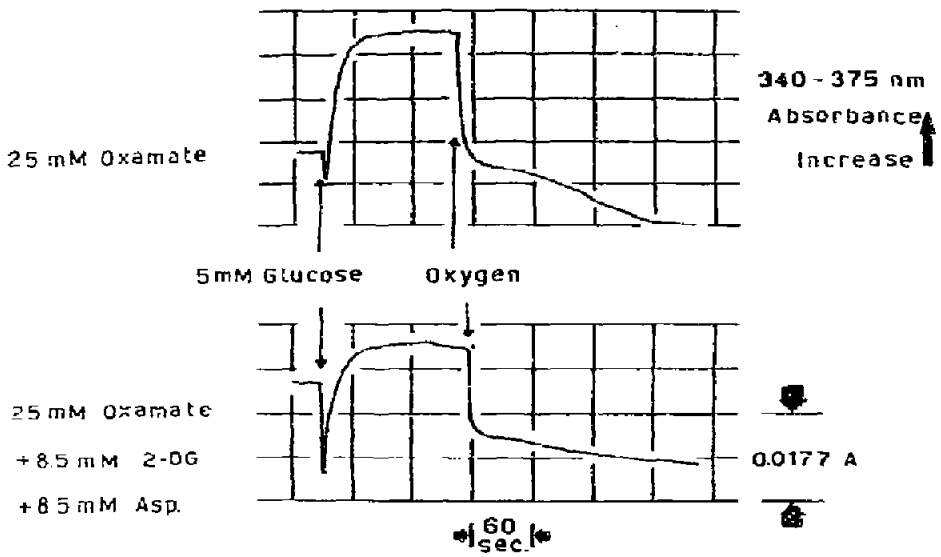

Fig. 2. Redox changes of nicotinamide nucleotides in Ehrlich Lettré (H–L) ascites tumour cells. The cells (4.5 mg dry wt./ml) were pretreated for 5 min with oxamate (upper trace) and oxamate *plus* 2-oxoglutarate (2-OG) *plus* aspartate (Asp.) (lower trace). After the cells became anaerobic, glucose was added to reduce cytosolic nucleotides. Oxidation of nucleotides was then obtained by adding an excess of oxygen.

phasic response, only a fast phase is observed in the H cells. The extent of this phase in the H cells is 0.023 A units and in the H–L cells is 0.026 A units. In the H–L strain the slow oxidation of the nucleotides, following after a lag period the rapid component, has an extent of 0.017 A units.

The data described above can be interpreted as follows: The smaller and slower reduction of cytosolic nicotinamide nucleotides in the H cells, as compared to H–L cells, may be due to the presence in the cytosol of H, but not of H–L cells, of an acceptor for glycolytic reducing equivalents different from pyruvate (in both the strains oxamate prevents reduction of pyruvate to lactate). A probable candidate for such a role is dihydroxyacetone phosphate which indeed is able to be reduced to α-glycerophosphate at the expenses of glycolytic NADH. In fact an accumulation of α-glycerophosphate has been shown to be present in glycolyzing H but not H–L cells (cf. fig. 5 of [2]). Such an hypothesis can explain also the presence of a slow oxidation of cytosolic nicotinamide nucleotides upon addition of oxygen in the H–L cells [1] but not in the H cells. In this strain, in fact, cytosolic nucleotides are already oxidized to a large extent and oxidation of reducing equivalents into the mitochondria occurs through the accumulated α-glycerophosphate, at a low steady state of nucleotides reduction in the cytosol, in agreement with previous data [2] indicating that the α-glycerophosphate shuttle is operating in the wild strain.

If indeed the higher redox state of cytosolic nicotinamide nucleotides in the H–L strain, with respect to the H strain, and their slow aerobic oxidation is due to the absence under anaerobic conditions of cytosolic sinks for reducing equivalents, the addition of a cytosolic oxidant, such as oxalacetate, would render more similar the kinetics of the H–L strain to those of the H strain. Since oxalacetate is not a good penetrant in ascites cells as such, a combination of 2-oxoglutarate and aspartate was used in the experiment shown in fig. 2. In fact the activity of the cytosolic glutamate–oxalacetate transaminase (EC 2.6.1.1) in these cells [3] is high enough to produce high levels of oxalacetate from 2-oxoglutarate and aspartate. The control experiment (upper trace) is not significantly different from the trace of fig. 1B. In the experiment shown in the lower trace H–L cells were preincubated for 5 min with 8.5 mM 2-oxoglutarate and 8.5 mM aspartate and allowed to become anaerobic. The addition of glucose brings about a reduction

Table 1
Steady-state levels of G-6-P and FDP under different metabolic conditions in H and H–L Ehrlich ascites tumour cells.

