ACTIVE MOLECULAR UNIT AND NADP CONTENT OF HUMAN GLUCOSE 6-PHOSPHATE DEHYDROGENASE

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SUMMARY

The active enzymatic species of human D-glucose 6-phosphate dehydrogenase has been shown to be a dimer with $S^{\circ}_{20,w}$ of 6.0 - 6.6 S at pH 8.0 and 7 S at pH 6.0. Dialysis equilibrium experiments indicate that the active dimeric enzyme contains one mole of NADP per mole of dimer.

Human glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: NADP oxidoreductase, EC 1.1.1.49) consists of several subunits (1, 2). Genetic and chemical evidence indicates that these subunits are identical and a single X-linked structural gene synthesizes this enzyme in man (3). The molecular weight determined at its isoelectric point (pH 6.0) was 235,000 - 240,000 ($S_{20,w}$ = 9.5 - 10 S) in a buffer of lower ionic strength (I = 0.05) (Ref. 1, 2), and 210,000 ($S_{20,w}$ = 9.0 S) in a buffer of higher ionic strength (I = 0.55) (Ref. 2). The molecular weight was estimated to be about a half ($S_{20,w}$ = 7 S) at its optimal pH (pH 8 - 9) (Ref. 2). Subunit molecular size determined in guanidine hydrochloride was 43,000 $\pm$ 3000 , and 45,000 $\pm$ 4000 (4), and that determined after the treatment with maleic anhydride was reported as 53,000 (2). The subunit molecular size estimated from the N-terminal and C-terminal amino acid content in the protein was approximately 55,000 - 60,000 (3). Considering the fact that dissociation (inactivation) and reassociation (reactivation) of the normal and variant human G6PD pro-

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vided one hybrid enzyme band besides the two original enzyme bands in starch gel electrophoresis at pH 8.5 (5), the enzyme should consist of an even number of subunits. It can be concluded that the enzyme molecules are in a dissociation-association equilibrium. Most of them are in the dimeric form at the optimal pH, while the more highly associated form (mostly tetramer) predominates at the isoelectric point. This work is an attempt to determine the molecular size of the enzyme which is functionally active in the reaction mixture at pH 6.0 and pH 8.0. An amount of NADP bound to the enzyme molecule was also determined.

MATERIALS AND METHODS

Human G6PD was purified and crystallized from normal human blood by the method described previously (1). The size of the active enzyme was estimated by the "active enzyme centrifugation" (AEC) method described by Cohen, Giraud and Messiah (6). Briefly about 25 μl of enzyme solution (0.5 - 5 μg per ml) was layered on the substrate solution which contains D-glucose 6-phosphate, NADP and buffer, through the use of a synthetic boundary cell. Movement of the band of enzyme was followed by the increase in absorbancy at 340 mμ as the NADP was converted to NADPH. The centrifuge runs were made at 60,000 rpm in a Spinco Model E Ultracentrifuge equipped with a photoelectric scanner and an external EAI X-Y recorder.

The sedimentation constant of the G6PD which was not functioning as an enzyme was determined by the moving boundary sedimentation velocity method by measuring the absorbancy at 280 mμ in the absence of D-glucose 6-phosphate in the buffer. The protein concentration used for this determination was about 0.5 mg per ml.

Radioactivity was measured with a Packard scintillation spectrometer. Protein was assayed by Lowry's method, using crystalline bovine serum albumin as standard.

EXPERIMENTS AND RESULTS

Sedimentation constants: The sedimentation constant ($S^{\circ}_{20,w}$) of the enzyme at its isoelectric point (pH 6.0) was 9.5 S, while at its optimal pH of 8.0, the $S^{\circ}_{20,w}$ was measured to be 6.26 S, confirming the previous findings. The sedimentation constant of active enzyme which was measured by the "AEC" method in the presence of D-glucose 6-phosphate and NADP was estimated to be 7 S at pH 6.0, and 6.0 - 6.6 S at pH 8.0 (Table I). The enzyme is most active at pH 8.0, and about 15% as active at pH 6.0. Thus, the dimer is the enzymatically active form.

