DISTINCTIVE PATTERNS OF NADP BINDING TO DIMERIC AND TETRAMERIC GLUCOSE 6-PHOSPHATE DEHYDROGENASE FROM HUMAN RED CELLS

A.Bonsignore, I.Lorenzoni, R.Cancedda and A.De Flora

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

Received February 24, 1970

SUMMARY

The opposite effects displayed by NADP and $NADPH_2$ on red cell G6PD (i.e., protection and inactivation) were elucidated in terms of a competitive mechanism between the oxidized and the reduced forms of the coenzyme. Dialysis equilibrium experiments showed that the dependence of the NADP binding on the initial NADP concentration followed a simple hyperbola when the enzyme exists as "pure" tetramer and conversely was sigmoid-shaped upon dissociation of the tetramers to dimers. The experimental data were consistent for six and three NADP equivalents bound per mole of tetramer and dimer, respectively.

Glucose 6-phosphate dehydrogenase (G6PD) from human red cells has been shown to undergo a partial autoinactivation, i.e. the active enzyme dissociates to catalytically inactive monomers when $NADPH_2$ accumulates as a product of the G6PD reaction itself, as well as of other NADP-dependent dehydrogenases (1, 2). Such dissociation may be due either to reduction of the "structural" NADP which is known to be essential to maintain the quaternary structure and the catalytic activity of G6PD, or to replacement of the "structural" NADP by external $NADPH_2$ (3): this view received further support by the ultracentrifugal demonstration of an active exchange occurring between the apoenzyme-bound NADP and the free NADP from the medium (4).

In this paper data are presented which provide kinetic evidence for the binding of both oxidized and reduced forms of NADP to the same site(s) of G6PD molecule. Moreover, the binding of labeled NADP has been shown to exhibit different features with the two catalytically active molecular species of the enzyme, namely dimers and tetramers. Thus, formation of the NADP-apoenzyme complex followed a sigmoid-shaped profile with the dimers and a simple hyperbola with the tetramers; the

number of NADP-binding sites was determined to be three for the dimeric form and six for the tetrameric one.

MATERIALS AND METHODS

Human red cell G6PD, type B^+, was purified by following the two procedures described by Cohen and Rosemeyer (5) and by Rattazzi (6), with some modifications (Lorenzoni, Cancedda and De Flora, in preparation), up to specific activities ranging between 40 and 120. G6PD activity was assayed according to Cohen and Rosemeyer (5) and expressed in international units (7). Protein was determined by the method of Lowry et al. (8). Measurements of radioactivity were performed with a Packard model 3214 Tri-Carb liquid scintillation spectrometer. Centrifugation on linear sucrose gradients was performed in a Spinco L-2 centrifuge according to Martin and Ames (9).

The degree of dissociation (expressed by ϕ) of the dimer-tetramer system was calculated as suggested by Cohen and Rosemeyer (10) on the basis of actual values of the sedimentation coefficient. This parameter was also taken to determine weight average molecular weights of the active enzyme, as previously described (4) and therefore actual molar concentrations of G6PD. To obtain homogeneous tetramers at pH values still consistently remote from the ones resulting in isoelectric precipitation (10), we used $MgSO_4$ at high concentrations (above 20 mM), which had preliminarily been shown to shift the equilibrium towards tetramers (Cancedda, Lorenzoni and De Flora, unpublished).

G6PD inactivation by $NADPH_2$ was evaluated as previously reported (1). The binding of NADP to the apoenzyme was investigated by means of the dialysis equilibrium technique, which at variance with the ultracentrifugation procedures (4), makes it possible to minimize the contribution given by the structural coenzyme. Equilibrium dialysis experiments were performed at 31°C with continuous stirring, by immersing a small dialysis bag containing 0.2 ml (usually 7-15 units) of the previously dialyzed enzyme solution in a 3 ml solution of radioactive NADP [(Nicotinamide (carbonyl-C^{14}) adenine dinucleotide, specific activity 55×10^{-6} cpm/umole, purchased from the Radiochemical Centre, Amersham, England]. Eight different concentrations of labeled NADP were routinely employed for each experiment. At different time intervals, 5-10 µl aliquots of both the external

and the enzyme solution were assayed for radioactivity and 2 µl aliquots of the enzyme were assayed for catalytic activity for 30 seconds.

RESULTS AND DISCUSSION

Kinetic evidence for competition between NADP and $NADPH_2$- Inspection of Fig. 1 shows that the protection afforded by NADP against the $NADPH_2$-induced inactivation is due to an increase of the apparent dissociation constant of the $NADPH_2$-G6PD complex, namely to a competitive mechanism between NADP and $NADPH_2$ for the same site(s). Attempts at determining the dissociation constant of the NADP-G6PD complex by evaluating the initial rate of G6PD inactivation with a set of varying amounts of NADP at two $NADPH_2$ concentrations, according to Dixon (11), yielded values of the order of 1-10 µM, namely below the corresponding Michaelis constant for NADP (25µM). This indicates binding of the coenzyme to both "structural" and "catalytic" sites (12).

