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A futile cycle in erythrocyte glycolysis

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The storage lesion which limits the shelf life of human blood in blood banking is associated with a metabolic loss of 2,3-diphosphoglycerate and ATP. This metabolic loss is driven by intracellular ATPases which are usually considered to include the ion pumps and the reactions which maintain the discoid shape of the human erythrocyte. Under the acidic conditions of blood storage, the energy-yielding reactions of the glycolytic pathway are restricted at the hexokinase and phosphofructokinase steps. We show here that under such circumstances the enzyme of the diphosphoglycerate shunt, diphosphoglycerate mutase/phosphatase and the glycolytic enzyme phosphoglycerate kinase can form a futile cycle with ATPase activity. This ATPase activity responds to 2-phosphoglycolate which is known to activate both diphosphoglycerate mutase and diphosphoglycerate phosphatase reactions. When the enzymes of the futile cycle are combined with the enzymes of the lower glycolytic pathway in a reconstitution experiment designed to represent conditions within the stored erythrocyte, the futile cycle does provide an ATPase activity which results in the metabolic loss of 2,3-diphosphoglycerate. An isotope incorporation experiment demonstrates that the futile cycle is active in glucose-depleted erythrocytes.

Introduction

The Rapoport–Luebering shunt [1] is a unique feature of erythrocyte glycolysis. The glycolytic intermediate 1,3-diphosphoglycerate is converted to 2,3-diphosphoglycerate by diphosphoglycerate mutase. The shunt is completed by the conversion of 2,3-diphosphoglycerate to 3-phosphoglycerate by diphosphoglycerate phosphatases. Both reactions are catalyzed by a single enzyme molecule [2–4] and are considered to be irreversible or nonequilibrium. The shunt by-passes the near-equilibrium phosphoglycerate kinase reaction [5], one of the two ATP-producing reactions in glycolysis. Since 2,3-diphosphoglycerate is an important intracellular modulator of hemoglobin function [6,7] regulation of the Rapoport–Luebering shunt balances the energy needs and oxygen-transporting function of the red cell.

For technical reasons, it has been difficult to measure the rate of the Rapoport–Luebering shunt relative to the rate of glycolysis. Estimates vary from 15% to 50% [8–10]. The actual rate will influence the ATP yield from glycolysis. If the shunt is inactive, the total glycolytic flux passes through the phosphoglycerate kinase reaction in the forward direction and the ATP yield from glycolysis is at a maximum. As shunt activity increases, the overall yield of ATP decreases until it reaches zero when shunt activity is equal to the flux through the glycolytic pathway. At this point there is no metabolic flow through the phosphoglycerate kinase reaction. If shunt activity were to exceed the glycolytic flux, the phosphoglycerate kinase reaction could operate in the reverse direction resulting in a futile cycle [11–16] with ATPase activity. It is this futile cycle activity which we examine here.

Consideration of this potential futile cycle is particularly pertinent to the conditions which prevail within the red cell during blood storage. Under the acidic conditions of blood storage, glycolytic flux is limited by the activities of hexokinase and phosphofructokinase [17]. Initially, the stored red cell is able to maintain ATP levels by metabolism of the high concentration of 2,3-diphosphoglycerate through the reactions of the lower glycolytic pathway, where ATP is generated at the pyruvate kinase step. Ultimately, when 2,3-diphosphoglycerate is depleted, ATP levels also fall and the stored red cell is no longer viable [18]. The rate of loss of 2,3-diphosphoglycerate will depend on the ATPase activity within the red cell, since this is the source of ADP necessary for the pyruvate kinase reaction.

Since the empirical conditions used for blood storage restrict glycolytic flux at the hexokinase and phosphofructokinase reactions and inadvertently favor the futile cycle described above, the futile cycle could make a significant contribution to the loss of 2,3-diphosphoglycerate and ATP associated with the blood storage lesion.

Materials and Methods

The diphosphoglycerate mutase and phosphatase reactions in human erythrocytes are catalyzed by the same protein [2–4]. The enzyme activities were partially purified from outdated human red cells using the procedure described by Kappel and Hass [4] omitting the second DEAE-cellulose and hydroxy-apatite chromatography steps and the heat treatment. The enzyme gave more than one band on polyacrylamide gel electrophoresis, but no interfering enzymatic activities were detected in control experiments. In particular, the enzyme did not cause the formation of ADP when incubated with ATP under the conditions described below for measurement of futile cycle activity, but omitting the other enzymes. Specific activity of the enzyme preparation used was 4.07 units/mg. Yeast phosphoglycerate kinase as a crystalline suspension in ammonium sulfate was obtained from Sigma Chemical Co., St. Louis, MO. Other enzymes and substrates were obtained from Sigma Chemical Co., St. Louis, MO.

