HUMAN ERYTHROCYTE GLUCOSE 6-PHOSPHATE DEHYDROGENASE. EVIDENCE FOR COMPETITIVE BINDING OF NADP AND NADPH.

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SUMMARY

The tetrameric form of human erythrocyte glucose 6-phosphate dehydrogenase (G6PD) was investigated in respect to interaction of coenzyme at its non-structural sites. 1:N^6-ethenoadenine dinucleotide phosphate (ε-NADP), although displaying a lower catalytic efficiency compared to NADP, showed identical binding patterns, i.e. four moles per tetramer with a K_{diss} of 1.0 μM. Furthermore, spectrofluorometric titrations with NADPH in the absence and in presence of varying NADP concentrations revealed a typically competitive mechanism of binding of NADP (four moles) and NADPH (two moles) at the non-structural sites of the tetramer.

We have previously reported that the tetrameric form of human erythrocyte glucose 6-phosphate dehydrogenase (G6PD) possesses two classes of four NADP-binding sites each (1, 2). One of these is responsible for combination with the tightly bound, or "structural" NADP. In addition, the enzyme has four sites for the loosely bound NADP fraction (K_{diss}=1.0 μM) or, alternatively, two sites for binding of NADPH (K_{diss}=0.1 μM).

These unusual features of interaction with the coenzyme led us to re-examine the binding of NADP(H) at the non-structural sites of tetrameric G6PD. Specifically, synthesis of 1:N^6-ethenoadenine dinucleotide phosphate (ε-NADP) allowed us to confirm the stoichiometry and the dissociation constant previously obtained with NADP (2), although the chemical change in the adenine moiety affects to some extent the efficiency of the coenzyme as substrate of G6PD. Morevover, evidence is presented for a competitive mechanism of binding of NADP and NADPH at the non-structural sites.

MATERIALS AND METHODS

Purification of G6PD (type B) from pooled erythrocytes was accomplished according to previously described procedures (3, 4). Final preparations were checked for homogeneity as described elsewhere (5). Assays of G6PD activity were performed according to Cohen and Rosemeyer (6). Protein was determined

by the procedure of Lowry et al. (7), or from absorbance at 280 nm, assuming that 1 mg/ml of homogeneous G6PD would give a value of 1.21 (8).

Removal of the loosely bound NADP and analysis of the tightly bound coenzyme were carried out as reported previously (1). Titrations of G6PD preparations, preliminarily submitted to either gel filtration on Sephadex G-25 or extensive dialysis (1, 2), with NADP, NADPH and ε-NADP were carried out with an Aminco-Bowman spectrofluorometer in conditions in which the enzyme is mostly tetrameric (1, 2). Sequential additions of 2-5 μl of standardized solutions of each ligand were made to 2 ml of enzyme solution in 0.075 M Na acetate, pH 6.0, containing 0.1 mM EDTA and 0.2% β-mercaptoethanol. Control experiments were run in parallel, in which the same additions of titrating solutions were made to the above buffer or to a solution of bovine serum albumin in this buffer, in order to correct the relevant emission values for changes in the intensity of the exciting radiation. The binding data obtained with each ligand were routinely plotted according to the equation (9):

$$\frac{K_{diss}}{1 - \alpha} = \frac{(L)_{total}}{\alpha} - (E_o)$$

where α, indicating the fractional saturation of the total concentration of ligand (L)-binding sites (E_o), is expressed by the ratio $\Delta fl/\Delta fl_{max}$. The parameter Δfl_{max} on its turn is the limiting value of the fluorescence difference (Δfl) approached at high concentrations of the ligand, L. Therefore, values of α range from zero, when all of the binding sites are vacant, through 1.0, when they are entirely occupied by the ligand.

ε-NADP was prepared essentially according to the procedure developed by Barrio et al. (10) for the synthesis of ε-NAD, by reacting for 10 days 100 micromoles of NADP with chloroacetaldehyde. After checking the complete disappearance of NADP by paper chromatography (11), the mixture was applied to a column (40x2 cm) of DEAE-Sephadex A-25 (Cl^-). Elution was performed with a linear gradient of LiCl from zero to 0.5 M. Fluorescent fractions were pooled and vacuum-concentrated, then precipitation was obtained by addition of 0.5 M $BaBr_2$ and 3 volumes of ethanol. The precipitate was repeatedly washed with ethanol and ether and then dried at 37°C. The UV absorption spectrum of the synthesized NADP derivative was identical to the one reported for ε-NAD (10). Paper chromatography (11) yielded a single fluorescent spot whose R_f was 0.60. Enzymic reduction with yeast G6PD and glucose 6-P led to appearance of a new UV-absorbing band centered at 340 nm. Assuming that the NADP derivative has the same absorption coefficient as ε-NAD, i.e., ε_{264}=10,200 M^{-1} x cm^{-1}, the absorption coefficient of its reduced form turned out to be ε_{340}=6,22 M^{-1} x cm^{-1}. The spectroscopic and analytical data were therefore consistent with identification of this compound with ε-NADP.

