DETECTION OF A NEW ABNORMAL VARIANT OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN HUMAN ERYTHROCYTES

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The kinetic and electrophoretic properties of preparations of glucose-6-phosphate dehydrogenase (G6PD), obtained from erythrocytes from healthy blood donors and patients with acute drug-induced hemolytic anemia caused by G6PD deficiency, purified 230-300 times, were investigated. A new abnormal variant of G6PD, not previously described in the literature, was isolated from the erythrocytes of a patient with acute drug-induced hemolytic anemia. The abnormal enzyme differs from the normal in having a lower Michaelis constant for glucose-6-phosphate and NADP, in its increased utilization of substrate analogs (2-deoxyglucose-6-phosphate and, in particular, diamino-NADP), low thermostability, the character of its pH dependence, and in the appearance of only one band of G6PD activity during electrophoresis in polyacrylamide gel.

KEY WORDS: glucose-6-phosphate dehydrogenase; abnormal variant of glucose-6-phosphate dehydrogenase in human erythrocytes

The investigation of the properties of glucose-6-phosphate dehydrogenase (G6PD) is interesting in connection with the discovery of abnormal enzymes in hemolytic anemias due to reduced G6PD activity [16-18]. The deficiency of enzyme activity is inherited as a trait linked with the X chromosome, and the appearance of anomalies is connected with a mutation of the locus of the X chromosome coding G6PD synthesis. As a result of mutations an enzyme differing from normal in its activity and its kinetic and electrophoretic characteristics is formed.

The object of this investigation was to obtain a G6PD preparation from a small number of human erythrocytes in order to investigate the kinetic and electrophoretic properties of the enzyme in healthy subjects and in patients with hemolytic anemia due to reduced G6PD activity.

EXPERIMENTAL METHOD

Experiments were carried out on partially (230-300 times) purified preparations of G6PD not containing hemoglobin or 6-phosphogluconate dehydrogenase (6PGD), the presence of which distorts the calculations of the kinetic characteristics. The work was done by the method suggested by the scientific group of the World Health Organization on standardization of methods of investigation of G6PD for the purpose of identifying abnormal variants of the enzyme [2].

Isolation and purification of G6PD from human erythrocytes were carried out by Kirkman's method [8] in the writers' modification [1]. Activity of G6PD and 6PGD [6, 10] and the protein concentration [9] were determined in the resulting preparation. When determining the Michaelis constants (K_m) for glucose-6-phosphate (G6P) and NADP the initial reaction velocities were measured for eight different concentrations of G6P (from 20 to 250 μM) and NADP (from 1.5 to 20 μM). The true concentrations of G6P and NADP were determined by an enzymic method.

The utilization of substrate analogs (2-deoxyglucose-6-phosphate, diamino-NADP, and NAD) was determined by the standard method of determination of G6PD activity using the above-mentioned analogs, in the same concentrations, instead of G6P or NADP. G6PD activity was expressed as a percentage of the level of activity obtained when G6P or NADP was used [5].

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TABLE 1. Comparative Kinetic and Electrophoretic Properties of G6PD of Erythrocytes from Healthy Donors and Patients with Hemolytic Anemia

G6PD	G6PD activity in hemolysate, % of normal	REM in TEB, pH 8.6, % of normal	REM in PAG (fractionation pH 8.9)		Ratio of G6PD activity, % (n = 6)		K_m for G6P, μM	K_m for NADP, μM	Utilization of 2-deoxy-G6P, % of G6P level	Utilization of diamino-NADP, % of NADP level	pH optimum	Critical temperature during incubation for 10 min in presence of 10^{-5} M NADP	Thermostability
			fraction 1	fraction 2	fraction 1	fraction 2							
Normal enzyme (control, n = 16)	100	100	0,29	0,36	36,68 ± 3,74	63,32 ± 3,74	35,0 ± 3	4,27 ± 0,3	< 4	55—60	9,0	52°	Normal
Isolated by present writers from erythrocytes of:													
S-r (son)	3	100	0,29	—	—	—	16,44	2,39	70	350	8,0	42°	Very low
S-r (mother)	59,2	100	0,29	0,36	31,5	68,5	22,64	2,16	5	63,3	9,5	> 50°	Slightly reduced
described in literature:													
G6PD B^+ [16]	100	100	—	—	—	—	50—70	2,9—4,4	< 4	55—60	9,0	—	Normal
Mediterranean [16]	0—7	99—100	—	—	—	—	19—26	1,2—1,6	23—27	350	Two optima	—	Low
Corinth [16]	0—7	100	—	—	—	—	19—26	1,2—1,6	23—27	55—60	" "	—	"
Orchomenos [16]	0—7	100	—	—	—	—	11	2,1	105	350	" "	—	"
Boston [11]	0—5	100	—	—	—	—	18—21	1,7—2,2	12		8,5	—	Slightly reduced
Toulouse [15]	3	97	—	—	—	—	55	1,1—3	13	220	Two optima	—	Very low