Diphosphoglycerate mutase activity was measured during the partial purification by the modified method of Rose [19] as described by Kappel and Hass [4].

For the measurement of futile cycle activity, a 0.25 ml reaction mixture containing 2 mM 2,3-diphosphoglycerate/1 mM ATP/0.2 mM 2-phosphoglycerate/0.25 units phosphoglycerate kinase/0.75 units diphosphoglycerate mutase in 0.1 M triethanolamine-HCl (pH 7.4)/0.1 M KCl/10 mM $MgCl_2$/10 mM KH_2PO_4/0.2 mM EDTA was incubated at 37°C. The ratio of enzyme activities used is such that the phosphoglycerate kinase activity vastly exceeds the diphosphoglycerate phosphatase activity. This is equivalent to the situation which pertains within the intact red cell [29]. The reaction was terminated by the addition of an equal volume of ice cold 1.2 M perchloric acid. The precipitate was removed by centrifugation at $8000 \times g$ for 4 min. The supernatant was neutralized by addition of 10 M KOH, recentrifuged and this supernatant filtered through a 0.22 μm nucleopore membrane.

The ATP and ADP contents of the filtered perchloric acid extract were determined by high-pressure liquid chromatography. The nucleotides were separated by reverse-phase chromatography on a Beckman Model 334 HPLC apparatus with isocratic elution by 0.1 M ammonium phosphate (pH 6.0) and 1% methanol at a flow rate of 1.5 ml/min. [20]. Quantitation was by peak height measurement and comparison to a standard curve.

To test the possible role of the futile cycle in stored red cells, the enzymes of the lower part of the glycolytic pathway were added to the futile cycle enzymes in a reconstitution experiment. One ml of the reaction mixture described above was supplemented with 0.2 mM NADH/4 units phosphoglycerate mutase/4 units enolase/3.6 units pyruvate kinase/8.8 units lactic dehydrogenase. Disappearance of NADH at room temperature was followed in a Gilford Model 2400 recording spectrophotometer at 340 nm.

In this assay conversion of NADH to NAD in the lactic dehydrogenase reaction requires two prior events to provide the substrates for the pyruvate kinase reaction, since these substrates are not present in the reaction mixture. A molecule of ATP must be converted to ADP in the futile cycle and a molecule of phosphoenolpyruvate must be

formed from 2,3-diphosphoglycerate in the diphosphoglycerate phosphatase, phosphoglycerate mutase, and enolase reactions. Only after both events have taken place, will the ADP be reconverted to ATP in the pyruvate kinase reaction and the other product of the reaction, pyruvate, become available as a substrate for the lactic dehydrogenase reaction.

To examine futile cycle activity in intact red cells an isotope incorporation experiment was carried out as follows. Stock ^{32}P-labelled inorganic phosphate was diluted to 2000 μCi/ml. 25 μl was added for each ml of red cells prepared a described below. 10 ml of venous blood was collected in heparin, washed three times in cold Krebs–Henseleit buffer [21] and preincubated for 30 min at 37°C in a shaking water bath under 95% air/5% CO_2. These conditions effectively remove glucose, and allow glycolysis to run down [22,23]. After the incubation the measured intracellular glucose concentration was 23 μM and the cells are effectively glucose-depleted compared to the normal intracellular concentrations. The inorganic phosphate labelled with ^{32}P was added and 1 ml aliquots taken at 5, 10, 15, 20, 25, 30 and 90 min. Reaction was stopped and protein precipitated by addition of 2 ml perchloric acid. Acid molybdate was added to the supernatant and inorganic phosphate extracted with iso-butanol [24]. The aqueous phase was neutralized, centrifuged and the supernatant separated by high-pressure liqiud chromatography. Fractions were collected and isotope incorporations measured by scintillation counting [20].

