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THERMAL INACTIVATION DIFFERENCES OF PHOSPHOFRUCTOKINASE IN ERYTHROCYTES FROM GENETICALLY SELECTED HIGH AND LOW DPG RAT STRAINS

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SUMMARY

Phosphofructokinase (PFK) has been previously suggested as being responsible for the differences in glycolytic 2,3-diphosphoglycerate (DPG) levels in two inbred populations of Long-Evans hooded rats. The enzyme's in vivo activity appears to be greater in animals with high levels of DPG and lower in those with low levels of this metabolite. In an initial attempt to characterize any structural differences in the red cell PFK enzyme of the two rat strains, we have performed a heat inactivation study. Our results show that the red cell PFK in animals with low levels of DPG is more heat labile relative to that in animals with high levels. This data provides further evidence that the genetic locus determining DPG levels may be the PFK structural gene.

INTRODUCTION

The oxygen affinity of hemoglobin (Hb) in the red blood cells of most mammals is modulated by two products of the cell's glycolytic metabolism, 2,3-diphosphoglycerate (DPG) and, to a lesser extent, adenosine triphosphate (ATP) (1,2,3,4). An increase in DPG shifts the hemoglobin-oxygen dissociation curve to the right and facilitates oxygen delivery to the body's tissues at any given partial pressure; a decrease in DPG results in a leftward shift of the curve and a decreased oxygen unloading at any given partial pressure. In addition to maintaining oxygenation homeostasis under normal conditions, DPG may also serve an adaptive capacity during situations of hypoxic stress (3). To study the genetic control of DPG-modulated oxygen transport by Hb, a population of Long-Evans hooded rats was selectively bred for high and low levels of red cell DPG (5). A single locus, two allele system was found to account for the major differences in DPG levels (6), and it was postulated that the locus involved the phosphofructokinase (PFK, EC.2.7.1.11) enzyme of red cell glycolysis (5). The PFK enzyme was implicated when all glycolytic intermediates were

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assayed. High DPG animals had low levels of PFK substrate, fructose 6-phosphate, and nearly a threefold greater concentration of product, fructose 1,6-diphosphate, than low DPG animals. The PFK in vivo activity in the high DPG rat line thus appears to be much greater than that of the low DPG line.

We provide further evidence here that the genetic locus determining DPG levels may be the PFK structural gene. In an initial search for evidence indicating possible structural differences in the PFK enzyme of the two types of animals, we have performed a heat inactivation study. Our results show that the PFK of erythrocytes of the low DPG line is more heat labile relative to that of the high DPG line. These data are supportive of the existence of structural differences in the red cell PFK of the two rat lines.

METHODS AND MATERIALS

Hooded Long-Evans rats (Simonsen Laboratories, Inc., Gilroy, California) of the previously developed high and low DPG lines (5,6) were used. All animals were male, 7-8 months of age.

Blood (3 ml) was drawn from each rat by cardiac puncture after anesthetizing with sodium pentobarbital. The blood cell preparation procedures were modifications of Wenzel et al (7). The plasma and buffy coat were removed by aspiration after centrifuging the blood at 1,000g. The packed red cells were subsequently resuspended and washed three times in 10 ml of cold isotonic saline and hemolyzed in 20 vols of a solution containing 10 mM Tris/HCl buffer (pH 8.0 at 37°C), 10 mM ammonium sulfate, 1 mM ethylenediamine tetraacetic acid, and 5 mM B-mercaptoethanol (added fresh). In an effort to maximize any heat stability differences, this hemolyzing solution was devoid of such stabilizing agents as fructose diphosphate and ATP. We found that leaving the stroma in the hemolysate gave more reproducible heat stability results, so the stroma were not removed. After stirring for 20 min at 4° C, 1 ml hemolysate aliquots were transferred to covered glass tubes and incubated in a 50 $\pm$ 1° C water bath. At suitable time intervals, the samples were immediately cooled on ice.

Determination of PFK activity was carried out according to the method of Beutler (8). The assay was initiated by the addition of ATP and monitored at 340 nm. Hemolysate Hb concentrations were determined by the cyanmethemoglobin technique. PFK activity is expressed as μmoles of fructose diphosphate converted per min per gram Hb at 37° C. Auxiliary enzymes necessary for the assay included α-glycerophosphate dehydrogenase (Sigma Chem. Co.) triosephosphate isomerase (Sigma Chem. Co.), and aldolase (Boehringer Mannheim Chem. Co.). The student t-test was used in all statistical analyses.

RESULTS

The results of PFK thermostability studies contrasting hemolysates from

high and low DPG animals are summarized in the Figure. The data are shown on both a logarithmic and arithmetic (inset) scale. The PFK from low DPG animals is significantly more heat labile relative to the high strain ($p < .01$) at all intervals after ten minutes.

There was no significant difference in Vmax of the PFK activities between the high and low DPG strains of rats ($p > .3$). The means and standard deviations of the Vmax of PFK activities of the high and low strains were 10.571 ± 3.053 and 11.640 ± 1.959, respectively.

