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Glycolysis is operative in amphibian oocytes

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Abstract

It is generally accepted that in frog full-grown oocytes glycolysis is absent and that carbon metabolic flux is largely directed to glycogen synthesis. Use of an anion exchange pellicular resin for analytical resolution of intermediates in perchloric acid extracts of oocytes has allowed us to observe the formation of labelled lactate after microinjection of [U-^{14}C]glucose. Further, formation of [^{32}P]ATP was observed after microinjection of ^{32}P-labelled glucose-6-P, fructose-6-P or fructose-1,6-bis-P, either in the presence or absence of 0.1 mM cyanide. The presence of phosphofructokinase activity, previously thought to be absent in oocytes, is also reported. These findings indicate that glycolysis to lactate is operative in frog oocytes.

Key words: Glycolysis; Phosphofructokinase, Oocyte

1. Introduction

Present views of carbon metabolism in oocytes and early amphibian embryos hold that they are largely glycogenic and that glycolysis is not operative in these cells (see [1] for a review). These conclusions stem from experiments showing that: (i) microinjection of [^{32}P]PEP[1] does not result in ATP labelling but instead in the rapid appearance of the radioactivity in glucose-6-P and/or glucose-1-P, and later UDP-glucose [2]; (ii) In vivo inhibition of glyceraldehyde-3-P dehydrogenase activity by iodoacetamide or iodoacetate or treatment of the cells with dinitrophenol results in the formation of [^{32}P]ATP from [^{32}P]PEP [2]. It was then concluded that oocyte pyruvate kinase is normally inactive in vivo but that conditions which inhibit glycogenic flux are able to activate the enzyme and, therefore, glycolysis. Likewise, reports from our laboratory [3] and others [4] have shown that after microinjection of [1-^{14}C]- or [6-^{14}C]-glucose, $^{14}CO_2$ production is observed only with the former, a result which is consistent with the view that glycolysis is inactive in oocytes. Further, measurements of enzyme activities in homogenates showed that pyruvate kinase is very active whereas PFK activity was very low or absent [5]. The recent availability of a protocol for the analytical separation and identification of intermediates of glucose utilization [6] has permitted the aquisition of the new evidence reported in this article, indicating that [^{14}C]lactate and [^{32}P]ATP are produced in frog oocytes after microinjection of [^{14}C]glucose or ^{32}P-labelled hexoses-P, respectively. The conclusion that glycolysis to lactate is operative in amphibian stage VI oocytes is important because it provides a proper reference to understand changes of energy metabolism during differentiation of these cells.

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Abbreviations: PEP, phospho*enol*pyruvate; PFK, phosphofructokinase; PCA, perchloric acid; DTT, dithiothreitol.

2. Materials and methods

2.1. Materials

Labelled compounds were from the Radiochemical Centre (Amersham). Before the experiment, aliquots were dried under N_2 to remove ethanol, carrier solutions were added, and resuspended in saline to achieve the desired concentration. Auxiliary enzymes, ATP and NAD^+ were mostly from Sigma. Mixtures for scintillation counting were from New England Nuclear.

2.2. Microinjection procedures and metabolic labelling

Oocytes (stage VI) from the frog *Caudiverbera caudiverbera* were used because of its local availability but mainly because its volume (about 3 μl, compared to the 1 μl *Xenopus* cells) which allows analysis of single oocytes instead of pooled cells. Nonetheless, a few experiments with *Xenopus* oocytes showed the same results (not shown). Injection techniques and handling of compounds were as described [5]. Briefly, approximately 50 nl of modified Barth saline [7] containing radioactive glucose (or other compounds) were injected. Groups of 5 oocytes were then incubated at 22°C under 100% O_2. After incubation, cells were frozen in liquid N_2, homogenized in cold 3% PCA and neutralized. After centrifugation, glycogen was discarded by ethanol precipitation and the supernatant fraction lyophilized and stored until analysis.

