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Hemolytic Anemia and G6PD Deficiency

Physiologic activity, not in vitro activity, of enzymes is related to the severity of genetic diseases.

Akira Yoshida

The discovery that primaquine-induced hemolytic anemia was associated with an inherited deficiency of glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: NADP oxidoreductase, E.C. 1.1.1.49) (G6PD) in red blood cells (*1*) led to many investigations of the genetic variants of this enzyme in man (*2*). This enzyme, G6PD, catalyzes the initial step in the hexose monophosphate oxidation pathway of carbohydrate metabolism causing reduction of NADP to NADPH. Under normal physiologic conditions glucose is metabolized primarily (more than 90 percent) via the Embden-Meyerhof pathway to produce lactate. During this process no net generation of NADH occurs since 1 mole of NAD is reduced to NADH by glyceraldehyde 3-phosphate dehydrogenase while 1 mole of NADH is oxidized to NAD by lactate dehydrogenase. Lactate can be oxidized by the aerobic oxidation pathway producing NADH and NADPH in the nucleated cells of various tissues. However, since the matured human red cells lack the oxidative enzymes of the Krebs cycle, the hexose monophosphate shunt pathway in red cells has a particular importance in generating NADPH. NADPH is required by the red cell glutathione reductase (E.C. 1.6.4.2) to maintain glutathione in the reduced state. Reduced glutathione appears to be necessary for maintaining sulfhydryl groups within the red cell and perhaps in the red cell membrane. Thus, severe genetic deficiency of G6PD is frequently associated with a low concentration of reduced glutathione and with hemolytic anemia.

More than 80 variants of G6PD, which are distinguishable from one another by their kinetic characteristics, electrophoretic mobilities, and substrate specificities, have been reported (*3*). The amino acid substitution has been elucidated in only two of these variant enzymes, the common Negro variant G6PD A$^+$ (*4*) and G6PD Hektoen (*5*) which is associated with overproduction of the enzyme (*6*). By analogy with the structure of many human hemoglobin variants, most of the G6PD variants are presumed to be caused by single amino acid substitutions.

About 40 variants have normal activity or mild enzyme deficiency in red cells and therefore are not associated with any clinical manifestations. Another group of variants causes severe enzyme deficiency in red cells, but requires exogenous agents such as drugs, infections, or fava beans for hemolysis to occur. Other variants (about 20) are associated with chronic nonspherocytic hemolytic anemia even in the absence of exogenous agents. Deficiency of G6PD is the commonest genetically determined enzymatic abnormality in human beings, probably affecting more than 100 million males. Because the gene determining the structure of the G6PD molecule is located on the X chromosome (*7*), almost twice as many females carry gene coding for variants of G6PD.

In contrast to genetic defects located on the autosomal chromosomes, the location of the gene for G6PD on the X chromosome results in males that carry a single G6PD gene. In the study of the molecular abnormality, enzymatic characteristics, and physiologic disorders caused by genetic mutation of G6PD, one can avoid some of the difficulties that arise when studying a heterogeneous mixture of normal and variant enzymes. Thus, G6PD deficiency has become a model system for understanding the molecular pathology of genetic diseases, and much valuable knowledge has been obtained which advances not only the understanding of G6PD disorders, but also the understanding of other genetic disorders [see, for example, the review by Kirkman (*8*)].

The degree of enzyme deficiency does not correlate well with the clinical severity of the disease, not only in the case of G6PD variants but also in other human enzyme abnormalities. This is one of the problems in the molecular pathology of genetic disorders. Thus, some variant subjects associated with severe G6PD deficiency, such as Gd Markham (*9*) and Gd Union (*10*), cause no hemolytic problem while other variants associated with less severe G6PD deficiency, such as Gd Manchester (*11*), Gd Alhambra (*12*), and Gd Tripler (*13*), cause chronic hemolytic anemia even in the absence of exogenous agents. Kinetic characteristics (affinity

The author is director, department of biochemical genetics, City of Hope National Medical Center, Duarte, California 91010.

for the substrate and coenzyme) of these variant enzymes reported in the literature cannot explain the reason for the different hemolytic manifestations in the affected subjects. To resolve this problem, one has to elucidate (i) the mechanism of function of normal G6PD, (ii) the mechanism by which the shunt pathway is regulated in red cells, and (iii) the physiologic activity of G6PD in the variant red blood cells.