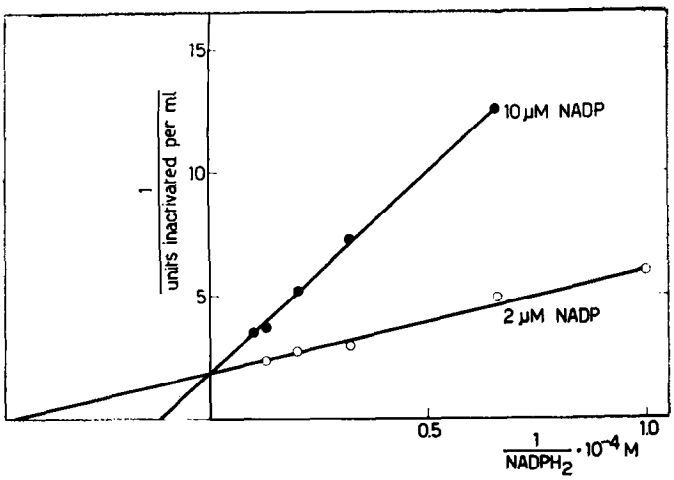

Figure 1: Effect of NADP on the initial rate of G6PD inactivation by $NADPH_2$- 1 ml of the incubation mixtures contained: 40mM glycine - NaOH, pH 8.8; 1.0 units of G6PD (specific activity, 52) previously dialyzed against 1,000 volumes of 40mM glycine - NaOH, pH 8.8; 0.1 M NaCl; NADP and $NADPH_2$ at the concentrations indicated. Controls lacking $NADPH_2$ were incubated in parallel. Incubation at 37°C in a water bath. At zero time and after 26 min (i.e., when the rate of inactivation is still linear under the above conditions), aliquots were assayed for G6PD activity.

Binding of labeled NADP to dimeric G6PD - Different G6PD preparations were subjected to dialysis equilibrium at increasing concentrations of C^{14}-NADP, as

described under "Methods".

In Fig. 2 a typical pattern of C^{14}-NADP incorporation is reported with a G6PD population whose actual sedimentation coefficient ($s_{20,w}$ = 6.74) indicated 70% dimers and 30% tetramers: under these conditions the incorporation of radioactivity versus increasing concentrations of C^{14}-NADP is represented by a sigmoid curve (Fig. 2, curve A). Occurrence of positive interactions between distinct NADP-binding sites is confirmed by the finding of two different dissociation constants, the first one (at low NADP) of the order of 20 μM and the second one (obtained at higher NADP) being 12 μM. Moreover, the actual number of NADP-binding sites extrapolated at infinite NADP was found to be 3.9 (Fig. 2, curve B). Accordingly, the Hill plot gave a value of n (1.23) which is consistent whith a low cooperativity between two different NADP-binding sites on the enzyme protein (Fig. 2, curve C).

Attempts at displacing further the equilibrium between tetramers and dimers in order to investigate the incorporation pattern of the "pure" dimeric form,