2.3. Separation of metabolic intermediates by ionic exchange chromatography

Separation and identification of metabolites was essentially as described [6]. Aliquots of lyophilized PCA extracts were submited to chromatograhy on a CarboPac PA1 column [8,9] controlled by a high performance liquid chromatography apparatus (LKB). The column was equilibrated with 100% solution A (50 mM NaOH) and 0% solu-

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tion B (1 M sodium acetate in 100 mM NaOH) and operated at a flow rate of 0.5 ml/min with a gradient program as follows: T_0 = 0%, T_9 = 0%, T_{10} = 10%, T_{20} = 15%, T_{21} = 20%, T_{51} = 50%, T_{65} = 50%, T_{66} = 100% (subscript numbers are time in minutes, percent figures refer to amount of solution B). Radioactivity readings were taken every 30 s by an on line Beckman 171 Radioisotope detector. Readings were processed with the ChromatoGraphics program supplied by Beckman. Labelled intermediates were identified by comparison of retention times with those of authentic commercial compounds, by spiking of the samples with authentic compounds or by incubation of PCA samples with specific enzymes [6].

2.4. Enzymatic identification of lactate

An aliquot (containing aproximately 100,000 cpm) of a PCA extract from [U-^{14}C]glucose-injected oocytes was incubated 4 h at 37°C in an assay mixture containing: 50 mM Bicine pH 9.4, 40 mM glutamic acid, 5 mM NAD^+, 4 units of glutamic-pyruvic transaminase, 27.5 units of lactic dehydrogenase in a final volume of 100 μl. The reaction was stopped by addition of 100 mM NaOH, the sample was spun and chromatographed as described. The elution time of an internal standard of [^{3}H]alanine was compared with that of the enzymatic product. The production of alanine by the enzyme system was checked in a parallel experiment using [^{14}C]lactate as substrate.

2.5. [^{32}P]fructose-6-P synthesis

1 mM unlabelled fructose was mixed with 0.1 mM unlabelled ATP, 200 μCi [γ-^{32}P]ATP, 100 mM Tris, pH 8.0, 5 mM $MgCl_2$ and 3.8 units of yeast hexokinase in a total volume of 50 μl. After 30 min incubation at room temperature the reaction was stopped by heating during 4 min at 95°C. After spinning, the supernatant was used as the labelled substrate, which was analyzed by HPLC on a CarboPac PA1 column prior to the experiments.

2.6. PFK activity

10 g of oocytes were homogenized in 7 ml of 20 mM Tris buffer, pH 7.5, containing 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 1 μM pepstatin and 0.1 mM phenylmethylsulfonyl fluoride. After centrifugation at 40,000 rpm for 1 h the supernatant fraction was applied to a DEAE-cellulose column equilibrated in the same buffer. The column was washed with 5 volumes of the buffer and the enzyme eluted with 100 mM KCl. Enzyme activity was determined by the coupled assay of Racker [10] in a Hewlett Packard 8452A Diode array spectrophotometer. The assay mixture contained 0.1 M Tris-HCl pH 8.2, 1 mM DTT, 0.2 mM NADH, 2 mM NH_4Cl, 1 mM fructose-6-P, 1 mM ATP, 10 mM $MgCl_2$ and a mixture of 40 μg aldolase, 3 μg of triose phosphate isomerase and 30 μg of α-glycerophosphate dehydrogenase in a volume of 1 ml.

3. Results and discussion

3.1. Separation and identification of labelled intermediates after [U-^{14}C]glucose microinjection

Chromatography of PCA extracts from oocytes microinjected with [U-^{14}C]glucose showed several radioactive peaks of intermediates (Fig. 1A). Criteria for identification were both coelution with authentic commercial compounds and specific enzymatic treatments prior to chromatography [6]. Identified intermediates are glucose-1-P, glucose-6-P, fructose-6-P, and fructose-1,6-bis-P. An additional fraction (designed as X) was also observed and will be dealt with below.