TABLE I

Sedimentation Constant of Human G6PD in buffers without one of the substrates and in reaction mixtures

Buffer	pH	NADP (M)	G6P	Enzyme Conc. per ml (initial)	$S^{\circ}_{20,w}$
Acetate 0.05 M	6.0	2×10^{-5}	---	Ca 0.5 mg	9.37
	6.0	---*	0.033	Ca 0.5 mg	9.67
Tris-HCl 0.1 M	8.0	2×10^{-5}	---	Ca 0.5 mg	6.26
Acetate 0.05 M + 0.1 M NaCl	6.0	2.5×10^{-4}	2.5×10^{-3}	5 µg	6.85
	6.0	5×10^{-4}	5×10^{-3}	0.5 µg	7.12
Tris-HCl 0.1 M + 0.1 M NaCl	8.0	5×10^{-4}	5×10^{-3}	5 µg	6.64
	8.0	2.5×10^{-4}	2.5×10^{-3}	0.5 µg	6.01
	8.0	5×10^{-4}	5×10^{-3}	0.5 µg	6.14

Experimental conditions ranged from 16.1° to 22.5° in different runs but no more than 0.5° within a run. Rotor speed was 60,000 rev. per min.

Moving boundary: Measurement of 1/2 height of each curve. Log X_i (X_i is value of X at 1/2 height) versus t curves were plotted and slopes calculated by linear least squares analysis.

Active enzyme band sedimentation velocities: Measurement of 1/2 height after subtraction of excess O.D. from previous curve. Then log X_i versus t was plotted and slopes calculated by linear least squares analysis.

*Since all NADP in the dialysis buffer was reduced to NADPH as a result of the enzyme reaction with G6P, 2×10^{-5} M of NADPH existed in the solvent.

Content of NADP bound to the Enzyme: The content of NADP in G6PD after dialysis against buffer containing radioactive NADP, was estimated from the difference in radioactivity of the enzyme solution and that out-

TABLE II

NADP Content in G6PD after Dialysis Equilibrium

NADP conc. in buffer (M)	NADP (μ mole) bound per mg of G6PD: Total 1) (from O.D. increase)	Exchanged 2) (From radioactivity)	Not exchanged 3) during dialysis
2.01×10^{-5}	0.01145	0.0066*	0.00485
1.15×10^{-5}	0.00985	0.00509	0.00476
0.541×10^{-5}	0.0083	0.0038	0.0045

The enzyme was dialysed against 0.05 M phosphate buffer, pH 6.8, containing 10^{-3}M EDTA, 10^{-3} M β-mercaptoethanol and various concentrations of radioactive NADP for 44 hours at 4°C. In order to correct an amount of NADP non-specifically bound to a protein, bovine serum albumen (approximately the same concentration as the enzyme) was also dialysed against the same buffer. The amount of NADP which can be attributed to non-specific binding was subtracted.

1) Total NADP bound to the enzyme was estimated from the difference of the increment of absorbancy at 340mμ between the enzyme solution (inside dialysis bag) and outside solution after adding excess glucose 6-phosphate. The details were described in a previous paper (4).

2) The amount of NADP which was exchanged with external NADP during the dialysis was estimated as the difference of radioactivity between the enzyme solution (inside the dialysis bag) and the outside solution.

   For example: Radioactivity of the enzyme solution = 4980 c.p.m. per 10 μl (subtracted non-specific absorption of NADP to protein using bovine serum albumin as control, 68 c.p.m.) Radioactivity of unbound NADP (outside dialysis bag) = 3433 c.p.m. per 10 μl. Concentration of NADP in buffer (measured from O.D. increase at 340 mμ) = 0.0201 x 10^{-3} M. Enzyme concentration in the dialysis bag (by Lowry's method) = 1.39 mg per ml.

   Amount of bound NADP per mg of the enzyme (μ mole/mg) = $\frac{4980 - 3433}{3433}$ x 0.0201 x 10^{-3} ÷ 1.39 = 0.0066*.