Mechanism of Function of Normal G6PD

The normal human G6PD consists of subunits each having a molecular weight of about 50,000 to 55 000. The subunit has pyroglutamic acid at the amino terminal and glycine at the carboxyl terminal (*14*). Because only one structural gene participates in the production of G6PD, the enzyme consists of one type of subunit (*15*). G6PD exists in various oligomeric forms depending upon protein concentrations, *p*H, and ionic strength of the solution (*14, 16*). The enzyme associated with NADP or NADPH is predominantly tetrameric (molecular weight, 210,000) at neutral *p*H and high ionic strength (> 0.1), and it is predominantly dimeric at its optimal *p*H (*p*H 8 to 9). It is presumed that the enzyme has a high oligomeric form at its isoelectric point (*p*H 6.0) when the ionic strength of the solution is low and when protein concentrations are high (> 1 milligram per milliliter). The functioning G6PD is predominantly dimeric (molecular weight, 110,000) in the presence of both NAPD and G6P at *p*H 6 to 8 (*16*). It can be concluded that G6PD is predominantly in the dimeric form and its concentration is 5 to 6 micrograms per milliliter, that is, 0.05 micromolar (dimeric enzyme) in red blood cells.

The dimeric G6PD is associated with either one molecule of NADP or one molecule of NADPH, but never one molecule of NADP and one of NADPH at the same time (*16, 17*). The dissociation constants of the G6PD-NADP complex ($1.7 \times 10^{-7} M$ at 37°C, $1.5 \times 10^{-9} M$ at 4°C) and the G6PD-NADPH complex ($4.9 \times 10^{-7} M$ at 37°C, $3.3 \times 10^{-9} M$ at 4°C) are very low and highly temperature-dependent (*17*). Removal of NADP (or NADPH) from the enzyme causes dissociation of the oligomeric enzyme into the enzymatically inactive monomer (subunit) (*17, 18*). The subunit can be reactivated by incubation with NADP at neutral *p*H. Therefore, when the normal and variant human G6PD or human and animal G6PD coexist during the dissociation-reactivation procedures, subsequent starch gel electrophoresis shows that a hybrid enzyme is formed while some proportion of the two original enzymes remain (*18, 19*).

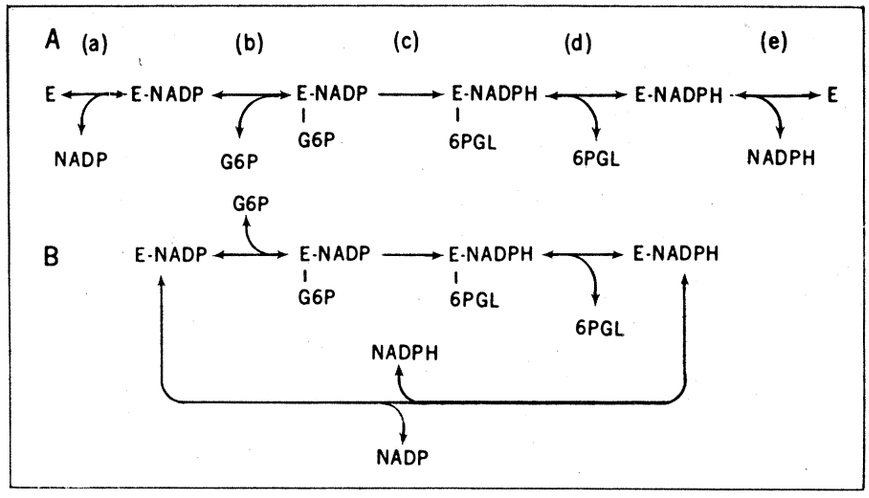

Fig. 1. (A) The compulsory sequence of reactions between the enzyme (*E*) and NADP and NADPH. (B) Direct substitution of NADPH by NADP without the production of free enzyme.

The G6PD without bound NADP and the G6PD-NADPH complex can not associate with G6P (*17*). Therefore, the association of G6P with the enzyme should occur in a compulsory sequence. On the assumptions (i) that there is a compulsory order mechanism for the enzyme action (Fig. 1A); and (ii) that all the reaction steps shown in Fig. 1A except (c) reach equilibria rapidly; and by using the kinetic equation of Alberty (*20*), the dissociation constant for the G6PD-NADP complex can be estimated to be of the order of $10^{-5} M$ at 25°C (*21*).