The appearance of these intermediates suggests that glucose is metabolized through the Embden–Meyerhof pathway but the suggestion would be strengthened by the observation of labelled pyruvate and/or lactate from microinjected [U-^{14}C]glucose. Therefore we sought the presence of either lactate or pyruvate in fraction X. Treatment of a neutralized PCA extract with lactic dehydrogenase and alanine aminotransferase followed by chromatography in the CarboPac PA1 column (Fig. 1B), moves about 65% of the radioactivity associated with fraction X to a different elution time (13 min) which corresponds to that of authentic labelled alanine. Control experiments with heat inactivated enzymes or with NAD^+ omitted from the reaction mixture did not show labelled alanine (data not shown). The presence of lactate in a purified preparation of fraction X was also confirmed by mass spectrometry (not shown). Experiments in progress suggest the presence of trioses phosphate in the minor unreacted portion of fraction X. Radioactivity in lactate after 5 min of incubation accounts for 6–8% of the total label microinjected but higher amounts have been observed at shorter times.

3.2. ATP formation in oocytes after [^{32}P]phosphoryl-hexoses microinjection

Another criterion for glycolytic activity is ATP production from ^{32}P-labelled glycolytic intermediates. Dworkin and Dworkin-Rastl [1,2] reported lack of [^{32}P]ATP formation in *Xenopus* oocytes after injection of [^{32}P]PEP, a result which they ascribed to a block of pyruvate kinase activity in vivo. We have studied this problem by microinjection of [^{32}P]phosphoryl-hexoses and detection of [^{32}P]ATP production by chromatography in the CarboPac column described above. Labelled fructose-6-P microinjected into oocytes produces a radioactive peak of ATP (eluting at min 73) after one minute that continues to increase up to 16 min, the longest time studied (Fig. 2). Other radioactive peaks were also observed and most of them identified as shown in Fig. 2. The same results were observed in oocytes co-injected with 0.1 mM cyanide and labelled fructose-6-P (not shown) indicating that [^{32}P]ATP production results from the operation of the glycolytic pathway rather than from oxidative phosphorylation. Oxygen consumption was completely inhibited in the presence of cyanide. Control oocytes showed a value of oxygen consumption of approximately 0.18 nmol/min/oocyte, very similar to the one reported by Cicirelli and Smith [4] for *Xenopus* oocytes.

Fig. 2 shows that microinjected [^{32}P]fructose-6-P is rapidly converted into labelled glucose-6-P (already at 5 s, the minimum time between injection and freezing). Minor peaks of fructose-1,6-bis-P and ATP are also visible at this time. At 1 min, P_i, glucose-1-P and 6-P-gluconate appears and the ATP peak increases. Between 4 and 16 min, the ATP peak has reached a maximum and has split into two peaks. The most retained peak is so far unidentified. Oocytes injected with [^{32}P]glucose-6-P or [1-^{32}P]fructose-1,6-bis-P, showed essentially the same behavior with respect to ATP production (data not shown).

