HUMAN ERYTHROCYTE GLUCOSE 6-PHOSPHATE DEHYDROGENASE. INTERACTION WITH OXIDIZED AND REDUCED COENZYME.

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SUMMARY

The complex between tetrameric glucose 6-phosphate dehydrogenase (G6PD) and four moles of structural NADP can bind four additional NADP equivalents with a K_{diss} of 0.85 μM. Alternatively, this complex shows a maximal binding capacity of two NADPH equivalents, with a corresponding K_{diss} of 0.1 μM. Therefore, a clear discrepancy has emerged from these spectrofluorimetric titrations with either the oxidized or the reduced form of the coenzyme, an "all-of-the-sites reactivity" being observed for NADP and a "half-of-the-sites reactivity" being conversely involved in NADPH binding.

We have recently isolated a complex between the tetrameric apoprotein of human erythrocyte glucose 6-phosphate dehydrogenase (G6PD) and four moles of tightly bound NADP (1). This complex represents the simplest molecular entity of G6PD associated with catalytic activity, since the tightly bound, or structural, coenzyme undergoes reduction to NADPH in presence of the substrate glucose 6-P.

The likely occurrence of additional NADP-binding sites on G6PD, suggested by previous kinetic experiments (2), prompted us to investigate the interactions between the apoG6PD-NADP complex with either NADP or NADPH. The results reported in this paper are consistent with the binding of four additional moles of NADP, while only two equivalents of NADPH can combine with the tetrameric enzyme containing four moles of structural NADP.

MATERIALS AND METHODS

Homogeneous G6PD was prepared from pooled human erythrocytes as previously described (3). Assays of catalytic activity were

carried out as indicated elsewhere (4). Protein was evaluated by the procedure of Lowry et al. (5), using crystalline G6PD from yeast (Boehringer) as standard protein. G6PD concentrations were usually referred to the monomeric species, assuming that 0.052 mg/ml corresponds to 1 μM enzyme, although all experiments were performed in solvent conditions resulting in a homogeneous population of tetramers (1).

Preparation of the apoG6PD-structural NADP complex was performed by dialyzing the G6PD preparations for 24 hrs at 2°C against 200 volumes of 0.075 M Na acetate, pH 6.0, containing 0.1 mM EDTA, 0.2% (vol/vol) β-mercaptoethanol, 5% glycerol (Buffer A) and also 2 mg Norit A per ml. This treatment was followed by a further 6-8 hours dialysis against Buffer A not containing Norit. The content of structural NADP was routinely evaluated as previously reported (1) and found to be constantly four moles dinucleotide per mole tetrameric apoprotein, while no bound NADPH was observed.

Titrations of the apoG6PD-structural NADP complex with either NADP or NADPH were performed with an Aminco-Bowman spectrofluorimeter. In either case binding was monitored by recording the quenching of protein fluorescence (excitation at 280 nm and emission at 350 nm). Binding of NADPH was also investigated by exploring the fluorescence emission of reduced dinucleotide (excitation at 350 nm and emission at 450 nm).

RESULTS

Interaction of the apoG6PD-structural NADP complex with NADP.

Fig. 1 shows the quenching of protein fluorescence which occurs on titrating with increasing amounts of NADP the tetrameric enzyme already containing four moles of tightly bound NADP. Although the decrease in fluorescence is not as strong as those reported to occur on binding of pyridine nucleotides to other dehydrogenases (6), it allows to determine both the stoichiometry of NADP binding and the relevant dissociation constant. Thus, the

intercept between the tangents to the initial and to the final parts of the titration curve corresponds to 2.3 μM NADP. The actual concentration of G6PD, referred to the monomeric species, was 2.4 μM, therefore indicating a stoichiometry of 0.96 moles NADP per mole of monomer. This finding demonstrates that the complex between tetrameric apoprotein and four moles of structural NADP is able to combine with four additional equivalents of NADP. Such stoichiometry was repeatedly observed in a number of similar experiments and was found to be unmodified when the complex was prepared by chromatography on Sephadex G-25, as described previously (1), rather than by extensive dialysis.

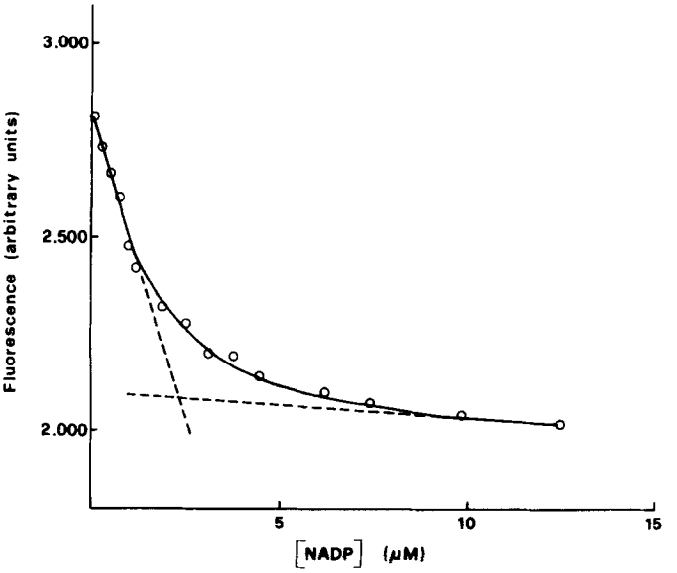

Fig. 1. Decrease in protein fluorescence following addition of NADP to the tetrameric apoprotein-structural NADP complex.-

Titration with NADP was performed at 23°C by sequential additions of a standardized NADP solution to 2.0 ml of a solution of G6PD (2.4 μM) preliminarily dialyzed against Buffer A, as described under Methods. This enzyme solution contained 2.3 μM NADP ("structural NADP") as analyzed according to De Flora et al. (1). Corrections were introduced for volume changes. In a control experiment a solution of bovine serum albumin giving the same fluorescence emission at 350 nm as the G6PD sample was similarly titrated with NADP, thereby providing the actual correction for inner-filter effects determined by the added dinucleotide.

