G6PD San Francisco: A New Variant of Glucose-6-Phosphate Dehydrogenase Associated With Congenital Nonspherocytic Hemolytic Anemia

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Congenital nonspherocytic hemolytic anemia in an adult male of Scandinavian ancestry was associated with virtual absence of G6PD activity in red cells. Characterization of G6PD purified from leukocytes using standard WHO techniques revealed diminished electrophoretic mobility, marked liability on heating at 46°C, normal pH optimum and utilization of alternate substrates (2-deoxy G6P, D-amino NADP), elevated K_m, absence of G6PD activity in vitro, while in leukocytes, where residual G6PD activity was approximately 60% of normal, hexose monophosphate shunt activity, oxygen consumption during phagocytosis, and bacterial killing were unimpaired. Thus, instability of the variant enzyme rather than its unfavorable kinetics appeared to be an important determinant of abnormal cell function.

OVER 180 variants of human G6PD have been distinguished on the basis of their physicochemical properties. Such biochemical diversity finds clinical expression in a wide spectrum of disorders, which includes episodic hemolysis, favism, neonatal hyperbilirubinemia, and congenital nonspherocytic hemolytic anemia. This report describes a new variant, G6PD San Francisco, discovered in a Scandinavian sailor with severe congenital nonspherocytic hemolytic anemia. An unusual feature of the variant enzyme was its complete inactivation by a brief period of dialysis during the purification procedure. Such instability has often prevented or limited efforts to characterize the properties of G6PD variants. Substitution of gel filtration chromatography for dialysis or addition of glyc erol to the partially purified enzyme stabilized G6PD Hamburg, G6PD Kessel Lo, G6PD Johannesburg, and G6PD Hillebrew sufficiently to allow their characterization. The purification and analysis of G6PD Torrance required very large amounts of NADP to stabilize the enzyme in its active form. After several unsuccessful attempts at purification, it was found that the mutant enzyme we studied resembled G6PD Torrance in its unusual requirement for NADP. By suitable modification of the purification procedure, we were then able to stabilize and characterize the variant.

MATERIALS AND METHODS

All studies to be described were approved by the Committee on Human Research at the University of California, San Francisco. The tetrazolium-linked cytochemical method was utilized to demonstrate G6PD activity in individual red cells from suspected female heterozygotes for G6PD deficiency. G6PD was purified from normal or mutant leukocytes and characterized as recommended by the World Health Organization. Just prior to characterization, the ammonium-sulfate-precipitated enzyme was dialyzed for 3 hr against 50 mM Tris glycine phosphate buffer, pH 6.5, plus 10 mM magnesium chloride, 1 mM mercaptoethanol, 200 μM NADP, and 1 mM EDTA. The K_m NADPH was determined in 50 mM Tris buffer, pH 7.3, with added 4 mM MgCl_2, 100 mM KCl, 60 μM glucose-6-phosphate, and various concentrations of NADP and NADPH. The stability of heated enzyme and the pH optimum were measured as previously described. To minimize enzyme instability during electrophoresis, the concentration of NADPH in the cathodic chamber and in the gel was increased from 10 to 50 μM. The voltage was increased to 8 V/cm, and the duration of the run was shortened to 5 hr. The relative mobilities of the normal B enzyme and the A variant under these electrophoretic conditions was identical to those obtained using lower voltage (4 V/cm) for a longer interval (12-16 hr).

The regeneration of reduced glutathione was studied in normal and mutant red cells using tertiary butyl hydroperoxide, as described by Srivastava, Awasthi, and Beutler. Red cells were washed and suspended in phosphate-buffered saline, pH 7.4, to which was added 0.75–1 mM tertiary butyl hydroperoxide. The complete oxidation of glutathione by tertiary butyl hydroperoxide was verified by assay of reduced glutathione concentration. Regeneration of reduced glutathione was then initiated by the addition of 5 mM glucose to the system and samples were obtained at frequent intervals for determination of red-cell-reduced glutathione, assayed according to the method described by Beutler.

For studies of granulocyte function, granulocytes were isolated from fresh heparinized whole blood by centrifugation on a Hypaque-Ficoll cushion and sedimentation with 5% dextran, using a modification of the technique of Steigbigel. Oxygen consumption of resting and stimulated (Latex beads) granulocytes was measured with a Clark membrane electrode by a modification of the method of Bachner. In vitro bactericidal activity of the patient's granulocytes against Staphylococcus aureus ATCC 25923 was measured...
by the technique of Michenberg. Additional granulocyte function studies included the quantitative reduction of nitroblue tetrazolium dye and the production of ¹⁴CO₂ from ¹⁴C-glucose in resting and stimulated cells to measure hexose monophosphate (HMP) shunt activity, as described by Root.