The real dissociation constant of

Table 1. Inhibition of G6PD and 6PGD by various metabolites. Concentrations in red blood cells were calculated from reported values (references in parentheses) with the assumption that such cells have a water content of 70 percent. For abbreviations, see (*2*). G6PD activity was measured in 0.05*M* tris chloride, *p*H 7.3, containing 4 m*M* $MgCl_2$, 0.1*M* KCl, 50 μM G6P, 10 μM NADP, and an inhibitor at 37°C. 6PGD activity was measured in the same buffer containing 4 m*M* $MgCl_2$, 0.1*M* KCl, 50 μM 6PG, 10 μM NADP, and an inhibitor of concentration specified.

Compound	Concentration (m*M*)		Inhibition (%)	
	In red blood cells	In reaction mixture	G6PD	6PGD
NADPH	50 (*26*)	50	53	77
NAD	100 (*26*)	100	0	10
NADH	60 (*26*)	100	0	10
ATP	1500 (*27*)	1500	40	40
ADP	180 (*27*)	1000	10	31
Glucose 6-phosphate	40 (*27*)	1000		33
Glucose 1-phosphate	?	1000	<10	0
Glucose 1,6-diphosphate	300 (*30*)	1000	0	20
Fructose 6-phosphate	15 (*27*)	1000	22	35
Fructose 1,6-diphosphate	7 (*27*)	1000	37	24
6-Phosphogluconate	?	1000	<10	
2-Phosphoglycerate	10 (*27*)	1000	0	0
3-Phosphoglycerate	70 (*27*)	1000	0	42
2,3-Diphosphoglycerate	1500 (*28*)*	1000	0	60
Glyceraldehyde 3-phosphate	6 (*27*)	1000	96	0
Glyceraldehyde 3-phosphate		100	29	0
Glyceraldehyde 3-phosphate		10	7	
Glyceraldehyde 3-phosphate		5	0	
Dihydroxyacetone phosphate	17 (*27*)	1000	25	55
Phosphoenolpyruvate	17 (*27*)	1000	19	0
Inorganic phosphate	500 (*30*)	1000	0	0
Reduced glutathione	3000 (*43*)	3000	0	<10

* Concentration of 2,3-diphosphoglycerate in free state (see text).

Table 2. G6PD and 6PGD activities under various conditions. Enzyme activities, expressed as nanomoles of NADPH produced per minute per 10^{10} red cells, were measured at 37°C and *p*H 7.3. The inhibitors added were NADPH (45 μM), ATP (1500 μM), and 2,3DPG (1500 μM); NADP, G6P, and 6PG were added as indicated. Visual interpolation was used when necessary. The maximum potential activity (V_{max}) was obtained from the enzyme activity measured in the absence of the inhibitors, and by extrapolating the data to infinite substrate and NADP concentrations.

NADP (μM)	G6PD		6PGD	
	G6P ∞ without inhibitor	40 μM G6P with inhibitors	6PG ∞ without inhibitor	10 μM 6PG with inhibitors
∞	2200 (V_{max})	88	1500 (V_{max})	60
10	880	30	600	26
5	545	16	340	14
2	250	7	150	6
1	135	3.5	80	3
0.5	70	1.8	40	1.5

the G6PD-NADP complex determined by the dialysis equilibrium method ($1.7 \times 10^{-7} M$ at 37°C) (*17*) is much lower than the hypothetical dissociation constant estimated by the kinetic analysis. Therefore the assumption that the reactions between the free enzyme and NADP, and between the free enzyme and NADPH, attain equilibria rapidly may not hold true for the G6PD reaction. Instead, the reaction may proceed without any free enzyme being produced, by way of direct substitution of NADPH by NADP as shown in Fig. 1B. Because of the low dissociation constants for the G6PD-NADP and G6PD-NADPH complexes, practically all of the enzyme would be associated with either NADP or NADPH in vivo as well as in the reaction mixture under usual assay conditions in vitro. The Michaelis constant (K_m) for NADP and the inhibition constant (K_i) of NADP do not represent dissociation constants of these coenzymes, but they are kinetic parameters which are determined by the dynamic balance between the G6PD-NADP and G6PD-NADPH complexes under the assay conditions.

