INHIBITION OF NORMAL RED CELL GLUCOSE 6-PHOSPHATE DEHYDROGENASE (G-6-PD) BY A VARIANT ENZYME, G-6-PD HAMBURG

Manfred GAHR and Werner SCHRÖTER

Universitätskinderklinik, Universität Göttingen, Humboldtallee 38, D 3400 Göttingen, West Germany

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1. Introduction

Deficiency of glucose 6-phosphate dehydrogenase (G-6-PD)* (EC 1.1.1.49) in red cells is often accompanied by a reduced survival time of these cells. The consequence of G-6-PD deficiency in erythrocytes is quite well understood, whereas the mechanism of reduced G-6-PD activity at the molecular level is not known. In two variants, G-6-PD A^+ [1] and G-6-PD Hektoen [2] single amino acid substitutions could be demonstrated. But till now this alteration of the primary structure could not explain the impaired function of this enzyme. Ratazzi et al. [3] have suggested, that in some G-6-PD variants amino acid mutations affect the association or a cooperative effect of the subunits of G-6-PD, whereas in others the structural abnormality affects the activity of the subunits themselves.

Modification of G-6-PD activity has been reported in single variants of red cell G-6-PD. In 1960 Rimon et al. [4] showed that stroma of erythrocytes with normal G-6-PD activity enhanced the low activity in some mutants of G-6-PD in patients of Jewish descent. These findings, however, were not confirmed later by immunological methods [5]. Recently Kahn et al. [6] observed an activation of the G-6-PD Beneventolike variant by incubating the enzyme with an antibody specific for normal G-6-PD.

We have reported recently a new G-6-PD variant associated with chronic nonsphaerocytic haemolytic anaemia, G-6-PD Hamburg [7]. In this paper we describe an inhibitory effect of this variant on normal G-6-PD activity and the results of several experiments to elucidate the nature of this inhibition.

* *Abbrevations:* G-6-PD, glucose 6-phosphate dehydrogenase; G-6-P, glucose-6-phosphate.

2. Materials and methods

2.1. *Determination of G-6-PD activity, enzyme constants and protein concentration*

G-6-PD activity was determined according to Beutler [8]. In a final vol of 1.0 ml the incubation mixture contained 0.2 μmol NADP, 0.6 μmol G-6-P, 10 μmol $MgCl_2$, 0.1 mmol Tris–HCl, pH 8.0, and varying amounts of haemolyzate or partially purified enzyme. K_M values and V_{max} were determined according to the method of Lineweaver and Burk [9]. Protein concentration was determined by the standard colorimetric method [10] using human albumin as a standard.

2.2. *Procedure of partial purification of G-6-PD*

Erythrocytes were haemolyzed by the addition of two vol 5 mM sodium phosphate buffer pH 6.4 with 1 mM EDTA, 1 mM mercaptoethanol and 20 μM NADP and 0.2 vol chloroform. G-6-PD was partially purified according to the recommendations of the WHO scientific group [11] and after the method of Ratazzi et al. [3]. Instead of DEAE-cellulose DEAE-Sephadex was used for the first step of purification. Then the enzyme was precipitated by 50% ammonium sulphate and dialyzed against 50 mM Tris–HCl pH 8.0 with 0.27 mM EDTA, 7 mM mercaptoaethanol and 10 μM NADP at least 24 h in the cold with change of buffer every 8 h. The specific activity of the partially purified normal enzyme was consistently in the range of 2 IU

per mg protein. G-6-PD Hamburg which had no measurable enzymatic activity in the haemolyzate had a specific activity of 0.0027 IU per mg protein after the purification procedure. The partially purified enzymes had no detectable 6-phosphogluconate dehydrogenase activity. The properties of the variant enzyme, G-6-PD Hamburg, have already been reported [7].

2.3. *Other methods*

For gel filtration with Sephadex G-25 a short column of 9 cm in length and 0.9 cm in diameter was used. The buffer was that of dialysis. Plasma of the patient was obtained after centrifugation of citrated blood for 15 min at 5000 *g* in the cold. 0.5 ml of plasma was added to the assay mixture for G-6-PD activity. Stroma of the patient's erythrocytes was prepared by haemolyzing his red cells in 0.27 mM EDTA, 10 mM mercaptoaethanol and 0.1 mM NADP. After centrifugation for 20 min at 20 000 *g* the stroma was resuspended in the haemolyzing solution. 0.5 ml of the stroma suspension was added to the assay mixture for G-6-PD when indicated.

