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GLYCOLYTIC AND ASSOCIATED ENZYMES OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) RED CELLS: *IN VITRO* AND *IN VIVO* STUDIES

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Summary

Studies were undertaken *in vitro* and *in vivo* to assess the maximal activities of 26 glycolytic and associated enzymes of rainbow trout (*Oncorhynchus mykiss*) red cells. The red cells possess a complete sequence of glycolytic enzymes capable of anaerobic oxidation of glucose to lactate. Red cell pyruvate kinase (PK) was inhibited by ATP ($I_{50} \approx 5\,mmol\,l^{-1}$), but was not sensitive to alanine inhibition or fructose-1,6-bisphosphate activation. The properties of red cell PK were similar to those of the muscle-type enzyme. Lactate dehydrogenase (LDH) from the trout erythrocyte resembled LDH from trout heart in terms of pyruvate inhibition *in vitro*. Enzymes associated with phosphagen and amino acid metabolism as well as the pentose phosphate shunt were also present. However, enzyme indicators of glycogenolytic and gluconeogenic potential were either absent or present at very low levels. The capacity for aerobic respiration *via* the tricarboxylic acid (TCA) cycle was suggested by the presence of citrate synthase activity.

The association of glycolytic enzymes with the particulate fraction of the red cell was assessed for six enzymes. No binding was detected for hexokinase, PK and LDH; low levels of binding by phosphofructokinase (5%), aldolase (1%) and glyceraldehyde-3-phosphate dehydrogenase (3%) were not altered by strenuous exercise. Glycolytic enzyme binding, therefore, does not appear to be an important regulator of energy metabolism in these cells. Intracellular glucose levels, in contrast, appeared to be regulated following exercise stress. An increase in intra-erythrocytic lactate levels at this time may reflect the importance of exogenously produced lactate as a substrate for ATP production. The description of trout red cell metabolism presented here provides a basis for further study of the relationships between organismic gas exchange and molecular-level adaptation of nucleated red cell function.

Introduction

The nucleated red blood cell of the rainbow trout (*Oncorhynchus mykiss*) is

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frequently employed in the study of hormone-sensitive ion transport processes involved in cellular pH regulation and the maintenance and/or optimization of cellular function (see reviews by Nikinmaa, 1986; Nikinmaa and Tufts, 1989). Following exhaustive exercise, for example, catecholamines are released into the vascular circulation of the trout and stimulate red cell membrane sodium/proton exchange such that intracellular pH is maintained in the face of an extracellular acidosis. Consequently, and despite a marked Root shift which is demonstrable *in vitro*, the arterial blood oxygen content of the animal is maintained constant *in vivo* (Primmett *et al.* 1986), thereby ensuring adequate oxygen delivery to the tissues.

Although the membrane ion exchange processes involved in adrenoceptor-mediated pH regulation by salmonid red cells are well known (Nikinmaa and Tufts, 1989; Motais and Garcia-Romeu, 1989), less is known about the nature or the response of the cellular metabolic pathways. The mature nucleated red cells of lower vertebrates (Hunter and Hunter, 1957) including rainbow trout (Eddy, 1977) display high oxygen consumption rates relative to those of mammals. Following catecholamine stimulation of salmonid red cells, Na^+/H^+ exchange is accelerated, erythrocyte O_2 consumption increases in proportion to the degree of intracellular pH regulation, and intra-erythrocytic ATP levels fall to a new, and lower, steady-state level (Ferguson and Boutilier, 1988; Ferguson *et al.* 1989). These responses are indicative of an increased cellular ATP turnover, which is related in part to enhanced membrane sodium pump activity in trout red cells (Bourne and Cossins, 1982; Boutilier and Ferguson, 1989). Despite such evidence of coupling between energy metabolism and membrane transport processes, very little is known about the pathways or enzymes involved in the energy metabolism of salmonid erythrocytes. Bachand and Leray (1975) have demonstrated that yellow perch (*Perca flavescens*) red cells possess many enzymes required for glycolysis as well as the catabolism of glucose *via* the pentose phosphate shunt. Very recently, Walsh *et al.* (1990) have reported that the erythrocytes of rainbow trout possess several enzymes that are indicative of metabolic capacities similar to those found for the perch red cell.

Given the importance of ATP as an allosteric modulator of salmonid haemoglobins (Weber, 1982; Bartlett, 1980), in addition to the reported links between energy metabolism and intracellular pH (pHi) regulation (see above), investigations on the enzymes involved in erythrocyte energy metabolism are indicated. To this end we have evaluated maximal activities and selected properties of various glycolytic and related enzymes of the rainbow trout erythrocyte.

Materials and methods

Animals

Freshwater-adapted rainbow trout (*Oncorhynchus mykiss*) were obtained from Linwood Acres Trout Farm, Campbellcroft, Ontario, and housed at the aquatic animal holding facilities of the Department of Biology, University of Ottawa. The

animals were held for at least 1 month prior to experimentation (December and January). They were maintained in dechlorinated Ottawa tapwater at 10±1°C under a 12 h/12 h light/dark cycle and were fed on commercially prepared trout pellets *ad libitum*. The fish were chosen randomly from a stock of male and female animals ranging in size from 200 to 300 g.

Chemicals

Biochemicals and coupling enymes were purchased from Sigma Chemical Company, St Louis, MO, or Boehringer Mannheim Corp., Montreal, Quebec. All other chemicals were of reagent grade. Solutions were prepared using distilled deionized water.