**CASE REPORT**

G.R. is a 27-yr-old male of Norwegian-Danish ancestry with a lifelong history of hemolytic anemia. Jaundice was initially noted on the first day of life and pallor at age 5 wk. Subsequently, 4-5-day episodes of scleral icterus, accompanied by low-grade fever, chills, dark urine, and sometimes vague bilateral lower abdominal pain, occurred throughout childhood. The hemolytic anemia was not favorably influenced by splenectomy, performed at 5 yr of age. A cholecystectomy was done at age 17, following several episodes of biliary colic and the demonstration of gallstones by oral cholecystogram. As an adult, G.R. fatigues easily but has been able to work full time, first as a merchant seaman and more recently as an electrician.

Serum total bilirubin has ranged from 6 to 8 mg/dl for many years, is predominately indirect reacting, and is unaccompanied by other biochemical evidence suggestive of liver disease. Jaundice is said to be exacerbated by lack of sleep and aspirin. A liver biopsy in 1965 was normal, and a bone marrow aspirate revealed profound erythroid hyperplasia.

Representative blood counts at age 27 were: hemoglobin 12.9 g/dl, PCV 39.9%, RBC 3.67 x 10¹²/liter, WBC 12.4 x 10⁹/liter, nucleated red cells 0.5 x 10⁷/liter, reticulocytes 840 x 10⁶/liter. Red cell morphology was normal except for changes characteristic of postsplenectomy reticulocyte-rich blood. Red cell G6PD activity was 0 and the activity of 12 other glycolytic enzymes was either normal or increased consistent with reticulocytosis. Hemoglobin electrophoresis, Coombs test, and the acid hemolysis test were normal. The unincubated osmotic fragility test was normal, but following incubation, approximately 15% of erythrocytes exhibited increased fragility. No other family member exhibited either anemia or reticulocytosis. Aside from the sister, who had received a blood transfusion at 8 mo of age following an infection, and a maternal aunt who had developed gallstones in childhood, there was no family history suggestive of hemolytic anemia. Red cell G6PD activity was borderline in the mother (1.69 μM/min/10⁸ RBC, normal range 1.71-3.11) and low in the sister (1.52 μM/min/10⁸ RBC). Cytochemical testing revealed a small population of G6PD-deficient cells in both affected females.

**RESULTS**

**Enzyme Purification**

Following partial purification of G6PD San Francisco from leukocytes, 48% of the enzyme activity initially present was recovered in the ammonium sulfate fraction, which compared favorably with the recovery of G6PD activity from normal leukocytes (44%). However, subsequent dialysis of the mutant enzyme in Tris buffer at pH 8 for 3 hr to remove ammonium sulfate led to complete loss of activity. Normal leukocyte enzyme, similarly dialyzed, lost little or no activity. A briefer period of dialysis at lower pH (7) and in the presence of small amounts of NADP (10 μM) preserved only a small amount of mutant enzyme activity, insufficient for further analysis. Lowering the pH to 6.5 and adding 10 mM MgCl₂, as well as large concentrations of NADP (200 μM), stabilized the enzyme sufficiently against inactivation by dialysis to allow its subsequent characterization. It was found that enzyme activity was best preserved by storage in ammonium sulfate plus Tris glycine phosphate buffer (pH 6.5) and 200 μM NADP. Aliquots of this ammonium sulfate enzyme preparation were dialyzed just prior to use. All studies were completed within 12 hr of dialysis, and the enzyme was completely characterized within 48 hr of obtaining blood.

**Properties of the Mutant Enzyme**

The properties of the mutant enzyme were compared to those of normal leukocyte G6PD purified and dialyzed in exactly the same way. G6PD San Francisco was extremely labile on heating at 46°C, while the normal B enzyme was stable. Heated mixtures of the normal and mutant enzymes yielded...
the expected algebraic sum of activities of each enzyme heated separately. Both normal and mutant enzymes exhibited optimal activity at pH 8 (Fig. 1). Near maximal mutant enzyme activity was found across a broad pH range from 6.5 to 9.0, while the pH range for the normal enzyme was narrower. Other physicochemical properties of G6PD San Francisco are shown in Table 1.