Results and Discussion

The phosphoglycerate kinase reaction was first studied in the reverse direction by incubating the enzyme with 2 mM 3-phosphoglycerate and 1 mM ATP. No detectable ADP was formed. From the equilibrium constant of $3.2 \cdot 10^3$ for the phosphoglycerate kinase reaction [25], the theoretical concentration of ADP formed at equilibrium would be 0.025 mM. This is at the limit of detection of the analytical system used. At the quantities of substrates used, phosphoglycerate kinase alone does not catalyse a significant hydrolysis of ATP to ADP.

When the potential futile cycle was completed by the addition of diphosphoglycerate mutase/phosphatase with 2 mM 2,3-diphosphoglycerate and 1 mM ATP as substrates, ATP was converted to ADP as shown in Fig. 1. From the previous experiment, even if all of the 2 mM 2,3-diphosphoglycerate has been converted to 3-phosphoglycerate by the phosphatase, no detectable ADP would have been formed.

We interpret the results as follows. The phosphatase converted 2,3-diphosphoglycerate into 3-phosphoglycerate which established the phosphoglycerate kinase equilibrium with ATP. Since the equilibrium constant is large, the quantities of ADP and 1,3-diphosphoglycerate formed were small. However, 1,3-diphosphoglycerate was converted to 2,3-diphosphoglycerate by the diphosphoglycerate mutase reaction and the ADP concentration was increased to maintain thermodynamic equilibrium at the phosphoglycerate kinase step. This cyclic process resulted in conversion of ATP and ADP.

We have demonstrated the catalytic role of 2,3-diphosphoglycerate in a separate experiment where the hydrolysis of 1 mM ATP was observed in the presence of only 0.2 mM 2,3-diphosphoglycerate. This demonstrates that the cyclic series of reactions functions as a futile cycle, where the overall result is the hydrolysis of ATP to ADP.

Rapoport–Luebering shunt enzymes and phos-

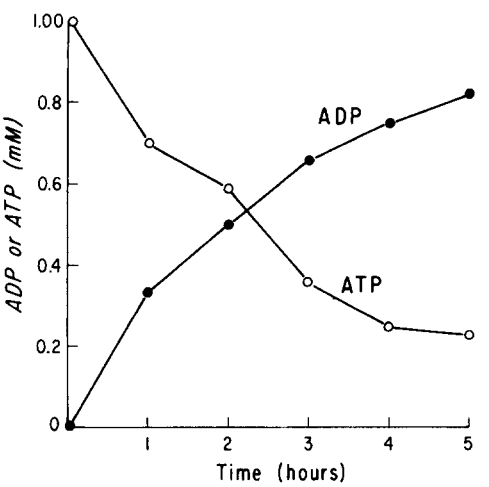

Fig. 1. Futile cycle activity measured by loss of ATP and production of ADP as a function of time. Each data point represents the average of three determinations.

phoglycerate kinase can function as a futile cycle. The futile cycle requires three enzyme activities, two of which, diphosphoglycerate mutase and phosphatase, are catalysed by a single protein. In separate studies of mtase [19] and phosphatase [26], Rose has shown that both activities are activated by anions, 2-phosphoglycolate being particularly effective. The influence of 2-phosphoglycolate on the ATPase activity of the futile cycle is shown in Fig. 2. ATPase activity increases up to 0.5 mM 2-phosphoglycolate which is consistent with the observed effect on the individual enzymes.

This experiment demonstrates that changes in the activities of diphosphoglycerate mutase or diphosphoglycerate phosphatase make the expected contribution to futile cycle activity. In previous studies the two activities have been measured separately, whereas in these experiments both activities must occur simultaneously. Since both activities are catalysed by the same enzyme molecule mutual interference is a possibility. Since the futile cycle ATPase activity increases with the concentration of a known activator of both mutase and phosphatase activity, mutual interference does not limit futile cycle activity.