Collection of blood and preparation of red cell homogenates

Trout were netted and immediately stunned by a blow to the head. An incision was made which severed the ventral aorta and blood was collected in vessels containing heparinized (50 i.u. ml^{-1}) teleost saline (Hoar and Hickman, 1967). The heparinized whole blood was centrifuged at 18000 *g* in a Hermle Z360K centrifuge for 5 min at 5°C. The plasma supernatant was removed and replaced with 2 vols of chilled heparinized teleost saline. The red cells were then resuspended and centrifuged to obtain washed red cell pellets which were frozen in liquid nitrogen until transfer to an ultralow-freezer (−80°C), where they remained until used for enzyme analyses.

Frozen red cell pellets were weighed and homogenized 1:4 (w/v) (3×10 s with a Polytron PT10 homogenizer) in ice-cold homogenization buffer: 20 mmol l^{-1} imidazole–HCl (pH 7 at 20°C) containing 30 mmol l^{-1} NaF, 1 mmol l^{-1} EGTA, 1 mmol l^{-1} EDTA and 15 mmol l^{-1} 2-mercaptoethanol was used routinely. This buffer was used for most enzymes but 2-mercaptoethanol was omitted for the determination of citrate synthase and glutathione reductase activities. For the measurement of phosphofructokinase activity, the frozen red cell pellets were homogenized in a stabilization buffer containing 100 mmol l^{-1} potassium phosphate (pH 7.5), 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA, 0.1 mmol l^{-1} fructose-1,6-bisphosphate, 0.1 mmol l^{-1} ATP and 10 mmol l^{-1} 2-mercaptoethanol. Homogenates prepared for the analysis of NAD^+-linked isocitrate dehydrogenase were made in 20 mmol l^{-1} imidazole–HCl (pH 7.2) containing 20 mmol l^{-1} 2-mercaptoethanol, 1 mmol l^{-1} ADP, 5 mmol l^{-1} citrate, 5 mmol l^{-1} $MgSO_4$ and 40 % (v/v) glycerol, as described by Storey and Fields (1988). Phenylmethylsulphonyl fluoride (PMSF) was added to all buffer solutions immediately before homogenization (final concentration in homogenate 0.1 mmol l^{-1}). Homogenates were centrifuged at 27000 *g* for 20 min at 5°C. The supernatant fractions were removed and samples (approx. 500 μl) were layered onto 5 ml columns of Sephadex G-25 equilibrated in homogenization buffer. Columns were centrifuged (IEC clinical centrifuge) for 1 min and the desalted eluent was used for the measurement of enzyme activities (Helmerhorst and Stokes, 1980). For glycogen

phosphorylase, red cell homogenates were not centrifuged or passed through a spun column; activity was measured directly in settled homogenates.

Determination of enzyme maximal activities

Enzyme activities in each red cell sample were determined in duplicate assays at 340 nm using a Gilford UV-VIS recording spectrophotometer. The assays were performed at 20° C so that maximal activity determinations were made within, and at the upper end of, the tolerated temperature range of this animal. For each enzyme, determinations of specific activities (as well as the corresponding non-specific activities) were performed on 3–4 independently prepared extracts. All reaction mixtures were buffered (pH 7.2) in 50 mmol l^{-1} imidazole–HCl unless otherwise indicated. Coupling enzymes were desalted before use. The volume of the reaction mixtures was set at 1.0 ml. To this volume, 20 μl or less of the homogenate was added. In the following descriptions, (*) will be used to designate the biochemical omitted to take into account non-specific activities. Optimal assay conditions were determined to be as follows (coupling enzymes, listed in terms of international units, were added in excess).

Hexokinase (E.C. 2.7.1.1): 10 mmol l^{-1} glucose (*), 0.2 mmol l^{-1} NADP, 2 mmol l^{-1} ATP, 5 mmol l^{-1} $MgCl_2$, 0.5 i.u. of glucose-6-phosphate dehydrogenase (G6PDH).

Phosphoglucoisomerase (E.C. 5.3.1.9): 2.5 mmol l^{-1} fructose-6-phosphate (*), 0.2 mmol l^{-1} NADP, 5 mmol l^{-1} $MgSO_4$, 0.5 i.u. of G6PDH.

Phosphofructokinase (E.C. 2.7.1.11): 10 mmol l^{-1} fructose-6-phosphate (*), 5 mmol l^{-1} ATP, 10 mmol l^{-1} $MgSO_4$, 0.15 mmol l^{-1} NADH, 50 mmol l^{-1} KCl, 0.5 i.u. of aldolase, 0.5 i.u. of triosephosphate isomerase, 2 i.u. of glycerol-3-phosphate dehydrogenase.

Aldolase (E.C. 4.1.2.13): 0.5 mmol l^{-1} fructose-1,6-bisphosphate (*), 0.15 mmol l^{-1} NADH, 0.5 i.u. of triosephophate isomerase, 2 i.u. of glycerol-3-phosphate dehydrogenase.

Triosephosphate isomerase (E.C. 5.3.1.1): 4 mmol l^{-1} glyceraldehyde-3-phosphate (*), 0.15 mmol l^{-1} NADH, 0.5 i.u. of glycerol-3-phosphate dehydrogenase.

Glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.9): 20 mmol l^{-1} glycerate-3-phosphate (*), 1 mmol l^{-1} ATP, 1 mmol l^{-1} EDTA, 5 mmol l^{-1} $MgSO_4$, 0.15 mmol l^{-1} NADH, 2 i.u. of phosphoglycerate kinase.

Phosphoglycerate kinase (E.C. 2.7.2.3): 10 mmol l^{-1} glycerate-3-phosphate (*), 1 mmol l^{-1} ATP, 1 mmol l^{-1} EDTA, 5.0 mmol l^{-1} $MgSO_4$, 0.15 mmol l^{-1} NADH, 1 i.u. of glyceraldehyde-3-phosphate dehydrogenase.