RESULTS

Titration with ε-NADP.

The quenching of protein fluorescence recorded on binding of NADP to the non-structural sites is rather low in extent (2). Therefore, we investigated the

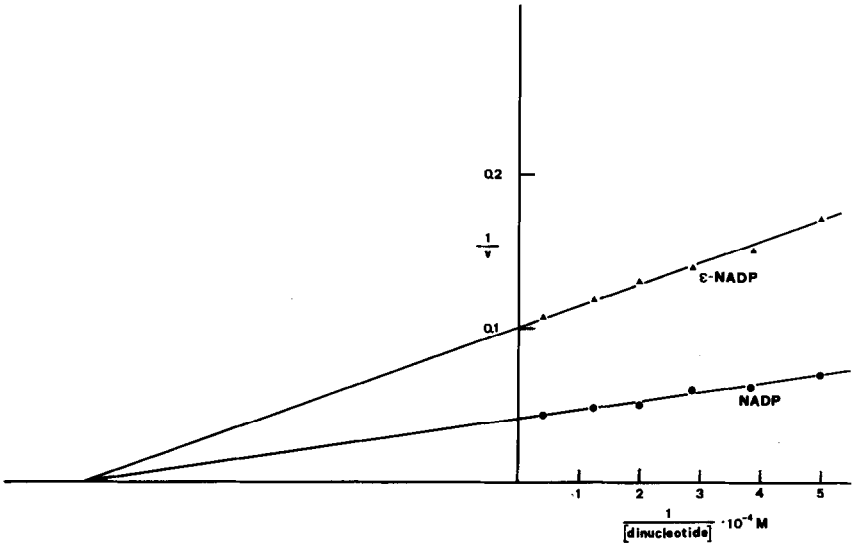

Fig. 1 Catalytic efficiencies of NADP and ε-NADP in the G6PD reaction.

Measurements of initial G6PD activities were made at 25°C in 0.2 M Na acetate, pH 6.0, containing 4 mM glucose 6-P and NADP or ε-NADP as indicated on the abscissa. Reaction was started by addition of G6PD.

patterns of coenzyme interaction at these sites using an analog, ε-NADP, which bears a fluorescent tag on the adenine.

Fig. 1 shows a double reciprocal plot of initial velocities versus varying concentrations of either dinucleotide, NADP or ε-NADP. The latter compound behaves similarly to the natural coenzyme in the hydride transfer reaction from glucose 6-P, yet at a consistently lower rate (the V_{max} being approximately 40% of that observed with NADP). The apparent K_m is identical for NADP and corresponds to 14 μM in the experimental conditions used.

Binding of ε-NADP to the non-structural sites of tetrameric G6PD is indicated by an enhancement of the fluorescence of this coenzyme derivative which amounts to about 3-fold compared with that of ε-NADP alone. This signal was exploited in order to calculate the stoichiometry and the dissociation constant of ε-NADP. Fig. 2 shows a typical experiment in which both parameters were determined by use of the Stinson-Holbrook equation (see Methods). Thus, a stoichiometry of one mole of ε-NADP per each polypeptide chain was found, with a K_{diss} of 1.03 μM. Both figures are superimposable to those observed with the natural coenzyme NADP bearing an unmodified adenine moiety (2).

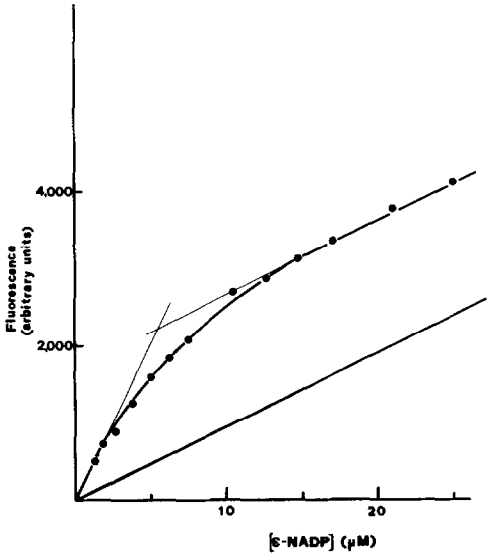

Fig. 2 Binding of ε-NADP to tetrameric G6PD determined by spectrofluorometry.