Other proposed futile cycles consist entirely of irreversible or non-equilibrium reactions in which case flux through the metabolic pathway and cycling can occur simultaneously. The futile cycle

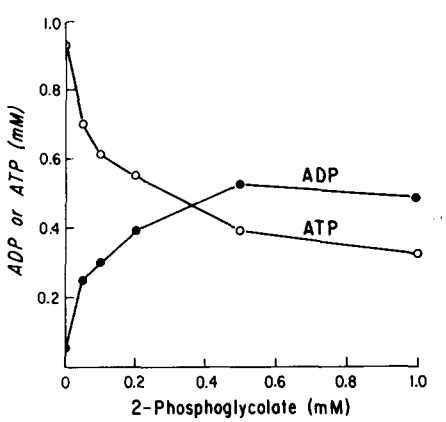

Fig. 2. The effect of 2-phosphoglycolate, an activator of diphosphoglycerate mutase and phosphatase, on futile cycle activity. The reaction mixture was incubated for 1.5 h with the 2-phosphoglycolate concentrations indicated. Each data point represents the average of three determinations.

examined here contains a reversible or near-equilibrium step in the phosphoglycerate kinase reaction. As indicated above, futile cycling will only occur if metabolism via the Rapoport–Luebering shunt exceeds the glycolytic flux. This conditions may exist if there are restrictions in metabolism through the upper part of the glycolytic pathway.

The following experiment suggests that futile cycling may be important in the storage lesion which limits the in vitro storage of human blood. Under the acidic conditions of storage, glycolysis is restricted by the activities of hexokinase and phosphofructokinase [17]. Initially, the cell is able to maintain ATP levels by metabolism of its high concentrations of 2,3-diphosphoglycerate through the lower glycolytic pathway, where ATP is generated at the pyruvate kinase step. Ultimately, when the 2,3-diphosphoglycerate reserves are depleted, ATP levels also fall and the stored red cell will not survive in the circulation following transfusion. The rate of loss of 2,3-diphosphoglycerate will depend on the ATPase activity within the cell, since this is the source of ADP necessary for the pyruvate kinase reaction.

We have reconstituted the enzyme reactions which are active during blood storage by adding the enzymes of the lower part of the glycolytic pathway to the futile cycle enzymes. This provides the pathway for maintaining ATP levels by metabolism of 2,3-diphosphoglycerate. The substrates added were ATP, 2,3-diphosphoglycerate and NADH. The reconstituted system does not contain any of the usually erythrocyte ATPases such as the ion pumps or 'shape' ATPases [27,28]. The ADP necessary for the pyruvate kinase reaction can only be formed from ATP by the futile cycling of a molecule of 2,3-diphosphoglycerate. Conversion of a second molecule of 2,3-diphosphoglycerate to phosphoenolpyruvate provides the second substrate for the pyruvate kinase reaction which results in the oxidation of a molecule of NADH at the subsequent lactic dehydrogenase reaction. The progressive loss of NADH and therefore 2,3-diphosphoglycerate in the reconstituted system is shown in Fig. 3. The reconstituted system mimics the metabolic changes observed during blood storage. The ATPase activity of the futile cycle can drain the metabolic reserves of 2,3-diphosphoglycerate and ultimately ATP. As

noted above, 2,3-diphosphoglycerate plays a catalytic role in the futile cycle, since with each turn of the cycle a molecule of 2,3-diphosphoglycerate is regenerated, and the only net change is loss of a molecule of ATP for each turn of the cycle. The loss of 2,3-diphosphoglycerate is a result of the simultaneous metabolism of 2,3-diphosphoglycerate to phosphoenolpyruvate, the substrate of the pyruvate kinase reaction where ADP is reconverted to ATP. The combined effect of both sets of reactions is to maintain the ATP concentration at the expense of the 2,3-diphosphoglycerate concentration. We conclude that the empirical conditions used for storing human blood in vitro inadvertantly favor futile cycle activity and that the futile cycle potentially contributes to the blood storage lesion.

The experiments described above demonstrate that diphosphoglycerate mutase, diphosphoglycerate phosphatase and phosphoglycerate kinase activities can combine to form a futile cycle where the only net effect is the hydrolysis of ATP. The futile cycle in combination with the reactions of the lower glycolytic pathway mimics the loss of 2,3-diphosphoglycerate observed in stored red cells.

