HUMAN ERYTHROCYTE GLUCOSE 6-PHOSPHATE DEHYDROGENASE. PHYSICAL PROPERTIES.

A.Bonsignore, R.Cancedda, I.Lorenzoni, M.E.Cosulich and A.De Flora

Istituto di Chimica Biologica dell'Università di Genova
Genova, Italy

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Summary

A number of physical properties of human erythrocyte glucose 6-phosphate dehydrogenase have been investigated under different experimental conditions yielding tetramers, (m.w. = 204,800), dimers (m.w. = 101,400) and monomers (m.w. = 51,300), respectively.

Glucose 6-phosphate dehydrogenase (G6PD) from human erythrocytes is an oligomeric protein requiring bound NADP for catalytic activity (1-7). Unequivocal evidence was recently provided that the active enzyme is a mixture of dimers and tetramers (6-8), while the individual monomers appear to be intrinsically inactive. The present investigation deals with some physical parameters of each of the three discrete G6PD species. These properties have been evaluated by appropriately displacing toward the individual forms (9) the two following equilibria:

$$\text{tetramer} \rightleftharpoons \text{dimer} \rightleftharpoons \text{monomer}$$

MATERIALS AND METHODS

Isolation of G6PD from human erythrocytes and assay of activity were as already described (10). Protein concentration was determined according to Lowry et al. (11). N-4-phage, albumin and hemoglobin were determined by absorption at 260, 280 and 413 mμ, respectively. Alcohol dehydrogenase (ADH) and catalase were assayed according to Beers and Sizer (12) and to Bonnichsen and Theorell (13), respectively.

A nearly homogeneous population of tetramers was obtained by suspending 10 I.U. of G6PD in 0.02 M phosphate buffer, pH 7.0, containing 0.05 M $MgSO_4$, 10 μM NADP and 0.2% β-mercaptoethanol (vol/vol). Dissociation to dimers for the experiments of gel chromatography was accomplished with 0.02 M Tris-HCl, pH 8.5, containing 0.1 mM EDTA, 10 μM NADP and 0.2% β-mercaptoethanol. To obtain a comparable extent of dissociation

with the ultracentrifugation experiments both a more alkaline pH (9.0-9.5) and a higher ionic strength (1.0) were required, which however result in a marked inactivation this discrepancy can be explained on the basis of the dissociating effect displayed by Sephadex itself on several association equilibria (14). Monomers were prepared by incubating for 60 min at 30°C the native enzyme with glucose 6-P from 20 to 100 μM in 0.02 M Tris-HCl, pH 8.0, containing EDTA, NADP and β-mercaptoethanol as above. After separating the monomers from residual active G6PD through centrifugation or gel filtration in the presence of G-6-P, they were detected by exploiting their ability to reassemble: reassociation was achieved by incubating 60 min at 37°C the corresponding fractions with 1 mM NADP and 0.2% β-mercaptoethanol, and followed as restoration of catalytic activity.

Sedimentation coefficients of the single molecular species were determined by three procedures: i) centrifugation on linear 5%-20% sucrose gradients (7, 10, 15); ii) centrifugation with a moving partition cell according to Yphantis and Waugh (16, 17) in a Spinco model E ultracentrifuge, as reported previously (8); iii) sedimentation velocity in a Spinco model E ultracentrifuge, using schlieren optics (10).

The Stokes radii of the three forms were evaluated by gel chromatography on Sephadex G-200.

The buoyant density of dimers was estimated by means of the equilibrium density-gradient centrifugation (18), using a 12-mm Kel-F cell in a Spinco model E ultracentrifuge. Centrifugation was at 25°C for 20-24 hours at 44,700 rpm. Banding of protein was evaluated by ultraviolet absorption and the films were scanned by means of a Beckman Analytrol densitometer.

Electrophoresis on polyacrylamide gel in the presence of SDS and β-mercaptoethanol was performed according to Weber and Osborn (19), with the following standard proteins: bovine hemoglobin, albumin (BSA, Sigma), yeast ADH, horse liver ADH and beef liver catalase (Boehringer). 10 μg samples of each protein were routinely used.

RESULTS

Sedimentation coefficients -

The following $s_{20,w}$ values were found for each of the three molecular forms: 4.04 S for the monomers; 5.75 S for the dimers; 9.18 S for the tetramers.

