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RED CELL PHOSPHOFRUCTOKINASE AND PYRUVATE KINASE ACTIVITIES CORRELATE WITH GENETIC VARIATION OF 2,3-BISPHOSPHOGLYCERATE IN RATS

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SUMMARY: Rat strains have been isolated which have low red cell 2,3-bisphosphoglycerate concentration and hemoglobin type *BB*, and high 2,3-bisphosphoglycerate concentration and hemoglobin types *BB* or *AA*. The genetic locus controlling red cell 2,3-bisphosphoglycerate concentration is closely-linked to the locus controlling hemoglobin type. Rats with low red cell 2,3-bisphosphoglycerate concentration also have relatively low red cell phosphofructokinase and pyruvate kinase activities. Kinetic studies suggest that the activity differences result from differences in *Vmax*. A proposed hypothesis suggests that an enzyme other than a glycolytic enzyme is coded by a gene closely-linked to the hemoglobin beta chain locus. Genetic variation in this enzyme then causes the activity differences in phosphofructokinase and pyruvate kinase between the low and high 2,3-bisphosphoglycerate red cells.

INTRODUCTION

Red cell oxygen affinity in rats can be influenced by genetic variation at a single locus: Rats with genotypes *DD* or *Dd* have red cells with higher 2,3-bisphosphoglycerate (2,3-P_2-glycerate) and ATP and lower oxygen affinity than rats with genotype *dd* (1,2). Rats also have a polymorphism for hemoglobin type, with two alleles (*A* and *B*) at a locus which probably codes for a minor beta chain (3,4). In Long Evans hooded rats, hemoglobin genotype *AA* was associated with the low 2,3-P_2-glycerate phenotype, while hemoglobin genotypes *BB* and *AB* were associated with the high 2,3-P_2-glycerate phenotype (4). This suggested the hypothesis that hemoglobin type directly influenced red cell 2,3-P_2-glycerate concentration in rats (4).

An alternate hypothesis proposed that a locus closely-linked to the hemoglobin beta chain locus codes for an enzyme which regulates red cell 2,3-P_2-glycerate con-

Abbreviations: 2,3-Bisphosphoglycerate, 2,3-P_2-glycerate; Hemoglobin, Hb; Phosphofructokinase, PFK; Pyruvate kinase, PK.

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centration (1,4). Data implicating phosphofructokinase (EC 2.7.1.11) came from studies of glycolytic intermediates (1). Glycolytic enzyme assays failed to show differences in maximal activity of phosphofructokinase (or any other enzyme) between hemolysates from high and low 2,3-P_2-glycerate rats (5,6). However, Noble and Tanaka (7) demonstrated greater ATP and 2,3-P_2-glycerate inhibition of phosphofructokinase from red cells with low 2,3-P_2-glycerate concentrations. This may account physiologically for the low 2,3-P_2-glycerate of those cells, but it does not prove that the cause is genetic variation at the phosphofructokinase structural locus (7).

I here report the isolation of a rat strain with hemoglobin type ßß and a low 2,3-P_2-glycerate phenotype. This disproves the hypothesis of one genetic locus causing both 2,3-P_2-glycerate and hemoglobin variation in rats. Enzyme assays show lower activities for both phosphofructokinase and pyruvate kinase (EC 2.7.1.40) in these low 2,3-P_2-glycerate rats. This has been interpreted to support the hypothesis that the locus responsible for the 2,3-P_2-glycerate variation in rats codes for an enzyme which regulates the activity of glycolytic enzymes.

METHODS

Rats were of the Long Evans strain (Charles River Breeding Laboratories, Wilmington, MA). Their ages ranged from three months to one year; for comparative purposes, pairs of rats with high and low red cell 2,3-P_2-glycerate were always matched for age and sex.

Rat hemoglobin type was determined as described previously (4), except that cellulose acetate electrophoresis with Titan III plates (Helena Laboratories, Beaumont, TX) was used instead of starch gel electrophoresis.