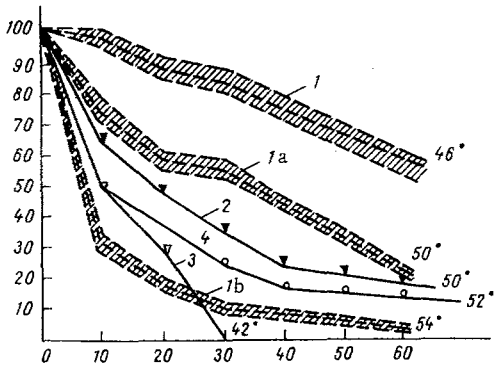

Fig. 1. Thermostability of G6PD isolated from erythrocytes of donors, of patient S-r, and of his mother, during incubation for 10 min in presence of 10^{-5} M NADP at different temperatures. 1, 1a, 1b) changes in activity of enzyme from donor's erythorcytes at different temperatures; 2) changes in activity of enzyme from erythrocytes of patient S-r (mother) at critical temperature; 3) changes in activity of enzyme from erythrocytes of patient S-r (son) at critical temperature; 4) changes in activity of enzyme from donor's erythrocytes at critical temperature. Abscissa, incubation time (in min); ordinate, activity (in %).

Thermostability was determined by measuring G6PD activity after incubation of the enzyme for 10 min at temperatures of 46, 50, 52, 54, and 58°C in the course of 60 min, and also for 5 min at temperatures of 45, 48, 51, 54, 57, and 60°C. G6PD activity was expressed as a percentage of its initial activity.

The pH optimum for G6PD was determined in 0.1 M Tris–0.1 M glycine–0.1 M $NaH_2PO_4 \cdot 2H_2O$ at pH 5.5-11.0 at intervals of 0.5 pH unit [5]. Activity of the enzyme at different pH values was calculated by taking the activity at pH 8.0 as a rule as 100%.

Electrophoretic analysis of the G6PD preparation was carried out in finely porous 7.5% polyacrylamide gel (PAG) without the use of gel of large pore size [7, 13].

Samples of the G6PD preparation with activity of 0.015-0.020 i.u. were mixed with 40% sucrose in the ratio of 1 : 1 and layered above electrode buffer on the gel with a micropipet in a volume of 0.1-0.2 ml. Electrophoresis was carried out with a current of 3 mA to the column for 2-2.5 h at 0°C, using 5 mM Tris-glycine buffer, pH 8.3, as the electrode buffer. To detect G6PD activity the gels were placed after electrophoresis in a reaction mixture containing 0.1 M Tris-HCl, pH 8.0, 3 mM G6P, 0.37 mM NADP, 0.98 mM phenazine metasulfate, and 0.12 mM nitro-BT for staining in darkness at 37°C. The relative electrophoretic mobility (REM) of zones of G6PD activity was calculated by the usual method [4]. Activity was estimated quantitatively by means of the DMU-2 densitometer (Toyo, Japan) at 620 nm [12].

The electrophoretic mobility of G6PD, which is essential when studying variants of G6PD, was determined by horizontal electrophoresis in starch gel at 0°C [14] in Tris–EDTA–boric acid (TEB) at pH 8.6. The current to the chamber was 30 mA, its voltage 150 V, and the duration of electrophoresis 16 h. The mobility of the mutant enzymes was expressed as percentages, taking as 100% the distance (to the center of the spot) moved by the control (normal) enzyme during parallel electrophoresis.

EXPERIMENTAL RESULTS

The results of investigation of the properties of G6PD from erythrocytes of 16 donors and patients with hemolytic anemia are given in Table 1.

The study of G6PD from erythrocytes of patients with hemolytic anemia caused by a deficiency of this enzyme revealed abnormalities. The physicochemical indices of G6PD from erythrocytes of a son and mother of Jewish race were studied. The investigation of the properties of G6PD from the erythrocytes of the mothers with hemolytic anemia is particularly interesting, for the patient could obtain the X chromosome with mutation in the G6PD locus only from his mother.