Conditions	H cells		H–L cells	
	G-6-P	FDP	G-6-P	FDP
	(μmoles/g dry weight)			
Anaerobic	5.2 ± 1.1 (20)	19.1 ± 1.1 (19)	4.0 ± 0.6 (19)	24.0 ± 1.5 (19)
Aerobic	9.6 ± 0.2 (10)	37.9 ± 2.6 (8)	9.1 ± 0.9 (8)	70.2 ± 3.5 (8)
Aerobic + TTFB	7.4 ± 1.5 (10)	29.4 ± 2.5 (10)	4.4 ± 0.9 (9)	30.5 ± 3.7 (10)
Aerobic + rotenone	10.0 ± 2.5 (8)	31.6 ± 1.0 (4)	4.6 ± 1.0 (8)	42.7 ± 4.7 (4)

Experiments have been performed as described in fig. 1. 25 mM oxamate was present in all the experiments. Samples were withdrawn from the spectrophotometric cuvette during kinetic measurements of nicotinamide nucleotides, after addition of glucose to anaerobic cells ('anaerobic') or oxygen to anaerobic cells plus glucose ('aerobic'). When present, TTFB (6 μM) and rotenone (3 μM) were added to anaerobic cells *plus* glucose together with oxygen ('aerobic + TTFB' and 'aerobic + rotenone'). The cell suspension concentrations were 7–16 mg dry wt./ml. The values represent the means ± S.E. (number of observations).

of nicotinamide nucleotides which is about one third of that of the control sample. The addition of excess of oxygen produces a rapid oxidation of nucleotides, half in extent of that of the control experiment. The slow phase starts immediately after the fast oxidation, not being preceded by the lag phase present in the control. In the pretreated cells the extent and the rate of the slow phase are very reduced. Therefore the experiment of fig. 2 indicates that addition to H–L cells of an oxidant for cytosolic reduced nicotinamide nucleotides renders this strain similar to H strain as far as nucleotides redox kinetics are concerned. Moreover the presence of the lag phase in the H–L cells indicates that under conditions of anaerobic glycolysis the shuttle for the reoxidation of cytosolic reducing equivalents is not fully operating and that, upon oxygen addition, intermediates of the malate–aspartate shuttle must be formed before cytosolic hydrogen is translocated into the mitochondria. This is confirmed by the lack of lag phase in the 2-oxoglutarate and aspartate treated cells.

In order to further analyze the characteristics of the shuttle mechanisms in the two strains of ascites cells the experiments described below were performed.

3.2. *The level of G-6-P and FDP in anaerobically and aerobically glycolyzing ascites cells*

The cytosolic NADH:NAD ratio may influence the flux of glycolytic intermediates by regulating the activity of the glyceraldehyde-3-phosphate dehydrogenase reaction. A decrease of this ratio causes a facilitation of the reaction [12]. Thus, the estimation of G-6-P and FDP in different metabolic conditions may give information on the change of the cytosolic redox potential and hence on the intramitochondrial oxidation of glycolytic NADH.

Table 1 shows measurements of such intermediates performed simultaneously with the kinetics of nicotinamide nucleotides, as described in Experimental. Samples were taken after nicotinamide nucleotides have reached steady-state levels upon addition of glucose to anaerobic cells, or excess O_2 to anaerobically glycolyzing cells in the absence or presence of TTFB and rotenone. As can be seen, oxygenation of the anaerobic cells causes, in both the strains, an increase in the level of G-6-P and FDP. This increase is, however, prevented in the H–L cells by TTFB, which causes also the disappearance of the slow phase of nucleotides oxidation, as already described [1]. The effect of the uncoupler on G-6-P and FDP is slight in H cells. Pretreatment of cells with rotenone has consequences similar to those of TTFB on the steady-state level of the two metabolites.

The experiments presented in table 1 indicate the following:
i) the level of G-6-P and FDP may change as a function of the aerobic oxidation of nicotinamide nucleotides in both strains, ii) such oxidation is not only concerned with the mitochondrial but also with the cytosolic nicotinamide nucleotides and iii) the intramitochondrial oxidation of cytosolic NADH is carried out in the two strains by shuttle mechanisms which differ for their sensitivity to inhibitors and uncouplers.

It appears from the data reported above that the operation of the α-glycerophosphate shuttle mechanism occurs at a low steady-state of reduction of cytosolic nicotinamide nucleotides, while the operation of the malate–aspartate shuttle requires a high reduction of nicotinamide nucleotides. Thus, it seems possible to conceive the choice between the two shuttle mechanisms, when both are present, as governed by the level of nicotinamide nucleotide reduction in the cytosol. In particular the malate–aspartate shuttle may permit high rate of nicotinamide nucleotide reduction, while the α-glycerophosphate shuttle would start to operate at lower levels of reduction, when a sufficient amount of α-glycerophosphate is formed at the expenses of reduced nucleotides and dihydroxyacetone phosphate.

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