Phosphoglycerate mutase (E.C. 2.7.5.3): 5 mmol l^{-1} 3-phosphoglycerate (*), 0.1 mmol l^{-1} 2,3-diphosphoglycerate, 5 mmol l^{-1} $MgSO_4$, 0.5 mmol l^{-1} ADP, 0.15 mmol l^{-1} NADH, 0.5 i.u. of enolase, 0.5 i.u. of pyruvate kinase, 0.5 i.u. of lactate dehydrogenase.

Enolase (E.C. 4.2.1.11): 1 mmol l^{-1} 2-phosphoglycerate (*), 0.5 mmol l^{-1} ADP, 5 mmol l^{-1} $MgSO_4$, 1 mmol l^{-1} KCl, 0.15 mmol l^{-1} NADH, 0.5 i.u. of pyruvate kinase, 0.5 i.u. of lactate dehydrogenase.

Pyruvate kinase (E.C. 2.7.1.40): 10 mmol l^{-1} phosphoenolpyruvate (*), 2.5 mmol l^{-1} ADP, 50 mmol l^{-1} KCl, 5 mmol l^{-1} $MgSO_4$, 0.15 mmol l^{-1} NADH, 0.5 i.u. of lactate dehydrogenase.

Lactate dehydrogenase (E.C. 1.1.1.27): 1 mmol l^{-1} pyruvate (*), 0.15 mmol l^{-1} NADH.

Glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49): 2.0 mmol l^{-1} glucose-6-phosphate (*), 5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} NADP.

Glutathione reductase (E.C. 1.6.4.2): 3 mmol l^{-1} glutathione (oxidized)(*), 0.15 mmol l^{-1} NADPH, 2.5 mmol l^{-1} EDTA.

Phosphoglucomutase (E.C. 2.7.5.1): 15 mmol l^{-1} glucose-1-phosphate (*), 0.20 mmol l^{-1} NADP, 5.0 mmol l^{-1} $MgSO_4$, 0.5 i.u. of G6PDH.

Glycogen phosphorylase (E.C. 2.4.1.1): 2 mg ml^{-1} glycogen, 25 mmol l^{-1} KH_2PO_4/K_2HPO_4 (*), 1 mmol l^{-1} AMP, 0.01 mmol l^{-1} glucose-1,6-bisphosphate, 15 mmol l^{-1} $MgSO_4$, 0.5 mmol l^{-1} NADP, 0.5 i.u. of phosphoglucomutase, 0.5 i.u. of glucose-6-phosphate dehydrogenase.

Fructose-1,6-bisphosphatase I (E.C. 3.1.3.11): 0.1 mmol l^{-1} fructose-1,6-bisphosphate (*), 0.2 mmol l^{-1} NADP, 5 mmol l^{-1} $MgSO_4$, 0.5 mmol l^{-1} glucose-6-phosphate dehydrogenase, 0.5 i.u. of phosphoglucoisomerase.

NAD^+-dependent isocitrate dehydrogenase (E.C. 1.1.1.41): 20 mmol l^{-1} isocitrate (*), 1 mmol l^{-1} ADP, 1 mmol l^{-1} NAD, 5 mmol l^{-1} $MgSO_4$.

$NADP^+$-dependent isocitrate dehydrogenase: (E.C. 1.1.1.42): 20 mmol l^{-1} isocitrate (*), 1 mmol l^{-1} ADP, 1 mmol l^{-1} NADP, 5 mmol l^{-1} $MgSO_4$.

Glutamate dehydrogenase (E.C. 1.4.1.2): 20 mmol l^{-1} α-ketoglutarate (*), 200 mmol l^{-1} ammonium acetate, 1 mmol l^{-1} ADP, 0.15 mmol l^{-1} NADH.

Citrate synthase (E.C. 4.1.3.7): 0.3 mmol l^{-1} acetyl coenzyme A, 0.5 mmol l^{-1} oxaloacetate (*) 0.5 mmol l^{-1} 5′,5′-dithiobis(2-nitrobenzoic) acid (assayed at 412 nm).

α-Glycerophosphate dehydrogenase (E.C. 1.1.99.5): 0.5 mmol l^{-1} dihydroxyacetone phosphate (*), 0.15 mmol l^{-1} NADH.

Malate dehydrogenase (E.C. 1.1.1.37): 0.5 mmol l^{-1} oxaloacetate (*), 0.15 mmol l^{-1} NADH.

Glutamate–oxaloacetate transaminase (E.C. 2.6.1.1): 40 mmol l^{-1} L-aspartate, 7 mmol l^{-1} α-ketoglutarate (*), 0.15 mmol l^{-1} NADH, 1 i.u. of malate dehydrogenase.

Glutamate–pyruvate transaminase (E.C. 2.6.1.2): 50 mmol l^{-1} L-alanine (*), 10 mmol l^{-1} α-ketoglutarate, 0.15 mmol l^{-1} NADH, 1 i.u. of lactate dehydrogenase.

Adenylate kinase (E.C. 2.7.4.3): 2 mmol l^{-1} AMP (*), 2 mmol l^{-1} ATP, 0.5 mmol l^{-1} phosphoenolpyruvate, 5 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} KCl, 0.15 mmol l^{-1} NADH, 0.5 i.u. of pyruvate kinase, 0.5 i.u. of lactate dehydrogenase.