Red cell 2,3-P_2-glycerate was assayed at the screening stage of this study by a kinetic-rate enzymic assay using dilute hemolysates (8). Subsequently, the endpoint enzymic assay of Keitt (9) was used on neutralized perchloric acid extracts of blood. When 2,3-P_2-glycerate was the only red cell intermediate to be assayed, two parts of blood were added to one part of cold 20% perchloric acid. When other intermediates were also to be assayed, blood was precipitated within one and a half minutes of drawing, using two parts of cold 6% perchloric acid to one part blood. Samples remained on ice twenty minutes prior to centrifugation and neutralization. Glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, and dihydroxyacetone phosphate were assayed fluorometrically (10). ATP (11) and other intermediates (12) were assayed spectrophotometrically. For calculations of intermediate concentrations, account was taken of the water content of blood (10).

For enzyme assays, white cells were removed from one hematocrit tube (60 µl) of blood using oil of density 1.06 (13). Red cells were then washed three times in saline, and lysed with 1 ml of hemolysing solution (10). Hemolysates were kept at room temperature, and adult rat hemoglobins did not precipitate. The activities of most enzymes under study did not change over the course of a day. Phosphofructokinase activity declined relatively quickly; it was assayed two minutes after hemolysis. It was eventually found that 0.2 mg/ml of fructose-1,6-bisphosphate in the hemolysing solution stabilized phosphofructokinase. Data of the kinetics study were obtained using this modified hemolysing solution.

For maximal activity studies, enzymes were assayed at 37° C, pH 8.0, using conditions of Beutler (10). Reagents were from Sigma Chemical Co. (St. Louis, MO) and Boehringer Mannheim (Indianapolis, IN). Phosphoenolpyruvate was obtained as the tri(cyclohexylammonium) salt, while other reagents were obtained as sodium salts Assays were initiated by the addition of undialyzed hemolysate. For kinetic studies of pyruvate kinase and phosphofructokinase, hemolysates from high and low 2,3-P_2-glycerate rats were dialyzed together against hemolysing solution containing fructose-1,6-bisphosphate. Assay conditions were as for the maximal activity studies, with the following modifications: For the pyruvate kinase assay, 0.3 mM fructose-1,6-bisphosphate was present in the reaction mixture to give Michaelis-Menton kinetics, and phosphoenolpyruvate concentration was varied between 0.4 and 10 mM. For the phosphofructokinase assay, the presence of 2 mM ammonium sulfate ensured Michaelis-Menton kinetics, and fructose-6-phosphate concentration varied between 0.5 and 5 mM. ATP was added to initiate the reaction after the fructose-1,6-bisphosphate present in the hemolysate had reacted completely.

RESULTS AND DISCUSSION

Isolation of Rat Strains With Low and High Red Cell 2,3-P_2-Glycerate

After a screening of Long Evans female rats (14), the majority were found to have hemoglobin genotype *BB* and high red cell 2,3-P_2glycerate: For 42 rats seven months of age, 2,3-P_2-glycerate concentration was 1.31 $\pm$ 0.18 moles per mole of hemoglobin. One rat had hemoglobin type *BB* and low 2,3-P_2-glycerate (0.78 moles per mole of hemoglobin at seven months). This female was bred with a typical male, and further matings produced a low 2,3-P_2-glycerate strain.

The original screening also turned up several *AB* animals. Two matings of the type *AB* X *AB* yielded 6 *AA* and 7 *BB* animals. At 11 weeks of age, their red cells had 2,3-P_2-glycerate concentrations of 1.34 $\pm$ 0.08 and 1.42 $\pm$ 0.11 moles per mole of hemoglobin, respectively. The difference was not significant. From crosses of this type, a strain of *AA* rats with high 2,3-P_2-glycerate was developed.