Regulation of the Shunt Pathway

Compared with the maximum potential activities (V_{max}), of G6PD and 6PGD in red blood cells, the actual activities of these enzymes in the shunt pathway is very low. Thus, under the saturation of NADP and substrates, 10^{10} red cells could oxidize about 2.2 μmole of G6P and 1.5 μmole of 6PG, producing 1.5 μmole of carbon dioxide in 1 minute at 37°C and *p*H 7.3; whereas only 2 to 3 nanomoles of CO_2 are actually produced by 10^{10} red cells suspended in an isotonic solution containing glucose at 37°C (*22*, *23*). This would imply that the activities of G6PD or 6PGD, or both enzymes, are strongly suppressed in red cells. It has been suggested that the low activity of G6PD in the shunt pathway could be attributed to its being inhibited by NADPH and ATP in red blood cells (*21*, *24*). However, no convincing mechanism has been demonstrated for the regulation of the shunt pathway.

To elucidate the nature of the regulation, the effects of various intermediate metabolites, ATP, coenzymes, and reduced glutathione on the activities of G6PD and 6PGD were examined (Table 1). Although several intermediate metabolites inhibit G6PD and 6PGD, the concentrations of these compounds in red cells indicate that the major inhibitors of G6PD under physiologic conditions are NADPH and ATP, and the inhibitors of 6PGD are NADPH, ATP, and 2,3DPG. Other metabolites could have only minor effect on the activities of G6PD in red cells (*25*). For example, glyceraldehyde 3-phosphate (1 mM) is a strong inhibitor of G6PD in vitro, but at the low physiologic concentration of glyceraldehyde 3-phosphate (about 6 μM) the inhibition of G6PD by this compound must be negligible.

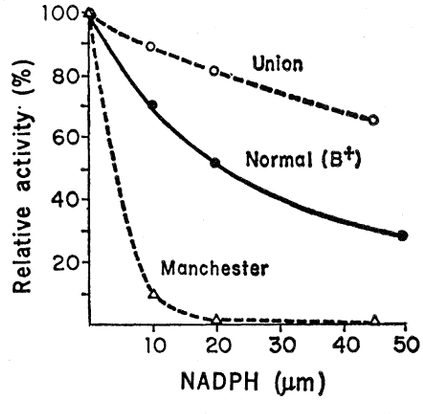

Fig. 2. Inhibition of normal and variant G6PD by NADPH. The reaction mixture contained 0.05M tris chloride, *p*H 7.3, 4 mM $MgCl_2$, 0.1M KCl, 60 μM G6P, 10 μM NADP, and various concentrations of NADPH. Measurements were made at 37°C. Values for relative activity were obtained on the assumption that the activity without NADPH is 100.

Kinetic analysis indicates that the inhibition of G6PD by NADPH is competitive with NADP (K_m for NADP is 12.5 ± 2.1 μM; K_i for NADPH is 9.1 ± 1.8 μM, at 37°C, *p*H 7.3 in the presence of 60 μM G6P) and the inhibition by ATP is competitive with G6P (K_m for G6P is 44 ± 4 μM; K_i for ATP is 1.03 ± 0.17 mM, at 37°C, *p*H 7.3 in the presence of 80 μM NADP). 6PGD is also strongly inhibited by NADPH. The inhibition is competitive with NADP (K_m for NADP and K_i for NADPH are estimated as 18 ± 3 μM and 7.7 ± 1.2 μM, respectively, in the presence of 10 μM 6PG at 37°C, *p*H 7.3). In contrast to G6PD, 6PGD is strongly inhibited by 2,3DPG of physiologic concentration. The mode of inhibition is competitive with 6PG (K_m for 6PG and K_i for 2,3DPG are estimated as 10 ± 2 μM and 0.12 ± 0.022 mM, respectively, in the presence of 80 μM NADP). ATP also inhibits 6PGD, that is, 40 percent inhibition of 6PGD occurs in the presence of 1.5 mM ATP in a reaction mixture containing 8 μM NADP, 10 μM 6PG, and 45 μM NADPH compared with the activity of 6PGD in the absence of ATP.