Creatine kinase (E.C. 2.7.3.2): 20 mmol l^{-1} creatine phosphate (*), 20 mmol l^{-1} glucose, 10 mmol l^{-1} $MgSO_4$, 1 mmol l^{-1} ADP, 0.5 mmol l^{-1} NADP, 15 mmol l^{-1} 2-mercaptoethanol, 0.5 i.u. of hexokinase, 0.5 i.u. of G6PDH.

Comparison of enzymes from red cells, heart, liver and white muscle

Samples of trout heart, liver and white muscle were collected after first killing the animal with a sharp blow to the head. The tissues were immediately dissected from the animal and placed in liquid nitrogen (this procedure took approximately 30 s). The frozen tissues were subsequently stored in a freezer at −80 °C. Tissue homogenates were prepared as described above for the red cells. Kinetic constants were determined as described in Plaxton and Storey (1984*a*,*b*) using a non-linear least-squares curve-fitting and statistical program developed in this laboratory (S. P. J. Brooks, in preparation).

Effects of strenuous burst exercise in vivo *on glycolytic enzyme binding to the particulate fraction of the rainbow trout red cell*

Trout were chronically catheterized by the method of Smith and Bell (1964) following anaesthesia in a fresh water: MS-222: $NaHCO_3$ mix (20000:2:4) (w:w:w). The fish were allowed to recover in blackened Perspex boxes, each with flowing fresh water, for at least 36 h. At the end of the recovery period, blood (approx. 1.5 ml) was drawn from the dorsal aorta of the resting fish (N=5). Each fish was then exercised in a 50 l aquarium by grasping its tail. After a period of approximately 5 min, the fish no longer responded to tactile stimuli with burst swimming. At this point the animal was placed back in the Perspex chamber and blood samples were again taken.

The dilution method of Clarke *et al.* (1984) was used to determine the degree of binding of glycolytic enzymes to the particulate fraction of red cell homogenates. Samples were prepared in a cold room at 5 °C by first spinning the whole blood in 1.5 ml Eppendorf tubes (Eppendorf microfuge). The plasma was discarded and the red cell pellet was homogenized 1:3 (w/v) in ice-cold homogenization buffer (250 mmol l^{-1} sucrose, 10 mmol l^{-1} 2-mercaptoethanol and 0.1 mmol l^{-1} PMSF) by two 15 s pulses from a Kontes micro-ultrasonic cell disrupter. A sample of the resulting homogenate was then transferred to a vessel in which it was diluted 1:4 (v/v) with stabilization buffer containing 100 mmol l^{-1} potassium phosphate (pH 7.5), 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA, 25 mmol l^{-1} NaF, 0.1 mmol l^{-1} fructose-1,6-bisphosphate, 0.1 mmol l^{-1} ATP and 10 mmol l^{-1} 2-mercaptoethanol. This preparation was used for the measurement of total activities of red cell glycolytic enzymes.

The remainder of the original homogenate was spun for 10 min in an Eppendorf microfuge. The resulting supernatant was removed and diluted (1:4 w/v) with stabilization buffer. This preparation contained the 'soluble' or 'free' red cell enzyme fraction. The bound fraction of red cell enzymes was then released by resuspending the pellet twice with stabilization buffer (the total volume used being equivalent to the volume of stabilization buffer used in the previous step), centrifuging for 10 min, and removing the supernatant. The two supernatant volumes were combined and the enzyme activities were then measured in the three fractions: bound, free and total. The last determination was made to verify a high

recovery of the enzyme of interest. Values of 'bound' and 'free' enzyme activities were used to determine the fraction of bound glycolytic enzyme using the following formula:

$$\% \text{ bound} = 100 \times \text{units bound} \times (\text{units free} + \text{units bound})^{-1}$$

Intracellular glucose and lactate concentrations of rainbow trout red cells

Blood (approx. 1 ml) was drawn *via* an indwelling dorsal aortic cannula from rainbow trout ($N=4$) at rest as well as 5 and 90 min following exhaustive exercise (see above). The blood was immediately prepared on ice for the determination of mean cellular haemoglobin (Hb) content [MCHC$=100\times$g Hb$\times$dl^{-1} whole blood$\times$ haematocrit (%)$^{-1}$], cellular and plasma water contents as well as plasma and cellular glucose and lactate concentrations.

Whole-blood haemoglobin content was determined spectrophotometrically on duplicate samples by the cyanomethaemoglobin method given in *Sigma Chemical Co. Bulletin no. 525*. Haematocrit values were obtained by spinning triplicate samples of whole blood in microcapillary tubes for 3 min in an IEC microcapillary centrifuge. MCHC (g Hb per 100 ml packed red cells) was calculated from these values.

Plasma and red cell water contents were determined by centrifuging approximately 500 μl of whole blood in dried, pre-weighed Eppendorf tubes for 4 min in an Eppendorf microfuge. Following centrifugation, the plasma layer was removed from the red cell pellet and placed in another dried and pre-weighed Eppendorf tube. Both tubes were weighed to determine the 'wet masses' of the samples prior to drying at 80 °C for 72 h. The dry mass values that were subsequently obtained were used in determining the percentage water content (g H_2O per 100 g red blood cells or plasma) of the samples.

To determine plasma and red cell glucose and lactate levels, 500 μl of whole blood was centrifuged, separated into plasma and red cell components, and weighed as above. The samples were then diluted 1:2.5 (w/v) with chilled 6 % (w/v) perchloric acid (PCA) containing 1 mmol l^{-1} EDTA. The resulting mixtures were sonicated for 15 s with a Kontes micro-ultrasonic cell disrupter and incubated on ice for 5 min prior to a 4 min centrifugation in an Eppendorf microfuge. The PCA supernatant was then removed and neutralized with one-third of their volume of 2 mol l^{-1} KOH containing 0.3 mmol l^{-1} Tris base and 0.4 mmol l^{-1} KCl. The prepared extracts were kept at -80 °C prior to analyses of glucose and lactate.