DISCUSSION

The first suggestion that PFK might be the enzymatic step responsible for the differences in DPG in the high DPG and low DPG rats resulted from observations of the red cell glycolytic intermediate concentrations (5). These glycolytic intermediate patterns suggested a relatively greater *in vivo* activity in the PFK of the high strain relative to the low strain. To investigate this relationship, thermostability measurements have been carried out and are reported here. The thermostability difference between the lines affords further biochemical evidence of apparent structural differences in PFK.

A Hb polymorphism in these same Long-Evans hooded rats has also been identified, involving a beta globin locus, referred to as *Hbb*[1] (9,10). The investigators found a clear association of high DPG levels with the *Hbb b* allele and of low DPG levels with the *Hbb a* allele, even in the random bred base population. The genetic mechanism causing the association of the beta globin locus with the DPG determining locus, referred to as *Dpg*[2], in Long-Evans hooded rats includes two alternative hypotheses. One is two distinct

[1]The symbol *Hbb* is used to refer to the beta globin locus in rats and mice. This locus in rats is made up of two alleles, designated as *Hbb a* and *Hbb b*.

[2]The symbol *Dpg* is used to refer to the genetic locus determining DPG levels in Long-Evans hooded rats. This locus in rats is thought to be made up of two alleles, designated *Dpg D* to refer to that determining high DPG type, and *Dpg d* to refer to that determining low DPG type.

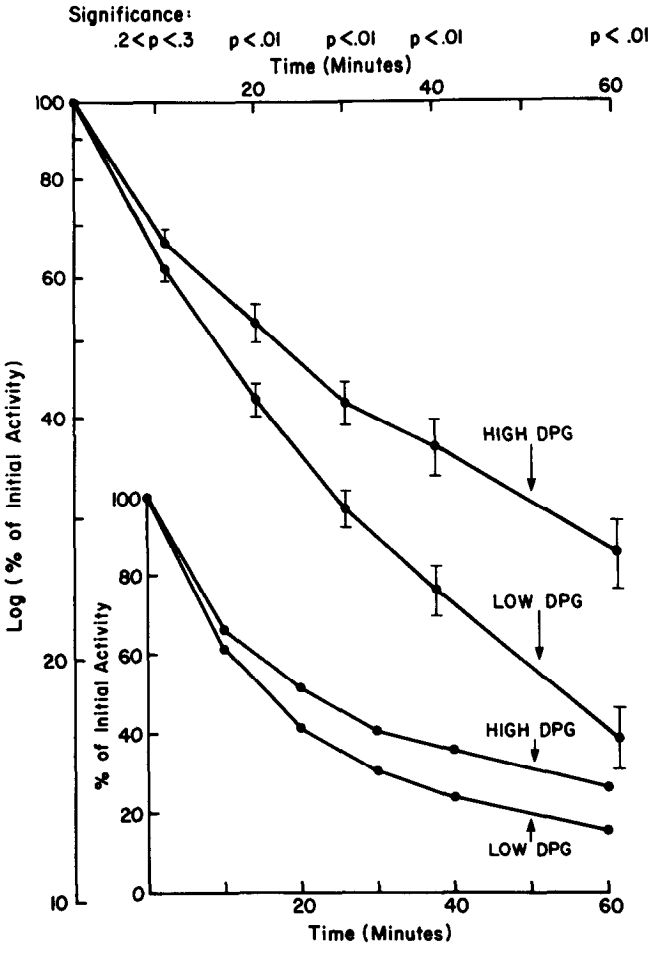

Figure 1. Contrast of heat stable PFK activity from red cell hemolysates of high and low DPG rats plotted as a logarithmic function (major figure) and as an arithmetic function (inset). The statistical significance of the differences in PFK heat stability at each time point is defined at the very top of the figure.

genetic loci, one for beta globin and one for DPG levels, which are closely linked and exhibit severe linkage disequilibrium such that Hbb b and Dpg D alleles are usually coupled, and Hbb a and Dpg d alleles are usually coupled. The second alternative is that only the Hbb polymorphism exists, and it also determines DPG phenotypes.

The present study provides evidence for the existence of a locus determining DPG type, distinct from the Hbb locus. We have shown that the PFK of

the two rat lines is different with respect to its resistance to heat stress. Such a difference is characteristic of a structural difference which is compatible with a polymorphism at the PFK structural genetic locus.

The results of the thermostability experiments are consistent with the existence of two isozymes comprising rat erythrocyte PFK (11,12). The biphasic nature of the curves for both enzyme types (see Figure, inset) is suggestive of differential heat inactivation rates of two isozymes. The rate of inactivation appears to be much more rapid for the low DPG enzyme during the first 20-30 minutes, after which time the slopes appear to be approximately equivalent. We speculate that only the more labile of the two PFK isozymes differ in the two types of rats. After this isozyme is inactivated, the remaining isozyme is equally labile in the two types of rats. Such an interpretation is consistent with the findings in human erythrocytes. In humans, a relatively unstable component of erythrocyte PFK has been characterized, and it is that isozyme which, if deficient, causes hemolytic anemia and a disease resembling MacArdle syndrome (13,14,15,16). If rat erythrocyte PFK is, indeed, analogous to human erythrocyte PFK, perhaps it is this labile PFK component which is different in the two DPG rat strains.

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