From the kinetic analysis and the measurements of enzyme activities under simulated physiologic conditions, an attempt was made to estimate the activity of G6PD and 6PGD in the red cell. The concentrations of NADP and NADPH in human red cells incubated in Ringer solution containing glucose were reported by Omachi *et al.* (*26*) to be 5 ± 3 nmole and 35 ± 4 nmole per milliliter of packed red cells. The same investigators reported a lower value for NAPD (1 nmole/ml) in washed fresh cells. Because complete protection against the oxidation of NADPH to NADP during the extraction process is very difficult, the actual concentration of NADP in red cells is presumably lower than the reported value. If we assume that the water content of red cells is 70 percent, the molar concentration of NADPH

should be about 50 ± 5 μM and that of NADP should presumably be lower than 2 μM. The concentrations of G6P, ATP, and 2,3DPG in fresh human red cells were reported as 27 ± 2.4 nmole, 1.1 μmole, and 4 μmole per milliliter of packed red cells (*27*), that is, 40 μM, 1.5 mM, and 5.7 mM, respectively, with the assumption of a 70 percent water content in red cells. Some of the 2,3DPG is associated with hemoglobin and presumably with red cell membrane (*28*). The concentration of 2,3-DPG in circulating red cells fluctuates depending upon the ratio of oxygenated to deoxygenated hemoglobins. However, it may not be unreasonable to assume that 70 percent of 2,3DPG is bound with hemoglobin and red cell membrane, and about 30 percent (1.5 mM) of it is in the free state. The concentration of 6PG in fresh human red cells, which is too small to be determined by the column chromatographic method (*28*), should be lower than 10 μM.

Table 2 shows the activities of G6PD in 10^{10} red cells under simulated physiologic conditions. Only 0.1 to 0.2 percent of the maximum potential activity (2 to 4 nmole per minute per 10^{10} red cells) of G6PD is expressed in red cells which contain 1 μM NADP, 45 μM NADPH, 40 μM G6P, 1.5 mM ATP, and 1.5 mM free 2,3DPG. This value is comparable to the actual activity of G6PD in the shunt pathway as measured by the generation of carbon dioxide. The activity of 6PGD is also strongly suppressed under physiologic conditions because of inhibition by NADPH, 2,3DPG, and ATP. Under physiologic conditions, if one assumes that the concentration of 6PG in red cells is several micromoles, only 0.2 percent of the maximum potential activity of 6PGD can be expressed. This value is also comparable to that of the activity in the shunt pathway.

Although the expected activities of G6PD and 6PGD are similar in red cells, a relatively high accumulation of G6P (40 μM) and a low accumulation of 6PG in red cells suggests that in normal red cells under physiologic conditions the rate-limiting enzyme in the shunt pathway is G6PD rather than 6PGD. The situation may be altered by slight changes in the concentrations of metabolites and coenzymes induced by genetic abnormalities, by exogenous reagents, or by storage of blood. Thus, 6PGD could become the rate-limiting enzyme under certain conditions. In these experiments very little, if any, of the G6PD and 6PGD was in a dormant state bound with red cell membrane. Consequently, these results indicate that the major regulatory factors for these two enzymes in red cells are the substrates, NADP, NADPH, ATP, and 2,3DPG, and that other phosphate compounds and intermediate metabolites have only minor effects on the activity of the shunt pathway.

Table 3. Michaelis constant (K_m) for NADP and inhibition constant (K_i) for NADPH of the normal and variant G6PD. K_m and K_i were measured in 0.05M tris chloride, pH 7.3, containing 4 mM $MgCl_2$, 0.1M KCl, 60 μM G6P, and various concentrations of NADP and NADPH at 37°C. Note that K_i of G6PD B^+ is higher in the presence of high concentration of G6P (24 μM) than in the presence of low concentrations of G6P (*17*).

Enzyme	K_m for NADP (μM)	K_i for NADPH (μM)
B^+ (normal)	12.5	9.0
A^+	7.2	6.7
A^-	8.0	13.0
Union	8.2	37.0
Markham	6.0	16.0
Mediterranean	3.8	9.5
Manchester	18.5	0.8
Alhambra	19.5	3.3
Tripler	80.0	2.6
Torrance	9.3	3.0

Association of G6PD Activity with Chronic Hemolytic Anemia

As mentioned previously, there is no linear relationship between the "red cell G6PD activity" (that is, the activity of G6PD in vitro) and the chronic hemolytic manifestations among hereditary disorders of this enzyme deficiency. The "red cell G6PD activity" of variant G6PD reported in the literature has always been determined in the presence of high concentrations of NADP and G6P and in the absence of any inhibitors at optimal pH (pH 8 to 9). To elucidate the correlations between G6PD deficiency and hemolytic manifestations, the physiologic activity of the enzyme in red blood cells should be deduced from careful kinetic analysis of the variant G6PD under simulated physiologic conditions in vitro.