**Red Cell and Leukocyte Metabolism and Function**

The ability of normal and G6PD-deficient erythrocytes to generate reduced glutathione was measured following treatment of erythrocytes with sufficient tertiary butyl hydroperoxide to oxidize all intracellular glutathione. In normal erythrocytes, there is a rapid rise in reduced glutathione concentration in the first 5 min following addition of glucose in the system and baseline levels are achieved within approximately 15–30 min. The baseline level of reduced glutathione in fresh G6PD-deficient erythrocytes was only half normal, and these cells were totally incapable of regenerating reduced glutathione in vitro following treatment with tertiary butyl hydroperoxide.

Leukocyte G6PD activity in the propositus was decreased to approximately 60% of that in a normal control. Resting hexose monophosphate shunt activity, measured with glucose 1-14C, was slightly less in the propositus than in the control (propositus, 10 nM glucose oxidized/5 × 106 WBC/hr; control, 13.5), but increased 20-fold following stimulation with methylene blue. Leukocytes from the propositus demonstrated a normal increase in oxygen consumption and NBT dye reduction when stimulated with polystyrene beads. The bacteriocidal capabilities of these leukocytes, as measured in vitro with *Staphylococcus aureus*, were also unimpaired.

**DISCUSSION**

The properties of G6PD San Francisco are summarized in Table 1. Other reported G6PD mutants with severe enzyme deficiency, chronic nonspherocytic hemolytic anemia, and reduced electrophoretic mobility can be distinguished from G6PD San Francisco on the basis of their kinetic properties. In addition, G6PD Hong Kong, G6PD Rotterdam, and G6PD Ashdod are more stable on heating than is G6PD San Francisco. G6PD Long Prairie, G6PD Yamaguchi, and G6PD Manchester have different electrophoretic mobilities. The activity of G6PD Alhambra, G6PD Freiburg, G6PD Johannesburg, G6PD Frankfurt, G6PD Atlanta, G6PD Manchester, and G6PD Tripler is significantly higher than that of G6PD San Francisco. G6PD Kurume is not inactivated by dialysis. It is important to note that the reported properties of G6PD San Francisco (Table 1) are those of the leukocyte enzyme and comparison is made to other mutants in which the erythrocyte enzyme was characterized. Leukocyte and erythrocyte G6PD are thought to have a common genetic origin and the physicochemical properties of both normal and mutant enzyme obtained from the two tissues have usually been identical. However, posttranslational modification of either or both enzymes may lead to subtle differences appreciated by isoelectric focusing, raising the possibility that other properties of mutant enzymes might be different in leukocytes and erythrocytes. Nevertheless, although no specific structural alteration has yet been defined in G6PD San Francisco, its kinetic, electrophoretic, and stability properties appear to be sufficiently novel for it to be classed as a new variant form of human G6PD.

G6PD San Francisco shares with G6PD Torrance the property of rapid inactivation by dialysis. The two mutants are clearly distinguishable, however, on the basis of their different electrophoretic mobility in phosphate buffer at pH 7. Under these conditions, the mobility of G6PD Torrance is slightly greater than that of the normal B enzyme, while G6PD San Francisco is considerably slower. Inactivation of G6PD San Francisco or of G6PD Torrance can be prevented or minimized by adding MgCl2 or NADP to the dialyzing buffer or by lowering the buffer pH to 7. NADP, magnesium chloride, and protons all favor the association of G6PD monomers in the active dimeric or
tetrameric configuration. A single tightly bound molecule of NADP, usually referred to as structural NADP, is required for stabilization of each G6PD dimer. In the absence of NADP, enzyme dissociates into inactive monomers. Decreased binding of NADP by mutant enzyme subunits is a logical, although as yet unverified, explanation for the instability of G6PDd San Francisco.

Little or no G6PD activity was found in the erythrocytes of the propositus. The amount of enzyme protein was not measured, so that the relative contributions of diminished enzyme synthesis, increased loss of enzyme, or enzyme inactivation cannot be assessed. However, the profound instability of G6PDd San Francisco, which made purification difficult and was so readily demonstrated by heating at 46°C, is likely to be a major cause of the virtual absence of G6PD activity in red cells. The Kₐ of NADPH of 2.7 μM is well below reported values for the intraerythrocytic concentration of NADPH, so that any residual functional enzyme would be significantly inhibited by NADPH under in vivo conditions. The lack of any significant G6PD activity in intact erythrocytes is demonstrated by their complete inability to reduce oxidized glutathione following the conversion of all glutathione to the oxidized form by tertiary butyl hydroperoxide. On the other hand, in leukocytes from the patient, where considerably more G6PD activity is present, the enzyme appears to function adequately despite its unfavorable kinetic properties. There was no increased susceptibility to infection, and in vitro tests of white cell function were all normal.

REFERENCES

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