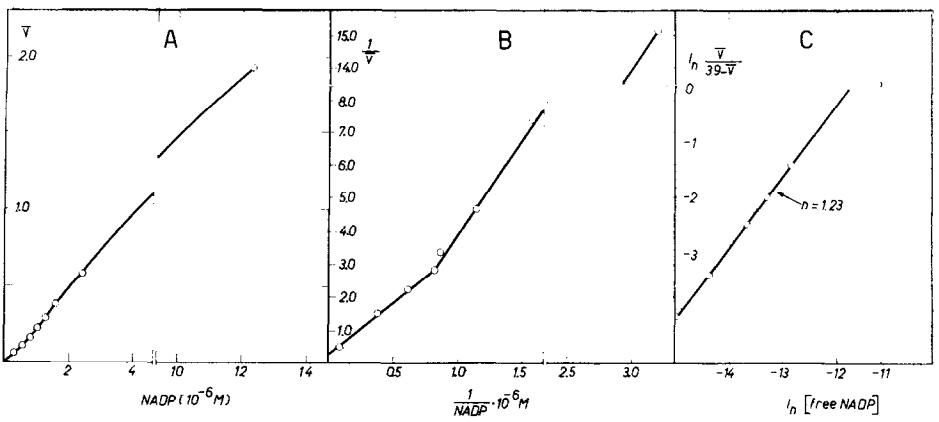

Figure 2: Incorporation of C^{14}-NADP into erythrocyte G6PD -

Sucrose gradient centrifugation of the enzyme (specific activity, 19.5) at the conditions of pH and ionic strength of the experiment, showed an $s_{20,w}$ value of 6.74 S (ϕ = 0.695), corresponding to a weight average molecular weight of 137,000 daltons. G6PD was previously dialyzed against 2,000 volumes (2 changes) of 0.05 M Tris-HCl, pH 7.5, containing 0.1 mM EDTA and β-mercaptoethanol (2μl/ml). Solutions of labeled NADP contained the same buffer with the addition of 0.15 M NaCl. The data obtained were treated to yield the number of NADP equivalents incorporated per mole of enzyme protein ($\bar{\nu}$) versus increasing NADP concentrations (curve A) and also plotted according to Klotz et al. (13) in order to calculate both the dissociation constants of the NADP-G6PD complex and the number of NADP-binding sites per mole of enzyme at infinite NADP concentrations (curve B). Curve C is a typical Hill plot, where n is the interaction coefficient of the NADP-enzyme system.

failed because of the known alkali-lability of the oxidized NADP as well as of susceptibility of G6PD to inactivation at high ionic strength [both parameters, i.e. high pH and high I, were found to result in a marked dissociation of the tetramers to dimers, confirming results reported by Cohen and Rosemeyer (10)] .

Binding of labeled NADP to tetrameric G6PD - At 25 mM $MgSO_4$, i.e. at a concentration resulting in pratically 100% tetramers (see "Methods"), the binding of C^{14}-NADP followed uncomplicated kinetics (Fig. 3), with a dissociation constant of 16 μM and a value of n (1.02), as derived from Hill equation, indicating a single class of NADP-binding sites. From the experiment depicted in Fig. 3, (curve B), one mole of tetrameric G6PD appears to have 6.2 equivalents for combination with NADP.

Distinctive features of NADP incorporation with dimers and tetramers are independent of the known property of Mg^{++} to form complexes with NADP (14): in fact, both the dissociation constant and the number of NADP-binding sites per mole of enzyme were unchanged with a concentration of $MgSO_4$ (40mM) consistently above the one leading to a homogeneous population of tetramers (25mM). Moreover

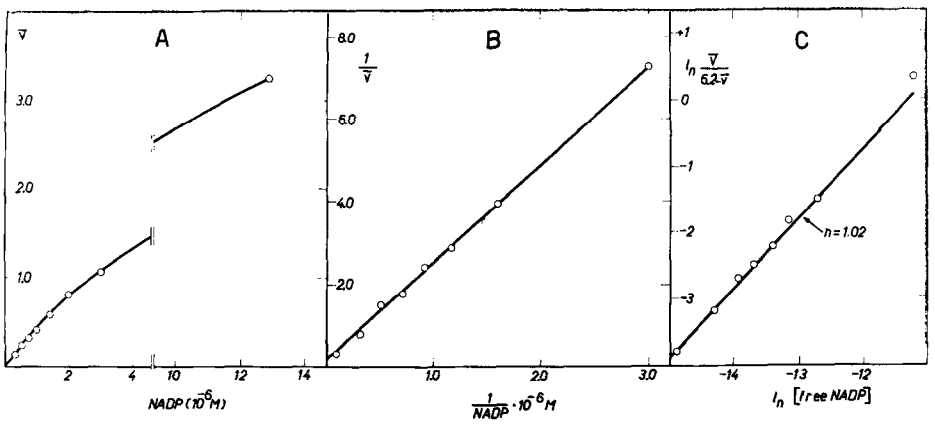

Figure 3: Incorporation of C^{14}-NADP into tetrameric G6PD -

Sucrose gradient centrifugation of the enzyme (specific activity, 62) at the conditions of the experiment, yielded an $s_{20,w}$ value of 8.96 s (ϕ = 0.011), corresponding to a weight average m.w. of 208,800 daltons. G6PD was previously dialyzed against 2,000 volumes (2 changes) of 0.02 M K phosphate buffer, pH 6.5, containing 0.1 mM EDTA and β-mercaptoethanol (2μl/ml). Solutions of labeled NADP contained the same dialysis buffer with the addition of 25 mM $MgSO_4$. For other details, see under Figure 2.

the patterns of incorporation were found to be uniform irrespective of the buffer used (K phosphate, or Tris-HCl) and of the degree of purification of G6PD (4).