The hemolytic variant enzymes (G6PD Manchester, G6PD Tripler, G6PD Alhambra, and G6PD Torrance) are strongly inhibited by NADPH (for example, G6PD Manchester shown in Fig. 2). The mode of inhibition of these variant enzymes by NADPH is competitive with NADP, as it has been found in the normal G6PD B^+ (Table 3). Therefore, in the presence of low concentrations of NADP and relatively high concentrations of NADPH as in the red cells, the hemolytic variants, such as Manchester, Tripler, and Alhambra, can scarcely function (Table 4) although their "red cell G6PD activity" is more than 20 percent of normal. Furthermore, these hemolytic variant enzymes are strongly inhibited by ATP of physiologic concentration (more than 55 percent inhibition by 1.5 mM ATP).

In contrast, the nonhemolytic variant enzymes associated with severe enzyme deficiency are resistant to inhibition by NADPH (for example, G6PD Union shown in Fig. 2). Such variant enzymes have either high K_i for NADPH (G6PD Union) or low K_m for NADP (G6PD Mediterranean) or both (G6PD Markham) (Table 3). These variant enzymes

Table 4. Activity of normal and variant G6PD under various conditions. Enzyme activity is expressed as nanomoles of NADPH per minute per 10^{10} red cells. The reaction mixtures contained NADP (concentration as indicated) and 40 μM G6P, 45 μM NADPH, 1.5 μM ATP, and 1.5 μM 2,3DPG.

Enzyme	V_{max}	NADP (μM)				
		10	5	2	1	0.5
Variants not associated with chronic hemolytic anemia						
B^+ (normal)	2200	30	16	7	3.5	1.8
A^+	2000	44	24	10	5	2.5
A^-	400	21	13	6	3	1.5
Union	60	8	5	2	1	0.5
Markham	60	7	4	1.6	0.8	.4
Mediterranean	80	5	3	1.2	.6	.3
Variants associated with chronic hemolytic anemia						
Manchester	700	< 0.7	< 0.4	< 0.2	< 0.1	< 0.1
Alhambra	400	< .7	< .4	< .2	< .1	< .1
Tripler	700	< 1.5	< .8	< .4	< .2	< .1
Torrance	50	< 0.3	< .2	< .1	< .1	< .1
Bangkok	120	< .7	< .4	< .2	< .1	< .1

are also more resistant to the inhibition by ATP (17 to 22 percent inhibition by 1.5 m*M* ATP). The G6PD activity of these variant enzymes in red cells in estimated to be more than 30 percent of the normal activity in the shunt pathway (Table 4), although their "red cell G6PD activity" of the variant is less than 5 percent of normal. In fact, activity of the hexose monophosphate shunt pathway in red cells from the Mediterranean variant subject, measured by carbon dioxide generation, is estimated to be 30 percent of that of the normal red cells (*31*).

Some Gd Mediterranean subjects have hemolytic problems induced by fava beans (favism). Because not all Gd Mediterranean subjects exhibit favism, certain other genetic factors or heterogeneity of the Mediterranean variant have been suggested (*32*). No hemolytic abnormality has been found in Gd Union and Gd Markham variant subjects. These three variants appear in large numbers in certain populations.

Two variants, G6PD A^+ and A^-, that show fast electrophoretic mobility, are common among Negroes. About 20 percent of Negro males are hemizygous for A^+ and 10 to 15 percent of Negro males are hemizygous for A^-. The "red cell G6PD activity" of Gd A^+ subjects is close to normal and that of Gd A^- subjects is 10 to 20 percent of normal. Gd A^- subjects suffer from primaquine-induced hemolytic anemia, but without oxidative stress, the variant subjects have no hemolytic problem. It has been reported that activity of the hexose monophosphate shunt pathway of red cells from Gd A^- subjects is close to normal (*23*). The G6PD A^- is more resistant to inhibition by NADPH than the normal G6PD, because of its lower K_m for NADP and higher K_i for NADPH (Table 3). Thus, under physiologic conditions in red cells, the variant enzyme is expected to express almost the same activity as that expressed by the normal enzyme B^+ (Table 4).