Glucose and lactate levels were determined spectrophotometrically at 340 nm (Pye-Unicam SP8–100 UV-VIS). Each sample was assayed in duplicate. Glucose determinations were made in 1 ml cuvettes containing Tris buffer (100 mmol l^{-1}, pH 8) with 5 mmol l^{-1} $MgCl_2$, 0.5 mmol l^{-1} ATP, 0.5 mmol l^{-1} NAD, 0.1 unit of glucose-6-phosphate dehydrogenase and 50 μl of neutralized extract. The change in absorbance following the addition of 0.3 units of hexokinase was used to calculate the amount of glucose in the extract. Lactate determinations were made with the addition of 2.5 units of lactate dehydrogenase to hydrazine sulphate

(200 mmol l^{-1}, pH 9.5) containing 0.5 mmol l^{-1} NAD and 10 μl of neutralized extract. Appropriate standards and blanks were used to validate the quantification procedures.

Determinations of intra-erythrocytic lactate and glucose concentrations were made using a correction factor of 2.5 % for plasma trapped in the red cell pellet (Motais *et al.* 1987; Houston, 1985).

Results and discussion

Glycolytic and related enzymes of the trout red cell

A full complement of glycolytic enzymes exists in the erythrocytes of *Oncorhynchus mykiss* (Table 1). Though we were unable to detect a gluconeogenic potential in the red cells of trout (fructose-1,6-bisphosphatase I activity is not present, Table 1) small amounts of intra-erythrocytic glycogen have been reported (Walsh *et al.* 1990). Nevertheless, a capacity for glycogenolysis appears to be lacking, since glycogen phosphorylase activity could not be detected and phosphoglucomutase activity was extremely low (Table 1). It is evident from these findings that glycogen metabolism does not play an important role in the overall energy metabolism of these cells. This contrasts sharply with frog erythrocytes, which store glycogen and show high glycogen phosphorylase and phosphoglucomutase maximal activities (Kaloyianni-Dimitriades and Beis, 1984).

Rainbow trout red cells have the enzymatic capability of phosphorylating endogenous glucose (see below for intracellular glucose levels) *via* the hexokinase (HK) reaction and deriving ATP equivalents through anaerobic oxidation. In addition to the glycolytic capabilities of these cells, they are able to catabolize glucose *via* the pentose phosphate shunt, as indicated both by the substantial glucose-6-phosphate dehydrogenase activity (Table 1) and by radio-labelling experiments (Walsh *et al.* 1990). This pathway is an important source of cellular NADPH which can be utilized by the erythrocyte to reduce glutathione (*via* glutathione reductase, Table 1) which, in turn, is involved in methaemoglobin reduction.

It is evident that these erythrocytes also have an appreciable degree of flexibility in their ability to store and utilize high-energy phosphate compounds, given the substantial activities of adenylate and creatine kinases. The glycolytic enzymes directly responsible for the production of ATP, namely phosphoglycerate kinase (PGK) and pyruvate kinase (PK), are present in much greater activities than HK and phosphofructokinase (PFK), which catalyse ATP-consuming reactions. Presumably, the low activities of the latter enzymes, in relation to those of the other enzymes involved in trout red cell glycolysis, reflect their well-known regulatory and/or rate-limiting roles in the glycolytic pathway.

In addition to the enzymes of the glycolytic and pentose phosphate pathways, the trout erythrocytes contain enzymes related to amino acid metabolism and the TCA cycle (Table 1). Malate dehydrogenase (MDH), in addition to playing a role in the TCA cycle of mitochondria, is found in abundance in the cytosol of various

Table 1. *Maximal activities of glycolytic and associated enzymes of rainbow trout red cells*

Enzyme	Maximal activity (units g^{-1} Hb)
Glycolytic enzymes	
Glycogen phosphorylase	ND
Phosphoglucomutase	3.22±0.89
Hexokinase	2.73±0.46
Phosphoglucoisomerase	112.7±2.95
Phosphofructokinase	1.56±0.03
Aldolase	4.28±0.78
Triosephosphate isomerase	139.36±15.82
Glyceraldehyde-3-phosphate dehydrogenase	22.37±2.55
Phosphoglycerate kinase	99.93±4.63
Phosphoglycerate mutase	9.79±0.62
Enolase	4.56±0.52
Pyruvate kinase	32.05±2.24
Lactate dehydrogenase	50.50±4.50
Others	
Glucose-6-phosphate dehydrogenase	14.06±3.11
Glutathione reductase	2.66±0.44
Fructose-1,6-biphosphatase I	ND
Glutamate–pyruvate transaminase	1.65±0.22
Glutamate–oxaloacetate transaminase	3.78±0.30
Glutamate dehydrogenase	3.27±0.03
α-Glycerophosphate dehydrogenase	0.70±0.08
Malate dehydrogenase	64.64±4.00
Citrate synthase	0.58±0.05
$NADP^+$-dependent isocitrate dehydrogenase	2.93±0.16
NAD^+-dependent isocitrate dehydrogenase	ND
Adenylate kinase	9.20±2.03
Creatine kinase	6.04±1.51

One unit of enzyme activity is defined as the amount of enzyme utilizing 1 μmol of substrate per minute at 20°C.

Mean haemoglobin (Hb) content of cell pellets was 0.2 g Hb g^{-1} wet mass.

Each value is the mean±1 S.E.M. of 3–4 independent determinations from separate samples of red cells.