The binding data shown in Fig. 1 were then plotted according to Stinson and Holbrook (7), in order to yield the actual dissociation constant for the titratable NADP. This was calculated to be 0.85 μM and showed similar values (from 0.5 to 1.0 μM) in other experiments.

Interaction of the apoG6PD-structural NADP complex with NADPH. Titrating with NADPH the tetrameric apoprotein associated with the structural NADP results in a binding of the reduced dinucleotide, as shown by, i) quenching of the protein fluorescence, ii) enhancement in the fluorescence emission of NADPH (Fig. 2). Inspection of Fig. 2 reveals that at a G6PD concentration as high as 7.6 μM (referred to the monomeric state), a sharp titration is ob-

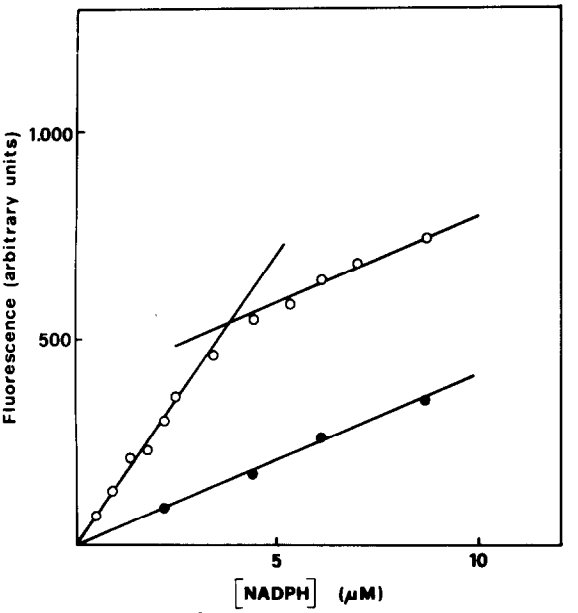

Fig. 2. Enhancement of NADPH fluorescence emission following addition of NADPH to the tetrameric apoprotein-structural NADP complex.-

Titration with NADPH was performed at 23°C by sequential increments of a standardized NADPH solution (the concentration of which had been measured by means of glutathione reductase) to 2.0 ml of a G6PD solution (7.6 μM) preliminarily dialyzed against Buffer A (open circles). The content of the structural NADP of this enzyme solution was 1.03 moles dinucleotide per mole of monomer. Filled circles refer to fluorescence values recorded on addition of NADPH to Buffer A. The observed values of fluorescence emission at 450 nm (excitation at 350 nm) were corrected for volume changes.

tained with an end point of 3.8 μM NADPH. These values correspond to an actual stoichiometry of exactly 2.0 equivalents of NADPH per mole of the complex between tetrameric apoprotein and structural NADP. This "half-of-the-sites reactivity" toward NADPH was confirmed in all titration experiments, also when binding of the reduced dinucleotide was monitored by means of the quenching of protein fluorescence.

Lowering the protein concentration allowed us to obtain titration curves smoother than the one reported in Fig. 2. By plotting the resulting fluorescence values according to Stinson and Holbrook (7), the dissociation constant for NADPH could be determined and found to be 0.1 μM.

DISCUSSION

Our recent investigations on the relationship between apoprotein and coenzyme have been focused on the tetrameric form of human G6PD, due to its peculiar stability and resistance to a number of disaggregating factors (1,4,8). This molecular species of G6PD contains two different aliquots of bound NADP, the first of which escapes removal during gel chromatography and extensive dialysis (1), therefore appearing as an integral part of the structure of the enzyme (four moles dinucleotide per tetramer). The second fraction, still comprising four NADP equivalents (Fig. 1), is apparently bound in a consistently looser way with respect to the structural NADP. The overall stoichiometry of NADP binding is therefore of eight moles NADP per tetramer and this figure is also supported by recent equilibrium dialysis experiments (unpublished data).

On the other hand, the complex between tetrameric apoG6PD and four moles of structural NADP can combine with a maximum of two equivalents of NADPH, as unequivocally shown by the results of spectrofluorimetric titrations (Fig. 2). Assuming a competition

of NADP and of NADPH for the same "titratable" regions of the complex (which is supported by earlier kinetic data (2,9)), these findings are consistent with a clearly distinctive response of these regions to the oxidized and the reduced forms of NADP, respectively. Thus, besides a quite higher affinity for NADPH, a half-of-the-sites reactivity is constantly observed toward NADPH, at variance with an all-of-the-sites reactivity toward NADP.

Although several oligomeric enzymes have been reported to have only half of their sites interacting with a substrate or an active site reagent (see 10), the co-existence of a half-of-the--sites reactivity with the product and of an all-of-the-sites reactivity with the substrate is quite unusual and difficult to be explained at the molecular level. For instance, Janin et al. (11) described a typical reactivity of only half of the sites of Aspartokinase I-Homoserine dehydrogenase I from E.coli K12 toward both NADP and NADPH. Future investigations will attempt to establish the reasons for the apparent half-of-the-sites reactivity of tetrameric G6PD toward NADPH and for the unexpected discrepancy with the patterns of NADP binding.

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