Conclusion

The hemolytic manifestation of some G6PD variants, such as in Gd Bat-Yam (*33*), Gd Ramat-Gan (*33*), and Gd Worcester (*34*) subjects, can be easily correlated with the extremely low activity of the variant enzymes (Table 5). The clinical manifestation can sometimes be explained by the unusually low affinity of the variant enzyme for its substrate or coenzyme. Kirkman pointed out that the hemolysis of red cells from a Gd Oklahoma variant subject could be attributed to a very low affinity (high K_m value) of the variant enzyme for G6P (*35*). Several variants (such as Ashdod and Freiburg) are also associated with a high K_m for the substrate (*33, 36*); therefore, these variant enzymes are expected to have little activity in the variant red cells. Hemolysis of red blood cells from Gd Albuquerque (*37*), Gd Milwaukee (*38*), Gd Clichy (*39*), and Gd Strasbourg (*40*) variant subjects can be explained by the combination of their low "red cell G6PD activity" (less than 5 percent of normal) and low affinity for the substrate or the coenzyme (Table 5).

However, several variant subjects (Gd Manchester, Gd Alhambra, Gd Tripler) with chronic hemolytic anemia are associated with neither very severe G6PD deficiency (more than 20 percent of normal) nor with variant G6PD which has unusually high K_m values for the substrate and coenzyme (Table 5). Because many G6PD variants' red cells associated with more severe enzyme deficiency (less than 5 percent of normal, for example, in Gd Union, Gd Markham, and Gd Mediterranean) cause no chronic hemolytic problem, it was difficult to explain the discrepancy.

The problem was resolved by estimating the physiologic activity of G6PD in red cells from the kinetic analysis of the variant enzymes. Taken into consideration were the physiologic concentration of the substrate, the coenzymes, and the various metabolites that can affect G6PD activity. The hemolytic variant enzymes are strongly inhibited by NADPH of physiologic concentration because of their high K_m for NADP or low K_i for NADPH, and they are more strongly inhibited by ATP than the normal enzyme. Thus, these variant enzymes cannot generate a sufficient amount of NADPH in red cells to maintain an adequate concentration of reduced glutathione. In contrast, the nonhemolytic variant enzymes are far less sensitive to the inhibition by NADPH. The physiologic activity of these nonhemolytic variant enzymes is estimated to be more than 30 percent of the activity of the normal enzyme, and this is adequate to maintain the red cells unhemolyzed.

Both G6PD and 6PGD are strongly suppressed in red cells and only 0.1 to 0.2 percent of their maximum potential

Table 5. Characteristics of normal and variant G6PD. The data were collected from references given in the text. Several other variants associated with nonhemolytic anemia have been reported, and more than ten variants associated with severe red cell enzyme deficiency but not associated with hemolytic anemia have been described (*3*). Relative enzyme activity in red blood cells is measured at near saturation of G6P and NADP at 25°C and *p*H 8.0 in the absence of inhibitors; K_m for G6P is measured at near saturation of NADP; K_m for NADP is measured at near saturation of G6P.

Enzyme	Activity (% of normal)	K_m for G6P (μM)	K_m for NADP (μM)	Heat stability	*p*H optima
Variants not associated with chronic hemolytic anemia					
B^+ (normal)	100	50–70	2.9–4.4	Normal	8–9
A^+	80	Normal	Normal	Normal	8–9
A^-	8–20	Normal	Normal	Normal	8–9
Union	< 3	8–12	3.6–5.2	Low	Biphasic
Markham	1.5–10	4.4–6.3		Low	Biphasic
Mediterranean	< 5	19–26	1.2–1.6	Low	Biphasic
Variants associated with chronic hemolytic anemia					
Bat-Yam	0	27		Very low	Biphasic
Ramat-Gan	0	35		Very low	Biphasic
Worcester	0	11.6	61	Very low	8.0
Oklahoma	4–10	127–200	20	Low	8.2
Ashdod	10	100		Slightly low	Biphasic
Freiburg	10–20	87–118	4		Biphasic
Albuquerque	1	115	11	Very low	8.2
Milwaukee	0.5	224			8.0
Clichy	2	178			9–10
Strasbourg	6	96	13	Low	9.0
Bangkok	5	60	5.3	Very low	8–85
Torrance	2.4	48–60	2.4	Very low	8–85
Manchester	25–30	64	6	Low	Biphasic
Alhambra	9–20	55	2.6	Low	Truncate
Tripler	35	30		Very low	Biphasic