ND, not detected.

vertebrate cells. In trout liver, for example, MDH activity in the cytosol exceeds that in the mitochondria 10-fold (Walton, 1985). Citrate synthase (Table 1), in contrast, is virtually restricted to the mitochondria (Walton, 1985) and is therefore a much more reliable index of TCA activity than is MDH. Activity of NAD^+-dependent isocitrate dehydrogenase (IDH) was not detected. This is probably a result of the highly labile nature of this enzyme in fish tissue and does not represent any modification of the TCA cycle to circumvent this reaction (Storey and Fields, 1988). Despite the failure to find NAD^+-dependent IDH activity, the presence of

citrate synthase and other enzymes related to aerobic metabolism (Table 1) is consistent with the high respiration rate exhibited by trout red cells (Eddy, 1977; Boutilier and Ferguson, 1989).

Our data are consistent with an ability of trout red cells to catabolize four- and three-carbon amino acids, namely aspartate (*via* glutamate-oxaloacetate transaminase) and alanine (*via* glutamate–pyruvate transaminase). The glutamate that arises from these reactions may be fed into the TCA cycle as α-ketoglutarate by the oxidative glutamate dehydrogenase reaction. Lungfish erythrocytes are known to oxidize glutamate preferentially over glucose (Mauro and Isaaks, 1990). It is not known whether rainbow trout red cells are similar in this respect. Though alanine oxidation by trout red cells has been demonstrated by Walsh *et al.* (1990), the rate was modest in comparison to those of glucose and lactate oxidation. It is evident that much more work is required to elucidate the major substrates involved in aerobic ATP production by these cells. Possibly, an additional role of glutamate–oxaloacetate transaminase is involvement in an erythrocytic malate–aspartate shuttle. In this case, cytosolic malate dehydrogenase and glutamate–oxaloacetate transaminase would shunt reducing equivalents (i.e. NADH produced by glyceraldehyde phosphate dehydrogenase) into the mitochondria. Whether this shuttle functions in the red cells of trout remains to be demonstrated. However, given the very low levels of α-glycerophosphate dehydrogenase (Table 1), it would appear that an α-glycerophosphate dehydrogenase/α-glycerophosphate oxidase shuttle is of little metabolic relevence to these cells.

Comparison of glycolytic isoenzymes in trout tissues

Some properties of erythrocyte glycolytic enzymes were assessed to determine their relationship to enzymes from other trout organs (Tables 2–4). Trout erythrocyte HK showed a low K_m for glucose, the range being similar to that of muscle hexokinase and well within the range of intra-erythrocytic glucose concentrations (Fig. 1 and Table 2). Erythrocyte HK is distinctly different from the high-K_m HK isoenzyme that is often called glucokinase. Glucokinase, in

Table 2. *Maximal activities and* K_m *for glucose of hexokinase/glucokinase from three different tissues of the rainbow trout*

	Maximal activity (units g^{-1} wet mass)	K_m (mmol l^{-1})
Liver	0.196±0.009	41.6±5.03
Erythrocyte	0.440±0.015	0.375±0.029
White muscle	0.054±0.003	0.204±0.021

Each value is the mean ±1 S.E.M. of determinations made on three independent tissue samples from different fish.

Note that the red cell enzyme activity is expressed in units per gram packed cells and differs from the value presented in Table 1.

The assay conditions are described in Materials and methods, with the exceptions that the optimal glucose concentrations were 300 and 12.5 mmol l^{-1} for liver and muscle, respectively.

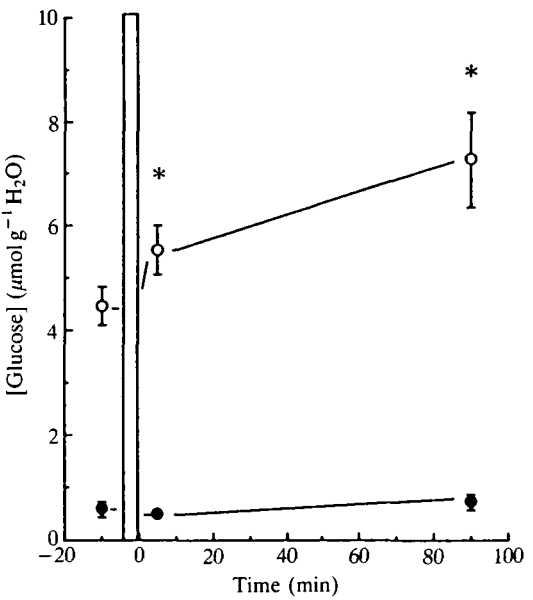

Fig. 1. Intra- (●) and extra- (○) erythrocytic glucose concentrations (expressed in terms of cell and plasma water) before 5 min of burst swimming activity and during recovery from exercise. The vertical column on the graph represents the 5 min period of burst exercise. Values are means±1 S.E.M. of determinations on four animals. An asterisk denotes a value significantly greater than the corresponding control value (paired *t*-test, $P<0.05$).

addition to a low-K_m HK, is known to be present in vertebrate livers. The low-K_m HK found in trout red cells appears to be well suited for utilizing intracellular glucose.

PK activity was lowest in the red cells (Table 3). The PK of trout erythrocytes quite closely resembles the enzyme from white skeletal muscle. The K_m for phosphoenolpyruvate was low and the enzyme was not sensitive to either L-alanine or fructose-1,6-bisphosphate as allosteric modulators. This is typical of the vertebrate M_1 isoenzyme. By contrast, trout liver PK showed strong inhibition by alanine and potent activation by fructose-1,6-bisphosphate (ninefold at optimum levels). Pyruvate kinase from human (Staal *et al.* 1971) and frog (Kaloyianni-Dimitriades and Beis, 1984) erythrocytes is also activated by fructose-1,6-bisphosphate. The absence of this effector for trout red cell pyruvate kinase may be due to phylogenetic or functional reasons that are not apparent at present.