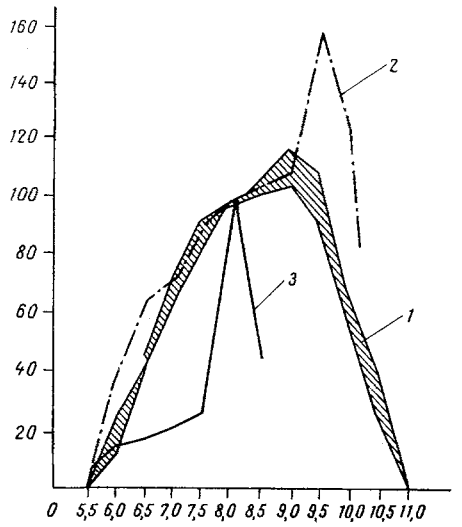

Fig. 2

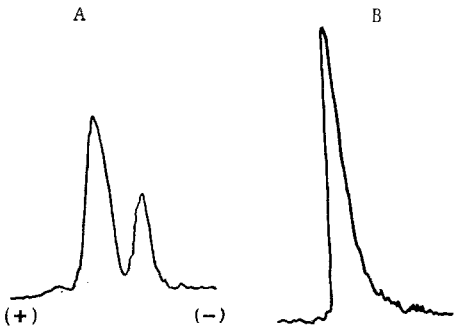

Fig. 3

Fig. 2. Effect of pH of medium on activity of G6PD isolated from erythrocytes of donors, patient S-r, and his mother. 1) Shaded region corresponds to observed deviation from mean level for 16 experiments (normal); 2) pH-dependence of G6PD from erythrocytes of patient S-r (mother); 3) pH-dependence of G6PD from erythrocytes of patient S-r (son). Abscissa, pH values; ordinate, activity (in %).

Fig. 3. Densitogram of G6PD from erythrocytes of donor and patient S-r (son). A) Densitogram of G6PD from donor's erythrocytes; B) densitogram of G6PD from erythrocytes of patient S-r.

During investigation of the enzyme from erythrocytes of patient S-r and his mother S-r who, like the son, also suffered from acute drug-induced hemolytic anemia, provoked by a variety of drugs, features of similarity and difference were found. In patient S-r (son) G6PD activity was not found in the hemolysate and 6PGD activity was normal: 0.0015 μM NADP during 1 min per milligram protein. After purification of the enzyme the G6PD activity in the resulting preparation was 0.017 μM NADP in 1 min per milligram protein (2% of normal). In the mother of patient S-r G6PD activity in the hemolysate was 0.00154 μM NADP per minute per milligram protein (59.2% of normal), whereas 6PGD activity was normal, namely 0.0015 μM NADP per minute per milligram protein. After purification of the enzyme by 286 times the G6PD activity in the preparation was 0.44 μM NADP per minute per milligram protein (51.8% of normal). The values of K_m for G6P and NADP were appreciably lower than normal for G6PD isolated from the erythrocytes of both the son and his mother. Utilization of substrate analogs (2-deoxy-G6P and diamino-NADP) was sharply increased for the son's abnormal enzyme, and a less marked increase, affecting diamino-NADP, was observed for the enzyme from the mother's erythrocytes. Neither mutant enzyme utilized NAD. The thermostability of G6PD from the son's erythrocytes was sharply reduced—the critical temperature during incubation for 10 min in the presence of 10^{-5} M NADP was 42°C, whereas for enzyme from the mother's erythrocytes it was above 50°C (Fig. 1). The optimum of enzyme activity from the son's erythrocytes occurred at pH 8.0; at pH 5.5 only 4% of the activity was exhibited, and with an increase in pH to 8.0 enzyme activity gradually increased to reach a maximum, and at pH 8.5 inactivation began. G6PD from mother S-r's erythrocytes had a pH dependence similar to that of the normal enzyme, but the maximum of its activity was observed at pH 9.5 (Fig. 2).

During electrophoresis in PAG the abnormal G6PD isolated from the erythrocytes of patient S-r (son) was manifested as a single band of G6PD activity (REM 0.29), evidently corresponding to the less active tetramer form of the enzyme (Fig. 3). The mutant G6PD from the erythrocytes of mother S-r was manifested as two bands of G6PD activity (REM of first band 0.29, of second band 0.36), corresponding to the dimer and tetramer forms of the enzyme.

During electrophoresis in starch gel both mutant enzymes were revealed as a single band of G6PD activity. The electrophoretic mobility of the abnormal enzyme from the erythrocytes of mother and son was indistinguishable from normal. The differences in the properties of the abnormal enzymes isolated from mother and son clearly reflect the presence of a mixture of different enzymes in the maternal erythrocytes: normal and abnormal.

Comparison of the properties of G6PD isolated from the erythrocytes of patient S-r (son) with the properties of known abnormal variants of this enzyme [3, 5, 16] showed that with respect to the deficiency of activity, the G6PD now isolated corresponds to class II of G6PD variants according to the WHO classification [2], but according to the characteristics studied, it differs from those described in the literature.

The variant of G6PD found in the erythrocytes of patient S-r (son) is a new abnormal variant of this enzyme, which we have called "Kremenchug" after the town where the proband lives.

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