activity is expressed under normal physiologic conditions. Under oxidative stress, the shunt pathway activity of red cells is stimulated (*41*) because the activity of both enzymes is almost linearly related to the concentration of NADP and inversely related to that of NADPH. The concentration of reduced glutathione is maintained unchanged in normal red cells under oxidative stress (*42*), presumably because of the stimulation of the shunt pathway activity described above. In the case of red cells with G6PD deficiency, stimulation of the shunt pathway activity is not sufficient (*23, 31*) to overcome the oxidative stress resulting in the decrease of reduced glutathione in red cells (*43*). This would explain the hemolysis of several G6PD variants associated with enzyme deficiency under various oxidative stresses. Study of the exact mechanism of hemolysis induced by antimalarial drugs in the Negro variant A^- is of particular importance, because the frequency of the variant is high (10 to 15 percent among the Negro population) and the development of adequate drugs is desirable.

The work described in this article indicates clearly that enzyme activity and kinetic charactertistics determined in vitro under unphysiologic conditions (at optimal *p*H, in the presence of high concentrations of the substrate and coenzyme, and in the absence of inhibitors) often provide misleading information concerning the real physiologic activity of the enzyme in the variant cells. Kinetic analysis under simulated physiologic conditions might provide insight into the molecular pathology not only of G6PD variants but also of genetic diseases in general.

Summary

The severity of enzyme deficiency often does not correlate well with the clinical severity of genetic diseases. Thus, some G6PD variants associated with severe enzyme deficiency, such as Union and Markham, cause no hemolytic problem, while some variants associated with less severe deficiency, such as Manchester, Alhambra, and Tripler, cause chronic hemolytic anemia. The kinetic characteristics of these variant enzymes have not explained the discrepancy. However, examination of the normal and variant enzymes under simulated physiologic conditions, with the effects of various intermediate metabolites and coenzymes in red cells being taken into consideration, reveal that the G6PD's from hemolytic variant subjects are strongly inhibited by a physiologic concentration of NADPH because of their high Michaelis constant for NADP or low inhibition constant for NADPH, and they are more sensitive to inhibition by ATP. These variant enzymes cannot generate enough NADPH in red cells to maintain an adequate concentration of reduced glutathione. The nonhemolytic variant enzymes are far less sensitive to the inhibition by NADPH because of their low Michaelis constant for NADP and high inhibition constant for NADPH. The physiologic activity of these nonhemolytic variant enzymes is estimated to be more than 30 percent of the activity of the normal G6PD, and this activity is adequate to maintain the red cells unhemolyzed.

References and Notes

1. P. E. Carson, C. G. Flanagan, C. E. Ickes, A. S. Alving, *Science* **124**, 484 (1956).
2. Abbreviations: G6P, α-D-glucose 6-phosphate; 6PG, 6-phosphogluconate; 6PGL, 6-phosphogluconolactone; P-, phosphate; 2,3DPG, 2,3-diphosphoglycerate; G6PD, glucose 6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44); NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide monophosphate; NADH and NADPH, reduced NAD and NADP, respectively; ATP, adenosine triphosphate; ADP, adenosine diphosphate; tris, tris(hydroxymethyl)aminomethane.
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25. No synergetic inhibitory effect is observed on G6PD activity in the presence of all these compounds in their physiologic concentrations in the reaction mixture. In contrast to the crystalline human G6PD preparation, which is free from other enzymes, the human 6PGD preparation used for these studies was partially purified and consequently contaminated with lactate dehydrogenase (E.C. 1.1.1.27), malate dehydrogenase (E.C. 1.1.1.37), diphosphoglyceromutase (E.C. 2.7.5.4), and glutathione reductase (E.C. 1.6.4.2). Therefore, it is impossible to assess the synergetic interaction of all these compounds for 6PGD activity. But the measurements of inhibition by various combinations of these compounds suggests no synergetic interaction.
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44. This research was conducted at the University of Washington School of Medicine, Seattle. I am grateful to Drs. E. Beutler, I. W. Delamore, and L. A. Malcolm who provided some of the variant enzymes, and to Mrs. Mei Lin, who ran many of the analyses. This work was supported by PHS grant GM 15253.