ATP was effective in inhibiting PK activity in crude homogenates prepared from skeletal (white) muscle, liver and red blood cells (Table 3). The I_{50} values of ATP found in this study are well within the range of intracellular ATP levels reported for trout liver, muscle (1.32 and 5.34 mmol ATP kg^{-1} wet mass, respectively; Dobson and Hochachka, 1987), and red cells (approx. 5 mmol l^{-1} red blood cells;

Table 3. *Maximal activities and some kinetic properties of pyruvate kinase prepared from rainbow trout tissues*

	Liver	Erythrocyte	White muscle
Maximal activity (units g^{-1} wet mass)	7.17±0.06	4.44±0.26	163.08±0.94
K_m (mmol l^{-1})	0.376±0.058	0.148±0.036	0.046±0.002
I_{50} ATP (mmol l^{-1})	0.561±0.041	5.16±0.12	2.80±0.33
I_{50} alanine (μmol l^{-1})	64±6	NE	NE
K_a F16P_2 (μmol l^{-1})	5.69±1.13	NE	NE

NE, no effect on pyruvate kinase (PK) activity at levels up to 10 mmol l^{-1} for L-alanine or 0.5 mmol l^{-1} for fructose-1,6-bisphosphate (F16P_2).

Effector values were determined at subsaturating phosphoenolpyruvate (PEP) levels. For K_a F16P_2, PEP was 0.05 mmol l^{-1} for liver and red cell PK and 0.03 mmol l^{-1} for white muscle PK; for I_{50} determination, PEP was 0.10 mmol l^{-1} for liver and red cell PK and 0.05 mmol l^{-1} for white muscle PK.

The optimal substrate conditions are described in Materials and methods.

See Table 2 for further details.

Table 4. *Maximal activities and some kinetic properties of lactate dehydrogenase prepared from rainbow trout tissues*

	Maximal activity (units g^{-1} wet mass)	K_m pyruvate (mmol l^{-1})	I_{50} pyruvate (mmol l^{-1})
Heart	527±15.5	0.056±0.013	7.14±0.38
Erythrocyte	8.64±0.58	0.143±0.013	8.51±1.30
White muscle	868±31.8	0.483±0.025	19.34±1.56

The assay conditions are described in Materials and methods with the exceptions that the optimal substrate (pyruvate) concentrations were 1.25 mmol l^{-1} for heart and 5 mmol l^{-1} for muscle.

See Table 2 for further details.

Ferguson *et al.* 1989), suggesting that the activity of this enzyme is tightly regulated by cellular ATP status in all these tissues.

LDH activity in trout erythrocytes was very low, only 1–1.6 % of that observed in white skeletal or cardiac muscle (Table 4). The erythrocyte isoenzyme had a relatively low K_m (approximately threefold less than that observed for the white skeletal muscle enzyme) and was strongly inhibited by pyruvate *in vitro* (inhibition becoming evident at pyruvate concentrations of 2.5 mmol l^{-1} and higher, data not shown). Overall, these properties resemble those of the H-type isoenzyme.

Effects of strenuous exercise on selected glycolytic enzyme binding in erythrocytes

Increased enzyme binding to cellular structural components and the formation of a 'glycolytic complex' in tissues relying predominantly or solely on glycolytic

Table 5. *Percentages of particulate-associated, 'bound' glycolytic enzymes in trout red cells before and 10 min after exhaustive exercise*

Enzyme	Percentage bound	
	Rest	Exercise
Hexokinase	ND	ND
Phosphofructokinase	4.60±1.69	5.36±1.28
Aldolase	1.06±0.46	1.59±0.98
Glyceraldehyde-3-phosphate dehydrogenase	2.78±0.81	4.22±1.12
Pyruvate kinase	ND	ND
Lactate dehydrogenase	ND	ND

Each value is the mean±1 S.E.M. of determinations on four fish.

Exercise stress promoted no significant differences in the percentage binding of any of the selected enzymes (paired *t*-test, $P>0.05$).

Enzymes for which no binding was detected are designated ND.

ATP production have been suggested to enhance carbon flow through consecutive glycolytic enzymes during periods of high energy demand (Tompa *et al.* 1986; Brooks and Storey, 1988). We tested the role of glycolytic enzyme binding in regulating the metabolic rate of trout red cells under conditions that stimulate adrenoceptor-mediated pHi regulation. Glycolytic enzyme binding to particulate material in the red cells prepared from resting animals (Table 5) is very much less than that observed for similarly prepared trout skeletal and cardiac muscles. In resting white muscle, the percentage of enzyme bound to the particulate fraction has been reported to range from approximately 25 (LDH) to 70 (PFK), whereas in cardiac muscle the range was somewhat lower (Brooks and Storey, 1988). In direct contrast to the large (approx. 50%) increases in the fractions of bound PFK, glyceraldehyde-3-phosphate dehydrogenase and aldolase in white muscle following burst exercise (Brooks and Storey, 1988), the low percentage binding of red cell glycolytic enzymes to the particulate fraction remained unchanged by exercise. The response of the red cells resembles that of trout cardiac muscle, which relies on aerobic ATP production following burst exercise; i.e. no increase in glycolytic enzyme binding is observed in the heart at this time (Brooks and Storey, 1988). Indeed, in trout red cells aerobic respiration predominates under these conditions (Boutilier and Ferguson, 1989). Therefore, it appears that glycolytic enzyme binding is not an important means of regulating metabolic rate during pHi regulation by these cells under the conditions of our study. Investigation of other means of enzyme modification, e.g. phospho-dephosphorylation, may shed more light on the molecular-level control of erythrocyte metabolism during stresses such as strenuous exercise.