3) Difference between total NADP and exchangeable NADP.

side the dialysis bag, and from the difference in the increment of 340 mμ absorbancy between the two solutions after adding excess D-glucose 6-phosphate to the solutions. Radioactivity measurements gave an amount of NADP which can be exchanged during the dialysis, while absorbancy measurements gave the total amount of bound NADP.

The enzyme was dissolved in 0.05 M phosphate buffer, pH 6.8, containing 10^{-3} M EDTA, 10^{-3} M β-mercaptoethanol and various concentrations of radioactive NADP (NADP-4-H^3, from New England Nuclear Corp.) and was dialysed against the same buffer for 44 hours at 4°C. After dialysis, the radioactivity, concentration of NADP and protein content in both the enzyme solution (inside the dialysis bag) and the solution outside the dialysis bag were assayed. From these values, the amount of NADP bound to the enzyme was calculated (see footnote of Table II).

In Table II, it is shown that about 4.5 μ moles of NADP are tightly bound to one gram of the enzyme and cannot be exchanged during the dialysis. The total amount of NADP in the enzyme is 8.3 - 11.45 μ moles/g (under dialysis equilibrium with 0.541 - 20.1 μ M NADP). These results imply that the active dimeric enzyme molecule (molecular weight about 110,000) contains one molecule of NADP in a reaction mixture which contains 10^{-6} - 10^{-5} M NADP.

DISCUSSION

It has been often questioned, particularly when an enzyme consists of several subunits, as to what is the size of the active enzyme molecule. Human G6PD is mostly tetrameric (part may be hexameric at low ionic strength) at its isoelectric pH (pH 6.0), while it is mostly dimeric at its optimal pH (pH 8.0 - 9.0). This would suggest that the dimeric form might be functionally active. However, it is also possible that the association of the enzyme with NADP and D-glucose 6-phosphate would induce polymerization of the enzyme (formation of tetramer or higher polymerized forms), and thus the functional enzyme molecule might have a greater molecular size. The determination of the sedimentation constant of the active enzyme described in this paper indicates that dimeric enzyme is enzymatically active at pH 6.0 and pH 8.0. It should be mentioned that the results do not exclude the possibility that the tetrameric form (or higher polymerized form) of the enzyme may also be enzymatically active at higher concentrations of enzyme in the reaction mixture. The sedimentation constant was measured at a protein concentration of about 0.5 mg per ml., while the active enzyme centrifugation method can only be applied at very low enzyme concentrations (0.5 - 5 μ g per ml.). Therefore, it is possible that the enzyme molecules are mostly dimeric, even in the absence of substrates at such low protein concentrations of the enzyme.

It has been suggested that G6PD dissociates to catalytically inactive monomers at pH 8.0 when NADPH accumulates as a result of enzymatic activity (7). The present experiments indicated that the enzyme was predominantly

in the tetrameric form (9.67 S) at pH 6.0 when the total NADP was reduced to NADPH in the presence of excess D-glucose 6-phosphate (Table I). This discrepancy could be attributed to the pH of the solvent and the concentration of the enzyme.

In the present work, it was found that the normal human G6PD had about one molecule of NADP per 110,000 g of the enzyme protein under the equilibrium with 2×10^{-6} - 10^{-5} M of NADP at pH 7.0 and 4°C. Similar results have been reported on the purified normal (2, 4) and the Negro variant A^{+} G6PD (4). This implies that the active dimeric enzyme has one molecule of NADP. Part of the bound NADP was so tightly bound to the enzyme that it is exchanged with great difficulty with external NADP during the dialysis, as was shown in the radioactivity assay described in the text. A higher content of NADP in human G6PD (maximum 6 molecules per 200,000 - 240,000 g of enzyme protein), i.e., three binding sites in the dimeric enzyme, has been reported using partially purified enzyme preparations (8, 9, 10). Since the experimental conditions used (purity of enzyme, pH, temperature, etc.) are not identical, it is difficult to decide which is the true number of specific binding sites.

The concentration of G6PD in human red cells is about 5 μg per ml., assuming a homogeneous distribution, and the concentration of NADP is on the order of 10^{-6} - 10^{-5} M. Therefore, it is reasonable to assume that dimeric G6PD with one molecule of NADP is functioning in human red cells.

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