3.3. Presence of PFK activity in frog oocytes

Reports from our laboratory [5] and others (for refer-

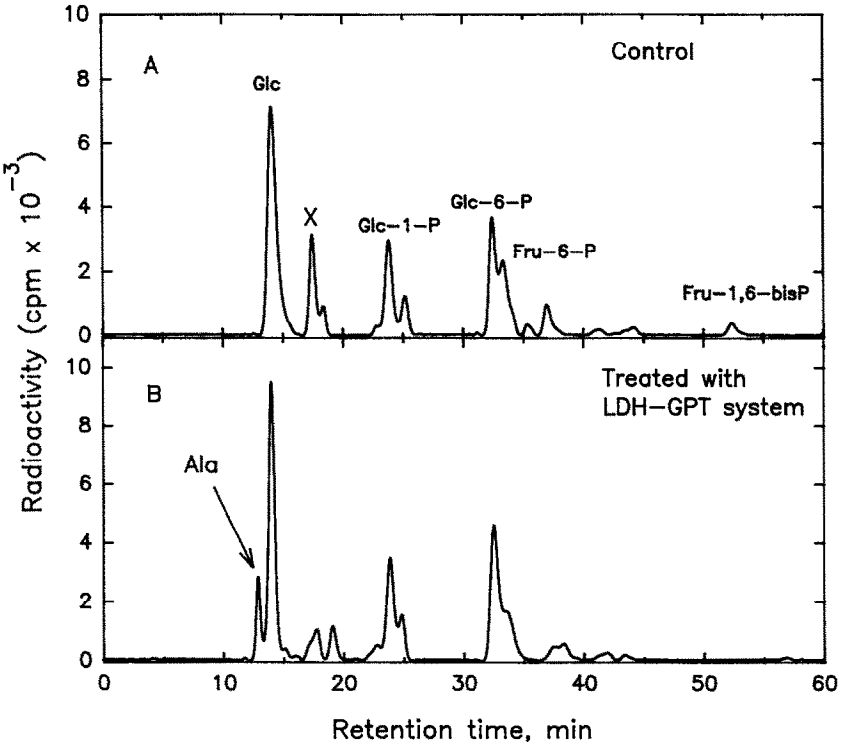

Fig. 1. The presence of lactate among intermediates of glucose utilization in frog oocytes microinjected with [U-^{14}C]glucose. Oocytes were microinjected with a saline solution containing 0.5 nmol of labelled glucose (about 50,000 cpm). After 5 min of incubation at room temperature, PCA extracts were prepared and processed as indicated in section 2. An aliquot of 70 μl was immediately chromatographed (A). Another aliquot was used for enzymatic identification of lactate as described in section 2 (B). Both samples were chromatographed for separation of intermediates in a CarboPac PA1 column. Identified compounds are marked. X refers to a peak containing lactate which is converted to alanine in the experiment depicted in panel B.

ences see [1]) have suggested the absence of PFK activity in stage VI oocytes. However, the formation of labelled lactate after [U-^{14}C]glucose microinjection suggests that this is not the case. The problem was reinvestigated by measurement of PFK activity in concentrated oocyte extracts in the presence of protease inhibitors. Values of about 4 milliunits of PFK activity per gram of oocyte were found, which are indeed very low compared with the activity of other enzymes involved in glucose utilization in these cells [5]. Moreover, some preparations were devoid of detectable enzyme activity. These results could explain the absence of PFK activity previously reported in these cells. Using a DEAE-cellulose semipurified enzyme preparation we observed a hyperbolic saturation curve with respect to fructose-6-P at pH 8 and 1 mM ATP. The apparent K_m value for fructose-6-P was 0.2 mM. Due to the very low activity of the preparations it was not possible to study saturation curves at neutral pH.

As reported in previous papers by our group [3,5,11] or by other workers [1,2,12,13], frog oocytes metabolize glucose to glycogen and, to a minor extent, through the pentose-P pathway. However, as shown in this report, glucose is also degraded to lactate with the concomitant production of ATP. The fate of lactate produced from glucose is unknown but further breakdown through the citric acid cycle can be ruled out since we [5] and others [4] have never observed $^{14}CO_2$ production from [6-^{14}C]glucose. The absence of ATP labelling from microinjected [^{32}P]PEP reported by Dworkin and Dworkin-Rastl [1,2] may be explained by the very low amount of injected substrate (0.2 pmol per oocyte) or by the poor resolution of the thin layer chromatography method used for separation of intermediates in their experiments. Also, different species (*X. laevis* vs. *C. caudiverbera*) were used but, as mentioned earlier, a few experiments performed with *Xenopus* oocytes gave essentially the same results as those in *Caudiverbera*.