Effects of strenuous exercise on red cell glucose and lactate levels

We examined intracellular levels of both glucose and lactate to determine whether the availability of these metabolic substrates is consistent with the known

Table 6. *Cell water content and transmembrane gradients of lactate and glucose for red cells of rainbow trout prior to (control) and following exhaustive exercise*

Treatment	Erythrocyte water content ($g H_2O\ 100\ g^{-1}$ red blood cells)	Lactate gradient	Glucose gradient
Control	63.98±0.69	1.47±0.08	9.01±1.56
After exercise			
5 min	70.77±2.08*	3.72±0.78*	11.29±0.50
90 min	72.09±3.36*	3.06±0.15*	11.56±1.85*

Each value is presented as the mean±1 S.E.M. of determinations on four fish.

Lactate and glucose gradient values represent the transmembrane gradients (extracellular/intracellular) calculated from the data presented in Figs 1 and 2.

An asterisk denotes a value significantly greater than control value (paired *t*-test, $P<0.05$).

metabolic response of the erythrocyte to organismic exercise stress and cellular catecholamine stimulation (Ferguson *et al.* 1989). Despite an exercise-induced increase in red cell water content (Table 6), which was reflected by a concomitant decrease in mean cellular haemoglobin content at this time (from 31.9±0.6 to 25.7±0.9 g Hb $100\ ml^{-1}$ packed red cells, $P<0.05$, paired *t*-test), red cell glucose concentration did not decrease significantly from the value observed for the control (pre-exercise) sample (Fig. 1).

Recent *in vitro* studies have shown that rainbow trout erythrocytes lack a cytochalasin-B-sensitive D-glucose transport system (Tse and Young, 1990). Indeed, the transmembrane glucose gradient is very high (Table 6) compared to the gradient across human red cells, for example, which has a value near unity (McDonald *et al.* 1964). Nevertheless, it would appear that the trout red cell membrane is not impervious to glucose. It is unlikely that red cell glucose (required to maintain intracellular concentration constant as the water content of the cell increases) arises intracellularly, given the absence of detectable glycogen phosphorylase and fructose-1,6-bisphosphate I activities (Table 1). A more reasonable explanation for our results is that glucose, or perhaps another blood sugar that can be rapidly converted to glucose, crosses the plasma membrane from the extracellular pool. The significance of this is that red cell metabolic pathways that utilize glucose are not limited by decreased substrate availability at this time.

We observed an increase in red cell lactate concentration following exhaustive burst exercise (Fig. 2). In addition, plasma lactate concentration increased, resulting in a higher transmembrane lactate gradient (Table 6). The lactate does not arise from red cell glycolysis, since arterial oxygenation remains high during this exercise protocol (Primmett *et al.* 1986) and erythrocytic lactate production remains low (Ferguson *et al.* 1989; Ferguson and Boutilier, 1989). Moon *et al.* (1987) have shown that lactate uptake by tuna *Katsuwonus pelamis* red cells consists of a significant diffusive component. In this study, lactate uptake by trout

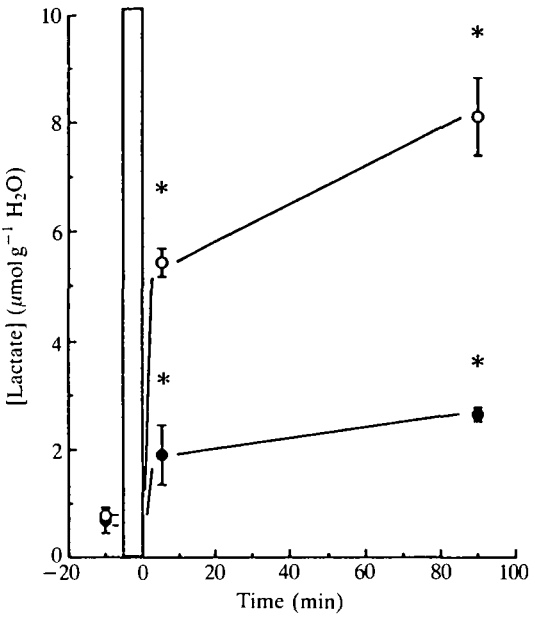

Fig. 2. Intra- (●) and extra- (○) erythrocytic lactate concentrations (expressed in terms of cell and plasma water) prior to and following 5 min of burst swimming activity by rainbow trout. Other details are given in Fig. 1.

red cells through diffusive pathways would most certainly be enhanced by the increased plasma lactate concentration and a decrease in the concentrations of intracellular impermeant anions (e.g. Hb and nucleotide triphosphates) which occurs as the red cells swell (Table 6). A role for stress hormones in modulating lactate uptake by the red cells is not known. Nevertheless, given the metabolic capabilities of trout erythrocytes, the abundance of intracellular lactate represents a substrate pool for aerobic ATP generation. Indeed, lactate oxidation by trout red cells is markedly accelerated following strenuous exercise (Wood *et al.* 1990). Thus, lactate is available as a fuel for metabolic ATP supply to, for instance, the plasma membrane sodium pump, which is accelerated during adrenoceptor-mediated pHi regulation (Bourne and Cossins, 1982; Ferguson and Boutilier, 1989). Evidently, the metabolic capabilities of trout red cells, as they have been demonstrated here, in addition to their 'pH regulatory' capabilities represent an exciting model for the study of the relationships between organismic oxygen transport and cellular (i.e. erythocyte) function at the molecular level.

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