Stepwise titration with ε-NADP was performed by recording the fluorescence emission at 410 nm with excitation at 300 nm. A 1.3 μM concentration of tetrameric G6PD ($s_{20°,w}$=9.2 S) was used, whose content of tightly bound NADP (1) was 4.15 moles per tetramer. Values were corrected for dilution. The straight line at the bottom refers to the fluorescence signal recorded by addition of ε-NADP to the buffer (see "Methods"). From the Stinson-Holbrook plot (9), a 5.4 μM concentration of ε-NADP-binding sites was calculated, with a dissociation constant, $K_{\varepsilon\text{-NADP}}$, of 1.03 μM.

Competitive binding of NADP and NADPH.

In an attempt to elucidate whether or not a competition exists between NADP and NADPH, the complex between apo-G6PD and tightly bound NADP was titrated with NADPH in the absence and in presence of NADP at different concentrations. The resulting values of the apparent dissociation constant for NADPH (K_{app}) were than plotted versus the actual NADP concentrations, this yielding a straight line of slope K_{NADPH} / K_{NADP} (Fig. 3). Such linearity indicates that the oxidized and reduced forms of NADP do compete for common sites on the tetrameric protein. From the intercept on the ordinate a dissociation constant for NADPH of 0.1 μM was obtained, in very good agreement with the previously reported value (2). Knowing K_{NADPH}, K_{NADP} was also calculated and found to be 1.0 μM: this compares with the macroscopic dissociation constant of the

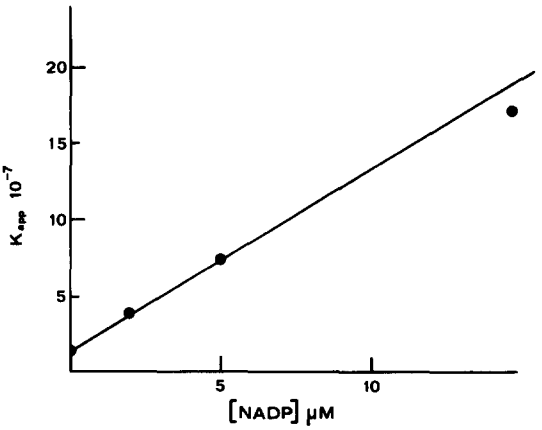

Fig. 3 Competition between NADPH and NADP.

The enzyme contained 3.7 moles of tightly bound NADP and had a sedimentation coefficient of 9.05 S. This preparation of G6PD, preliminarily titrated with NADPH at a 1.65 μM tetramer concentration, was found to bind 3.4 μM NADPH: conversely, its NADP-binding capacity at the same concentration of tetramer, was 6.5 μM.

Stepwise titrations with chromatographically purified NADPH were carried out as described previously (2) and under "Methods", at a 0.72 μM concentration of tetrameric enzyme, without and with purified NADP at the concentrations indicated. Emission values were recorded at 450 nm on excitation at 350 nm.

"titratable" sites for both NADP (2) and ε-NADP. These data indicate that a mutually exclusive combination of NADP and NADPH exists at the non-structural sites of the tetramer, yet with distinctive stoichiometries of four moles of the oxidized coenzyme form and of two moles of the reduced form, respectively.

DISCUSSION

The experiments with ε-NADP suggest that binding of the coenzyme at the titratable sites is unaffected by changes in the adenine moiety which still lower the catalytic efficiency of the dinucleotide. On the other hand, it has been previously shown in our laboratory that adenosine 2',5'-P_2 is the basic fragment of NADP involved in the binding (11) and equilibrium dialysis experiments (umpublished) support this conclusion. Therefore, the structure of adenine per se appears not to be essential to recognition neither to combination of the coenzyme.

A competitive relationship between NADP and NADPH had been reported for human G6PD on the basis of indirect evidences including kinetic data (12) and stabilization-destabilization experiments (13). The data reported in this communication provide a direct demonstration that a reciprocally exclusive binding of the oxidized and the reduced coenzyme forms really exists at the non-structural sites of tetrameric G6PD. Inhibition by NADPH is probably a major regulatory mechanism of G6PD activity and therefore of the pentose phosphate pathway in the erythrocyte (12). With reference to this, the interplay between NADP and NADPH appears to be critical in affecting the in vivo functioning of normal (type B) G6PD as well as of a number of genetic variants of the enzyme. In fact, evidence is available that the structural abnormality following mutation at the G6PD locus often affects in a reciprocal way the affinities for NADP and for NADPH, respectively (14, 15): furthermore, such genetically modified interactions of the enzyme with NADP and NADPH appear to correlate with the clinical severity of G6PD deficiency (14-17). The availability of procedures for rapid purification of human G6PD (4, 18, 19), coupled with use of simple spectrofluorometric titrations, can now provide a way of directly measuring the real dissociation constants of G6PD variants toward NADP and NADPH, thereby overcoming uncertainties inherent to evaluation of kinetic parameters (20, 21) and allowing a better understanding of the physiology of mutant erythrocytes.

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