3. Results

3.1. *Mode of inhibition*

Addition of haemolyzate, prepared from erythrocytes of the patient with G-6-PD Hamburg, or partially purified G-6-PD Hamburg preparation to normal G-6-PD inhibits its activity immediately. The inhibition could be observed between pH 6.5 and pH 9.0 always by the same amount. The degree of inhibition depends on the amount of G-6-PD Hamburg either as haemolyzate or as partially purified preparation added to the normal enzyme. The effect of increasing amounts of partially purified G-6-PD Hamburg on the catalytic activity of the normal partially purified enzyme is shown in fig.1. The resulting curve is sigmoid shaped. Similar curves were seen after the addition of haemolyzate with G-6-PD Hamburg to partially purified normal enzyme or of partially purified G-6-PD Hamburg to haemolyzate containing normal enzyme.

The inhibitory effect could be demonstrated on partially purified G-6-PD preparations from six different blood donors. On the other hand, purified G-6-PD A^- and purified preparations of two new G-6-PD variants (not yet published) did not inhibit

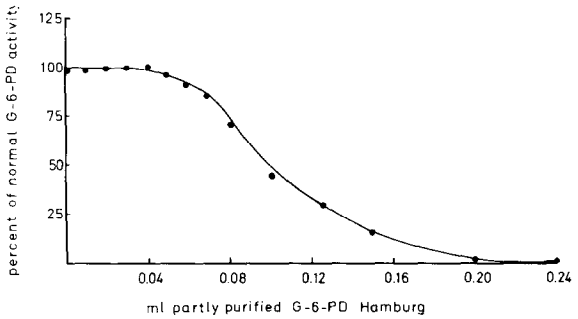

Fig.1. Inhibition of normal G-6-PD by G-6-PD Hamburg. Different amounts of partially purified G-6-PD Hamburg were added to a constant amount of partially purified normal G-6-PD. Normal G-6-PD activity without G-6-PD Hamburg added is taken as 100%. The specific activity of partially purified G-6-PD Hamburg was 0.0027 IU per mg protein. The protein concentration was 270 μg/ml.

normal, partially purified G-6-PD nor G-6-PD activity in normal haemolyzates.

In order to characterize the nature of this inhibition

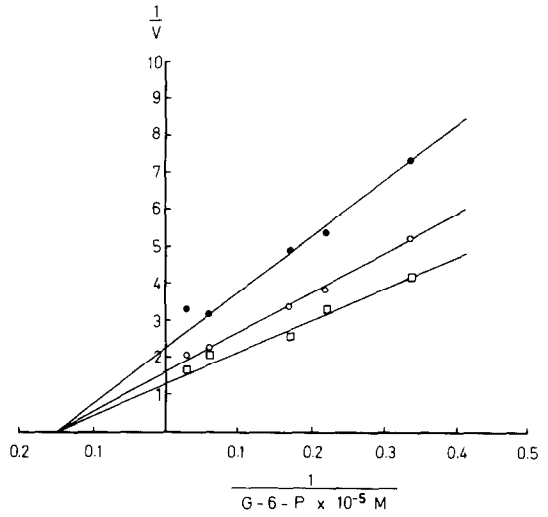

Fig.2. Lineweaver–Burk plot for the determination of the type of inhibition of normal G-6-PD by G-6-PD Hamburg. Michaelis–Menten kinetics for G-6-P of normal partially purified G-6-PD were determined without and in the presence of two different amounts of partially purified G-6-PD Hamburg. (□——□) without G-6-PD Hamburg; (○——○) with 0.06 ml G-6-PD Hamburg; (●——●) with 0.07 ml G-6-PD Hamburg added. The graph demonstrates a non-competitive inhibition.

Table 1
Effect of treatment of partially purified preparations of G-6-PD Hamburg on the inhibition of normal partially purified G-6-PD by G-6-PD Hamburg

Treatment of partially purified G-6-PD Hamburg	Activity of normal G-6-PD[a] in the presence[b] of G-6-PD Hamburg before and after the indicated treatment of G-6-PD Hamburg	
	Before	After
Column chromatography with Sephadex G-25	0.39	0.42
Dialysis for 24 h	0.44	0.46
Addition of diisopropylfluorophosphate (5×10^{-5} M)	0.48	0.39
Addition of ε-amino caproic acid (2×10^{-3} M)	0.47	0.46
Heating (65°C for 30 min)	0.42	1.04
Heating (40°C for 30 min)	0.37	0.40

[a] Enzyme activities are expressed in IU per mg protein.
[b] The amount of G-6-PD Hamburg added produced a 50% inhibition of normal G-6-PD activity.

the K_M value for G-6-P and the V_{max} of the reaction of the normal, partially purified enzyme in the presence of different amounts of partially purified G-6-PD Hamburg was determined. Fig.2 shows that the K_M value for G-6-P is not altered by the addition of G-6-PD Hamburg, whereas the maximal velocity was diminished; this indicates that the inhibition is non-competitive. As a result, high concentrations of the substrate G-6-P (0.2 M) could not overcome the inhibitory effect. The same experiment with the other substrate NADP also revealed a non-competitive inhibition.