We have carried out an isotope icorporation experiment to demonstrate that futile cycling can occur in intact red cells. The cells were first washed then preincubated at 37°C to remove glucose and allow glycolysis to run down. These procedures eliminate the metabolic flux through the upper reactions of the glycolytic pathway. In particular there will be no activity of the unidirectional phosphofructokinase reaction. 2,3-Diphosphoglycerate breakdown will contribute intermediates to the lower reactions of the glycolytic pathway and generate ATP at the unidirectional pyruvate kinase reaction. The glycolytic reactions between phosphofructokinase and pyruvate kinase can operate either in the forward or in the reverse directions. Under the conditions of this experiment, although there is no net flux through the glyceraldehyde dehydrogenase reaction, there will be some activity in both the forward and reverse directions. This allows the possibility of labelling 1,3-diphosphoglycerate by isotope exchange with ^{32}P-labelled inorganic phosphate. If the labelled 1,3-diphosphoglycerate participates in the phosphoglycerate kinase reaction, either by isotope exchange or because of a metabolic flux through the

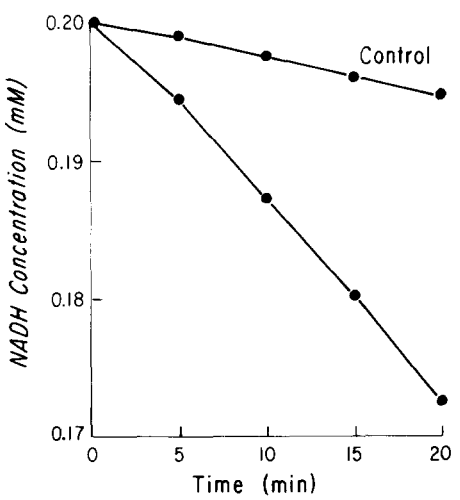

Fig. 3. Changes in NADH concentration in the reconstituted system. The control measured the spontaneous hydrolysis of ATP under the conditions of incubation by the coupled pyruvate kinase and lactic dehydrogenase reactions with phosphoenolpyruvate as a substrate.

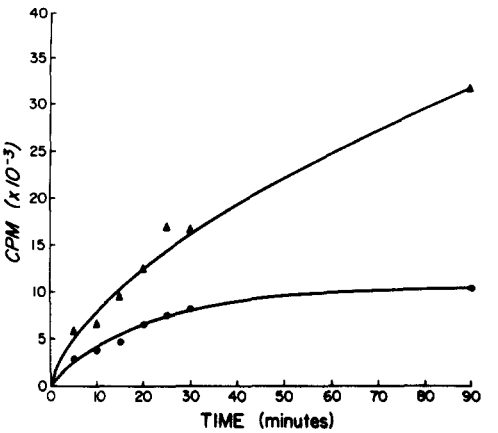

Fig. 4. A demonstration of futile cycle activity in intact glucose-depleted red cells. The incorporation of ^{32}P-labelled inorganic phosphate into 2,3-diphosphoglycerate is a measure of intracellular 2,3-diphosphoglycerate mutase activity and futile cycle activity under these experimental conditions. (▲——▲, counts incorporated into 2,3-diphosphoglycerate, ●——●; counts incorporated into ATP.) The results are expressed as counts per min, since this provides a relative measure of incorporation into 2,3-diphosphoglycerate and ATP. Since the intracellular concentration of 2,3-diphosphoglycerate is approx. 5 times that of ATP, specific activity calculations would tend to minimize the actual 2,3-diphosphoglycerate mutase activity.

reaction in the forward direction, the label will be transferred to ATP.

In these glucose-depleted cells, 2,3-diphosphoglycerate breakdown is taking place. Since the 2,3-diphosphoglycerate mutase reaction is unidirectional, transfer of label from 1,3-diphosphoglycerate to 2,3-diphosphoglycerate would demonstrate that the mutase reaction is taking place. The simultaneous activity of 2,3-diphosphoglycerate mutase and phosphatase under conditions of glucose depletion is only possible if futile cycling is occuring.

The results of the isotope incorporation experiments are shown in Fig. 4. The transfer of label from inorganic phosphate to 2,3-diphosphoglycerate in these glucose-depleted cells is evidence for futile cycle activity catalysed by the 2,3-diphosphoglycerate mutase, 2,3-diphosphoglycerate phosphatase and phosphoglycerate kinase reactions under these intracellular conditions. As noted above the labelling of ATP can occur by isotope exchange in the phosphoglycerate kinase reaction even though the net flux in the reaction is in the reverse direction. This does serve as an internal control, since continued glycolysis would result in preferential labelling of ATP. As shown in Fig. 4 the majority of the isotope is incorporated into 2,3-diphosphoglycerate.

We conclude that 2,3-diphosphoglycerate mutase, 2,3-diphosphoglycerate phosphatase and phosphoglycerate kinase can form a futile cycle with ATPase activity and that this futile cycle is active under the appropriate intracellular conditions.

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