Glycolytic Intermediate and Enzyme Differences Between Low and High 2,3-P_2-Glycerate Red Cells with Hemoglobin *BB*

Matched pairs of rats with low and high 2,3-P_2-glycerate were compared for concentrations of red cell glycolytic intermediate compounds. Data are presented

Table 1 Concentrations of Red Cell Glycolytic Intermediate Compounds for High and Low 2,3-P_2-Glycerate Rats

Glycolytic Intermediate	Concentrations for Low 2,3-P_2-Glycerate Red Cells	Concentrations for High 2,3-P_2Glycerate Red Cells
Glucose-6-phosphate	26 ± 8	25 ± 10
Fructose-6-phosphate*	7 ± 5	2 ± 2
Fructose-1,6-bisphosphate*	2 ± 1	4 ± 1
Dihydroxyacetone phosphate*	15 ± 3	27 ± 6
2,3-Bisphosphoglycerate*	4900 ± 300	7800 ± 310
3-Phosphoglycerate	130 ± 17	130 ± 27
2-Phosphoglycerate	13 ± 9	13 ± 5
Phosphoenolpyruvate	170 ± 44	160 ± 45
Lactate*	2600 ± 500	1900 ± 500
AMP	45 ± 21	37 ± 13
ADP	160 ± 19	160 ± 14
ATP*	740 ± 120	1200 ± 100

Concentration units are micromoles/(liter of blood) for pyruvate and lactate and micromoles/(liter of red cells) for all other intermediates. Mean ± S.D. are shown (data are from 8-10 pairs of animals for each intermediate). * indicates that the difference between high and low 2,3-P_2-glycerate red cells is significant ($p < .01$, except for fructose-1,6-bisphosphate, for which $p < .05$). Significance for data of this table, and in every other comparison reported in this paper, was calculated by the t-test (unpaired version).

in Table 1, which shows higher fructose-6-phosphate and lower fructose-1,6-bisphosphate and dihydroxyacetone phosphate for low 2,3-P_2-glycerate red cells. The data agree with results of Noble and Brewer (1), which suggested lower phosphofructokinase activity in low 2,3-P_2-glycerate red cells.

That hypothesis was tested for these rat strains by assays of red cell phosphofructokinase and other glycolytic enzymes. Significant differences in phosphofructokinase activities were found: Red cells with low 2,3-P_2-glycerate concentrations had activity of 36.5 ± 3.2 IU/gHb, compared to 49.6 ± 3.5 for high 2,3-P_2-glycerate red cells (eight pairs of rats).

Surprisingly, red cell pyruvate kinase activity was also significantly lower in low 2,3-P_2-glycerate red cells: 23.1 $\pm$ 1.6 IU/gHb, compared to 33.2 $\pm$ 4.3 IU/gHb for high 2,3-P_2-glycerate red cells (eight pairs). Two other kinases were examined and showed no significant differences between high and low 2,3-P_2-glycerate rats: Red cell hexokinase (EC 2.7.1.1) activities were 5.2 $\pm$ 0.7 IU/gHb and 5.0 $\pm$ 0.6 IU/gHb, respectively (5 pairs); red cell phosphoglycerate kinase (EC 2.7.2.3) activities were 350 $\pm$ 50 IU/gHb and 370 $\pm$ 40 IU/gHb (5 pairs).

These data differ from results of previous studies on *AAdd* compared to *BBDD* rats (5,6), in which no glycolytic enzyme differences were noted. Perhaps the phenotype of low red cell 2,3-P_2-glycerate reported here is due to different genetic factors than the phenotype reported earlier. Another theoretical possibility is that the strains developed for this study have differences in genetic factors which have nothing to do with the 2,3-P_2-glycerate genetic differences. This possibility is shown not to be correct by data of the next section.

Specific Crosses to Test Genetic Linkage of 2,3-P_2-Glycerate Variation, Glycolytic Enzyme Differences, and Hemoglobin Type

Female rats of hemoglobin genotype *AA* and high red cell 2,3-P_2-glycerate concentration were mated to males of hemoglobin genotype *BB* and low 2,3-P_2-glycerate. Backcrosses were then made to male parents. Since the gene for high 2,3-P_2-glycerate is dominant (Gilman, unpublished observations), one expects these backcrosses to yield 50% high and 50% low 2,3-P_2-glycerate rats, as well as 50% with *AB* hemoglobin and 50% with *BB* hemoglobin.