Table I

Determination of the Stokes radii of G6PD tetramers, dimers and monomers according to Ackers (20).

Protein	a (Å) (with ref.no)	K_d	r (Å)	a found (Å)
N-4 phage	-	-	-	-
Catalase	52.2 (21)	0.280	207	-
BSA	35 (22)	0.44	203	-
ADH (liver)	34.3 (23)	0.476	216	-
ADH (yeast)	45.5 (24)	0.356	211	-
Hb	24 (20)	0.610	218	-
tetramers	-	0.297	211	51.5
G6PD dimers	-	0.396	211	40.7
monomers	-	0.512	211	30.7

A K 25 column (Pharmacia) equipped with flow adaptor and containing Sephadex G-200 (89 x 2.5 cm) was used: the gel was previously equilibrated with the same buffer in which the enzyme was suspended. The flow rate was 7.5 ml/hour and fractions of 2.5 ml were collected using upward flow. Standards were N-4 phage (to calculate V_o), crystallin bovine hemoglobin (N.B.C.), bovine serum albumin (Sigma), beef liver catalase, horse liver alcohol dehydrogenase and yeast alcohol dehydrogenase (Boehringer). The Stokes radii of the standard proteins were directly obtained from literature or calculated according to the Stokes-Einstein equation (25):

$$a = \frac{KT}{6\pi\eta D}$$

Symbols a and r refer to the Stokes radii of the various proteins and to the pore size of the gel, respectively. The average value of r was 211 Å. K_d is the "distributi coefficient" of the single molecular species (20) and is defined as follows:

$$K_d = \frac{V_e - V_o}{V_t - V_g - V_o - V_{tu}}$$

where V_e is the effluent peak volume of the proteins used, V_o is the void volume of the column, V_t is the total volume of the gel bed, V_g is the net volume of the gel grains (8.8 ml in our experiments) and V_{tu} is the volume of the outlet tubing.

Stokes radii and diffusion coefficients -

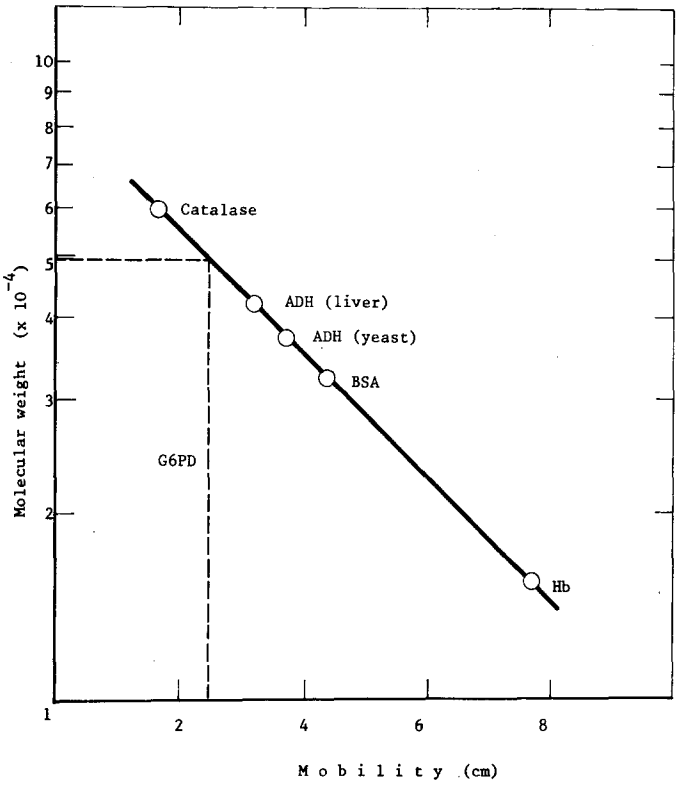
The results of gel filtration on Sephadex G-200 are reported in Table 1. Each of the G6PD species was chromatographed at a time after controlling that the gel pore parameter, r, was unchanged.

Diffusion coefficients (6.95×10^{-7}, 5.26×10^{-7} and 4.15×10^{-7} cm^2/sec for the monomers, dimers and tetramers, respectively) were calculated from the Stokes radii by means of the Stokes-Einstein equation (25).