3.2. *Experiments characterizing the nature of the inhibitory factor*

Column chromatography with Sephadex G-25 and dialysis did not remove the inhibiting action of the partially purified preparation of G-6-PD Hamburg on the normal, partially purified enzyme (table 1). Heating, however, of the partially purified G-6-PD Hamburg preparation at 65°C for 30 min abolished the inhibitory effect. Dialyzate of G-6-PD Hamburg had no inhibitory effect on the activity of normal, partially purified G-6-PD, when the mutant enzyme prior to dialysis was heated 30 min at 65°C. Both, haemolyzate and the partially purified fraction of G-6-PD Hamburg, inhibited the activity of normal G-6-PD. Whether or not the degree of inhibition is a function of its state of purification, i.e. of an increase in specific activity, cannot be answered, since there was no measurable activity in the haemolyzate of G-6-PD Hamburg. However, after partial purification of G-6-PD Hamburg the inhibiting effect per mg protein increased. Since the activity of G-6-PD is determined by the amount of NADPH formed, an enzyme oxidizing this compound in the erythrocytes of our patient could be responsible for the observed inhibition of normal G-6-PD activity. To test this possibility partially purified G-6-PD Hamburg was incubated under conditions used for the assay of G-6-PD activity as described under Material and Methods, except that NADP was omitted and was replaced by 0.2 μmol NADPH. Oxidation of NADPH was not observed in the presence of G-6-PD Hamburg. Plasma or stroma of the erythrocytes from the patient with G-6-PD Hamburg had no inhibitory effect on normal G-6-PD activity. Inhibitors of proteases (diisopropylfluorophosphate, ε-amino caproic acid) did not abolish the inhibiting action of G-6-PD Hamburg on normal G-6-PD (table 1).

4. Discussion

In this paper we describe the inhibition of normal G-6-PD activity by a partially purified preparation of a mutant enzyme, G-6-PD Hamburg. This inhibition is a non-competetive one. The true nature of the inhibitor could so far not be elucidated. Since the inhibiting action is not eliminated by dialysis or gel filtration, it appears to be linked to a protein. It seems that the inhibition of the partially purified preparation of G-6-PD Hamburg on normal G-6-PD activity is not altered by the process of purification and thus intrinsic to the enzyme protein. So a possible explanation for the observed effect could be an exchange of subunits. For normal G-6-PD, a polymeric enzyme composed of identical subunits, it has been shown that at pH 8.0 and in the presence of NADP an equilibrium exists between the dimeric, catalytic active and the monomeric form, which has no enzymatic activity [12]. By the assumption, that also the variant enzyme G-6-PD Hamburg at the same pH and the same molar concentration of NADP exists partly in the monomeric form, the inhibition could be due to an exchange of monomers between normal and variant enzyme. There are some arguments against this hypothesis: (1) The inhibition is observed in the same range after lowering the pH to 6.5. At this pH normal G-6-PD exists as a stable tetramer which cannot exchange subunits [12]. (2) The inhibition could be demonstrated very rapidly after the addition of mutant to normal enzyme. This fact also does not support the hypothesis of exchange of subunits as the basis for the observed inhibition, because hybridization of G-6-PD subunits takes a longer time to occur [12,13].

There are other possible reasons for the observed inhibition of normal G-6-PD activity by the partially purified preparation of G-6-PD Hamburg. Absorption of an inhibitor to the variant G-6-PD molecule, whose action is abolished by the process of heating, could be responsible for the inhibition. Removal of 'structural' NADP, which is a regulating factor for the equilibrium between dimers and monomers [14], results in the dissociation of the dimeric enzyme to monomers [15]. To explain the inhibition of normal G-6-PD by G-6-PD Hamburg on this basis, we have to assume that G-6-PD Hamburg has such an effect on the 'structural' NADP of the normal enzyme. This would result in an increased number of catalytic inactive monomers from normal G-6-PD and so to a decreased total catalytic activity. This hypothesis, however, cannot be supported by facts. The non-competetive inhibition of normal G-6-PD activity by the partially purified preparation of G-6-PD Hamburg might be explained by factors destroying the normal G-6-PD molecule. The presence of a proteolytic enzyme specific for G-6-PD activity could be compatible with the reported results. Inhibitors of proteases, however, were not able to prevent the inhibition.

To elucidate the true mechanism of the observed inhibition it is necessary to obtain the variant enzyme G-6-PD Hamburg in a highly purified state. This, however, is not possible by the use of currently available techniques.

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