Results for 42 offspring are shown in Fig. 1. Twenty-one rats had hemoglobin *BB*, and low red cell 2,3-P_2-glycerate (1.01 $\pm$ 0.06 moles per mole of hemoglobin). Twenty had hemoglobin type *AB* and high red cell 2,3-P_2-glycerate (1.37 $\pm$ 0.11 moles per mole of hemoglobin). One *BB* rat had 2,3-P_2-glycerate bordering on the high range (1.21) and may therefore be a recombinant. On the whole, however, the data demonstrate close linkage of the 2,3-P_2-glycerate locus to the hemoglobin locus.

Data of Fig. 1 also show a close association of red cell 2,3-P_2-glycerate concentration with phosphofructokinase activity (correlation coefficient 0.85).

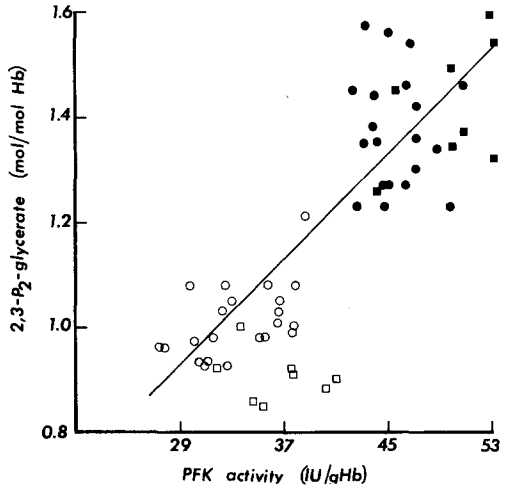
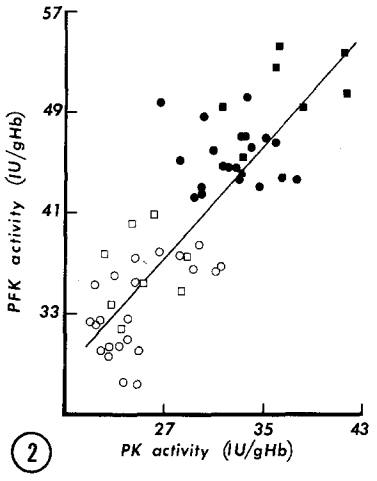


Fig. 1. Graph of red cell 2,3-P_2-glycerate concentration against red cell phosphofructokinase activity for 42 rats of a genetic backcross (details in text). Data on eight low and high 2,3-P_2-glycerate rats of hemoglobin type *BB* are also shown. The solid line represents the least squares fit to the backcross data. Animals from the backcross: ○, hemoglobin type *BB*; ●, hemoglobin type *AB*. Animals of the matched pairs: □, low 2,3-P_2-glycerate strain; ■, high 2,3-P_2-glycerate strain.

High 2,3-P_2-glycerate red cells had phosphofructokinase activity of 45.6 $\pm$ 2.3 IU/gHb, while low 2,3-P_2-glycerate red cells had activity of 32.5 $\pm$ 2.9 IU/gHb. Among rats of hemoglobin type *BB* there was a modest correlation of 2,3-P_2-glycerate concentration with phosphofructokinase activity (correlation coefficient 0.52). Among rats of hemoglobin type *AB* there was essentially no correlation.