Molecular weights -

The molecular weights of the three species (Table II) were calculated by substituting values of the Stokes radii and of $s_{20,w}$ in the Svedberg equation, with a partial specific volume of 0.731 ml g^{-1} as determined from the amino acid composition (4).

Electrophoresis of the SDS-treated enzyme on polyacrylamide gel (19) yielded constantly one single band of protein, the mobility of which, as compared with that of standard proteins, is consistent with a m.w. of 49,700 (Fig. 1).



Buoyant density -

The buoyant density of the dimers was found to be 1.344 g ml^{-1}, which indicates a preferential hydration of 0.052 g H_2O/g of protein under the conditions of the experiment. Requirement of highly concentrated CsCl solutions made it impossible to evaluate the buoyant density of the tetramers, which are completely dissociated to

Table II
Physical parameters of human G6PD

Molecular species	Monomer	Dimer	Tetramer
$s_{20,w}$ (10^{-13} sec)	4.04	5.75	9.18
Stokes radius (10^{-8} cm)	30.7	40.7	51.5
$D_{20,w}$ (10^{-7} cm^2/sec)	6.95	5.26	4.15
Molecular weight$_{s,D}$	52,900	101,400	204,800
Molecular weight SDS-enzyme	49,700	-	-
f/f_o	1.27*	1.33	1.328
Buoyant density (g/ml)	-	1.344	-

* For calculation of the frictional ratio of the monomers, a molecular weight of 51,300 daltons was taken, averaging between those obtained by sedimentation-diffusion and by SDS-electrophoresis.

dimers at the ionic strength used (1.8-2.0). Under these particular conditions also attempts at further dissociating dimers to monomers by "autoinactivation" (7) met several difficulties, possibly due to some alterations of the charge in the salt.

The physical characterization of the three species of the enzyme is summarized in Table II.

DISCUSSION

Values of frictional ratios of monomers, dimers and tetramers (Table II) may be interpreted in terms of hydration and (or) of shape (26). Table III reports the extent of asymmetry (i.e., the axial ratio), the volume and the mass of each G6PD species for some arbitrary values of hydration, according to a partial specific volume of 0.731 ml g^{-1} (4). If the shape of a prolate ellipsoid is assumed, it may reasonably be expected, on the basis of the arguments quoted by Yue et al. (27) for

Table III

Relationship between hydration and some molecular parameters of the G6PD species

	δ_1	axial ratio		volume	mass
	g H_2O/g protein	prolate ellipsoid	oblate ellipsoid	ml(10^{-20})	g(10^{-20})
Monomer	0	0.178	6.0	6.23	8.51
	0.2	0.26	4.23	7.93	10.2
	0.6	0.481	2.09	11.34	13.61
	0.79	1.0	1.0	12.87	15.25
Dimer	0	0.163	6.93	12.3	16.8
	0.2	0.204	5.1	15.7	20.2
	0.6	0.364	2.88	22.4	26.95
	0.985	1.0	1.0	28.9	33.4
Tetramer	0	0.162	6.83	24.9	34
	0.2	0.216	5.05	31.6	40.8
	0.6	0.374	2.8	35.9	57.4
	0.98	1.0	1.0	58.1	67.4

yeast G6PD, that the identity of f/f_o values of dimers and of tetramers reflects an axial ratio approaching 1.5 for both species: from this, the hydration may be computed around 0.9 g H_2O per g of protein. Furthermore, the dimer-dimer aggregation may b envisaged as yielding a cyclic or a tetrahedral structure rather than linear oligomer

Dissociation of the native enzyme during the SDS-mercaptoethanol electrophoresis to yield one single band of protein (m.w. = 49,700) is consistent with presence of one polypeptide chain per subunit and with identity, or close similarity, of the individual subunits. In this connection, it is important to remind that also maleyla (6), and reaction with pCMB (De Flora, unpublished), guanidine-HCl (4) and urea (28) lead to one single species of monomer; moreover, hybridization in vitro of human and rat G6PD (29) as well as of different human variants (30) produced one species only showing intermediate features as compared with the native enzymes. Finally the finding of only a tyrosine residue as amino-terminal and of a glycine residue as carboxy-terminal (28) strongly supports the view that G6PD monomers are identical: this problem has a marked genetic relevance and the above results point to a single locus on the X chromosome being involved in the synthesis of G6PD.

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