Although it is well established that glycogen is the main product of glucose metabolism in frog oocytes, the experiments presented in this report show a considerable flow of carbon to lactate during the first minutes after glucose microinjection. Likewise, phosphate from injected fructose-6-P is transferred to ATP which implies the use of the carbohydrate moiety in glycolysis instead of glycogen synthesis. These facts are at variance with the accepted view that polysaccharide synthesis directly starts from glucose-6-P derived from glucose phosphorylation. Although the details of lactate utilization by oocytes have not yet assessed, the lack of $^{14}CO_2$ production from [6-^{14}C]glucose renders an oxidative metabolism of lactate unlikely and would favor the assumption that it is finally converted into glycogen through the gluconeogenic pathway. The understanding of the bene-

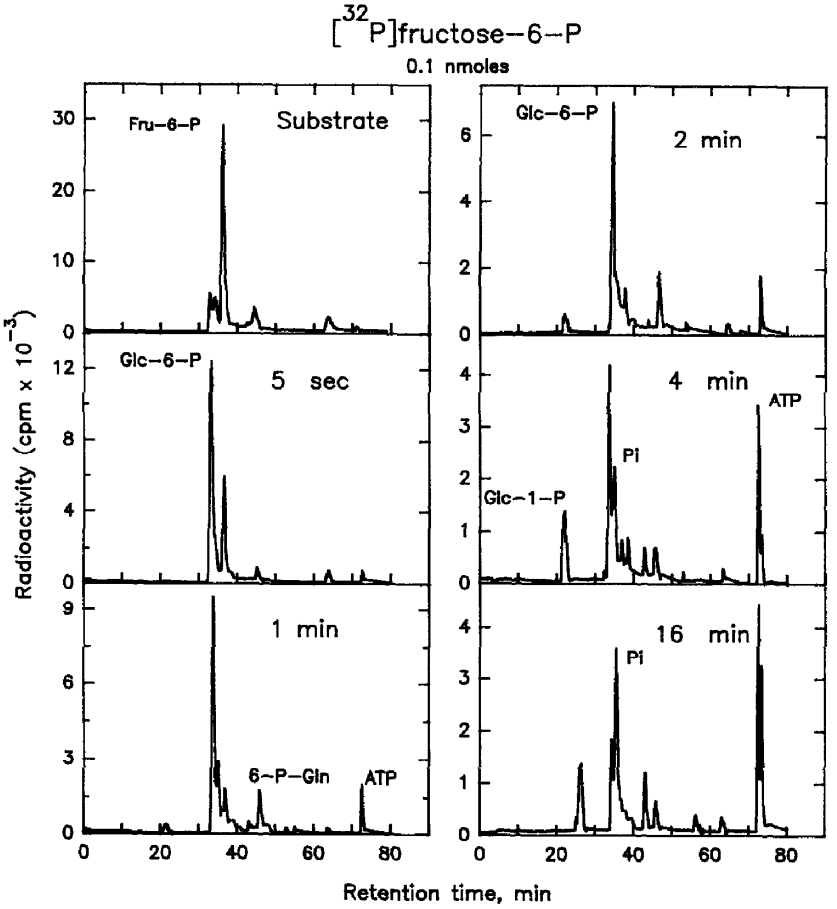

Fig. 2. ATP production in frog oocytes microinjected with [32P]fructose-6-P. Oocytes were microinjected with 50 nl of saline solution containing 0.1 nmol of labelled fructose-6-P (about 200,000 cpm). After incubation at the indicated times, PCA extracts were prepared and processed in a CarboPac column. Identified compounds are marked but, for the sake of simplicity, the labels are indicated only in a few cases.

fits of such an 'indirect' pathway of glycogen formation from glucose needs a better knowledge of oocyte metabolic organization than is presently available. Since the interpretation of tracer experiments performed in a single cell is easier than in multicellular systems (because of the possibility of compartmentation between cells), oocytes provide a useful model for this kind of experiments.

Finally, we would like to stress that the results presented in this report should emphasize the importance of detailed metabolic studies in frog oocytes vis-à-vis the present widespread use of these cells for the analysis of almost any kind of problem in cell biology.

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