The same sample used to assay red cell phosphofructokinase activity was used for the pyruvate kinase assay. Results of Fig. 2 show that activities of the two enzymes were highly correlated (correlation coefficient 0.80 for all rats). Among rats of hemoglobin genotype *BB* there was a correlation coefficient of 0.58, while there was no correlation among rats of hemoglobin *AB*. These data, and those of Fig. 1, suggest that the 2,3-P_2-glycerate locus (or one closely-linked to it) may cause the phosphofructokinase and pyruvate kinase activity differences. Variation in red cell 2,3-P_2-glycerate concentration among high 2,3-P_2-glycerate rats is unrelated to maximal activity differences in these enzymes. Since enzyme assays were conducted at non-physiological conditions designed to maximize activities,



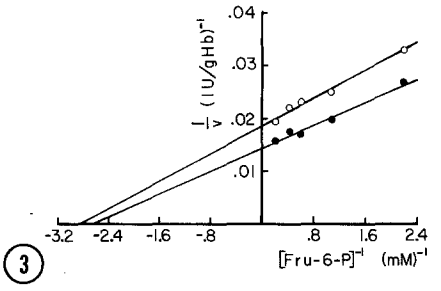


Fig. 2. Graph of red cell phosphofructokinase activity against red cell pyruvate kinase activity for 42 rats of the genetic backcross. Data on eight low and high 2,3-P_2-glycerate rats of hemoglobin type *BB* are also shown. The solid line represents the least squares fit to the backcross data. Animals from the backcross: ○, hemoglobin type *BB*; ●, hemoglobin type *AB*. Animals of the matched pairs: □, low 2,3-P_2-glycerate strain; ■, high 2,3-P_2-glycerate strain.

Fig. 3. Lineweaver-Burk plot of fructose-6-phosphate kinetics for phosphofructokinase of dialyzed hemolysates. ○, low 2,3-P_2-glycerate hemolysate; ●, high 2,3-P_2-glycerate hemolysate.

the data do not show the physiological mechanism by which the 2,3-P_2-glycerate locus controls red cell 2,3-P_2-glycerate concentration, but only suggest which enzymes may be involved.

Preliminary Kinetic Studies of Enzymes From Low and High 2,3-P_2-Glycerate Rats

Fig. 3 presents a Lineweaver-Burk plot of fructose-6-phosphate kinetics data for phosphofructokinase of low and high 2,3-P_2-glycerate hemolysates (dialyzed three hours). *Vmax* values are 54 IU/gHb and 70 IU/gHb, while K_M values are 0.35 and 0.39 mM, respectively. The correlation coefficients are 0.99 for each data set.

The same samples were used to obtain data on phosphoenolpyruvate kinetics of pyruvate kinase. *Vmax* was 34 IU/gHb and 55 IU/gHb for low and high 2,3-P_2-glycerate samples, while K_M values were 0.10 and 0.14 mM, respectively. In this experiment, *Vmax* values for both enzymes were 40% higher than activities of undialyzed hemolysates. The reason is unknown, but typical ratios of activities for low and high

2,3-P_2-glycerate hemolysates were maintained. Another experiment on hemolysates dialyzed 24 hours gave more typical values: For pyruvate kinase, *Vmax* was 27 IU/gHb and 40 IU/gHb, respectively, while K_M values were 0.15 and 0.13 mM, respectively.

These data suggest that differences in *Vmax* are the primary causes of activity differences for pyruvate kinase and phosphofructokinase between low and high 2,3-P_2-glycerate red cells, under the assay conditions of this study. K_M values are so low that they could not account for the demonstrated activity differences

CONCLUSION

Simultaneous changes in maximal activities of two glycolytic enzymes under genetic influence suggests that the 2,3-P_2-glycerate locus codes for an enzyme which regulates the activities of both enzymes. Phosphorylation appears to be a possible mechanism, because pyruvate kinase and phosphofructokinase can be phosphorylated in human red cells (15, 16). This leads to increased K_M for phosphoenolpyruvate in the case of pyruvate kinase (15), but no kinetic change for phosphofructokinase (16). Phosphorylated rat liver phosphofructokinase, however, shows increased ATP inhibition (17), which is an effect similar to that seen for rat red cell phosphofructokinase from low 2,3-P_2glycerate red cells (7). If phosphorylation is the mechanism which causes this, one must hypothesize different kinetic effects on rat red cell pyruvate kinase and phosphofructokinase than are known for human red cells.

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