Fig. 4 clearly shows that the number of NADP-binding equivalents is a function of the actual quaternary structure of the enzyme and that extrapolation to "pure" dimers indicates a number of 3 sites for combination with NADP. Since both Yoshida (15) and Cohen and Rosemeyer (10) obtained evidence for two moles of NADP tightly bound per mole of tetramer and because of identity, or close similarity, of G6PD monomers according to fingerprinting methods (16), to electrophoretic (10, 17) and ultracentrifugal (10, 18) criteria, it appears fairly reasonable that each subunit has one "catalytic" site, two other sites of "structural" NADP being involved in the assembly of four subunits; accordingly,

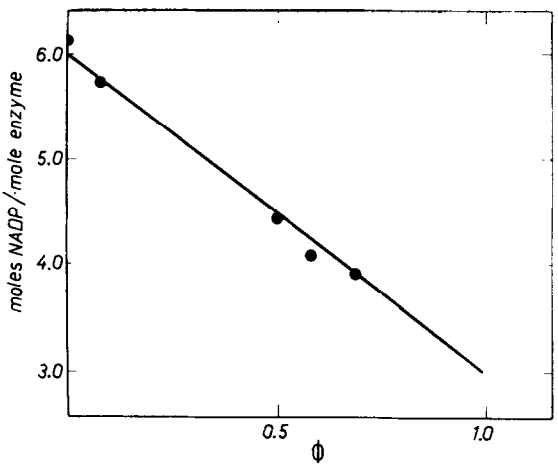

Figure 4: Effect of the quaternary structure of G6PD on NADP binding - Values of ϕ were calculated (10) by means of the sedimentation coefficients of the molecular species involved, namely

$$\phi = \frac{s_t - \bar{s}}{s_t - s_d}$$

where s_t and s_d are the sedimentation coefficients of the tetramers and of the dimers (9.0 S and 5.6 S, respectively, see 10) and $\bar{s}$ is the actual $s_{20,w}$. On the ordinate, the reciprocal values are reported of the intercepts with the ordinate obtained by plotting the dialysis equilibrium data according to Klotz et al. (13). The points are experimental values. The theoretical line fits the model of three and six NADP equivalents bound per mole of dimer and of tetramer, respectively.

each dimer should have two catalytic and one structural NADP-binding sites, which corresponds to the dialysis equilibrium data (Fig. 4).

Reasons accounting for absence of NADP-NADP cooperativity with the tetrameric species are still unknown, although the higher polymeric structure may determine masking of the above interactions which are evident for the dimer. Also, the nature of the two interacting classes of sites still awaits elucidation. Finally it is noteworthy that the patterns of NADP incorporation observed with the dimers are closely similar to the kinetic results obtained by Luzzatto (19) under experimental conditions where a significantly larger proportion of dimers with respect to tetramers is expected.

ACKNOWLEDGEMENTS

This work was supported by the Consiglio Nazionale delle Ricerche, Rome. The expert technical assistance of Mr. F. Giuliano is gratefully acknowledged.

REFERENCES

1. Bonsignore, A., Lorenzoni, I., Cancedda, R., Silengo, L., Dina, D. , and De Flora, A., Ital. J. Biochem., 17, 346 (1968
2. Bonsignore, A., Lorenzoni, I., Cancedda, R., Silengo, L., Dina, D., and De Flora, A., Abstract no. 664, 6th FEBS Meeting, Madrid (1969)
3. De Flora, A., Ital. J. Biochem., 17, 365 (1968)
4. Bonsignore, A., Lorenzoni, I., Cancedda, R., Morelli, A., Giuliano, F., and De Flora, A., Ital. J. Biochem., 18, 439 (1969)
5. Cohen, P., and Rosemeyer, M.A., European J. Biochem., 8, 1 (1969)
6. Rattazzi, M.C., Biochim. Biophys. Acta, 181, 1 (1969)
7. World Health Organization, Techn. Rep. Ser. no. 366 (1967)
8. Lowry, O., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. Biol. Chem., 193, 265 (1951)
9. Martin, R.G., and Ames, B.N., J. Biol. Chem., 236, 1372 (1961)
10. Cohen, P., and Rosemeyer, M.A., European J. Biochem., 8, 8 (1969)
11. Dixon, M., Biochem. J., 55, 170 (1953)
12. Frieden, C., Biochim. Biophys. Acta, 47, 428 (1961)
13. Klotz, I.M., Walker, F.M., and Pivan, R.B., J. Am. Chem. Soc., 68, 1486 (1946)
14. Mangiarotti, G., Garrè, C., Acquarone, M.E., and Silengo, L., Ital. J. Biochem., 15, 67 (1966)
15. Yoshida, A., Biochem. Genetics, 1, 81 (1967)
16. Yoshida, A., in "Hereditary disorders of erythrocyte metabolism", edited by E. Beutler, pp. 146 (1968)
17. Beutler, E., and Collins, Z., Science, 150, 1306 (1965)
18. Yoshida, A., J. Biol. Chem., 241, 4966 (1966)
19. Luzzatto, L., Biochim. Biophys. Acta, 146, 18 (1967)