HUMAN ERYTHROCYTE GLUCOSE 6-PHOSPHATE DEHYDROGENASE. CONTENT OF BOUND COENZYME

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SUMMARY

Tetrameric glucose 6-phosphate dehydrogenase (G6PD) purified from human erythrocytes contains four moles of tightly bound NADP and lower and variable amounts of NADPH. This finding represents the basis for differentiating "structural" from "catalytic" sites involved in coenzyme binding.

A typical property of human erythrocyte glucose 6-phosphate dehydrogenase (G6PD) is tight association with NADP to form an apoenzyme-coenzyme complex (1-5). Kinetic evidence has also been presented, however, for interaction of this complex with additional equivalents of NADP (6), to which a "catalytic" role has been attributed as a main function.

The tightly bound, or "structural" NADP is thought to act as a conformation primer for the individual polypeptide chains and is involved, probably through such mechanism, in stabilizing an oligomeric, catalytically active structure (for a review, see 7). The important effects of NADP on both the structure and the activity of human G6PD, as well as the serious uncertanties concerning the content of apoenzyme-bound NADP (1,2,8,9), prompted us to re-examine the stoichiometrical relationship between the structural coenzyme and the apoprotein under accurately defined conditions. The data reported in this paper provide unequivocal evidence for presence of four moles of tightly bound NADP per mole of tetrameric G6PD. In addition, the tetramer can bind variable amounts of NADPH which can however be completely removed without any consequence on both the state of aggregation and the catalytic activity of the enzyme.

MATERIALS AND METHODS

G6PD (type B) was purified from pooled human erythrocytes according to our rapid procedure (10), which is essentially based on the affinity chromatography on NADP-Sepharose columns. Purity of the enzyme was routinely checked by disc gel electrophoresis (11) and by ultracentrifugation on a Spinco Model E analytical ultracentrifuge. Assays of catalytic activity were performed as reported previously (12). Protein was determined according to Lowry et al. (13), using crystalline yeast G6PD (Boehringer) as standard protein. Molar concentrations of G6PD (referred to the monomeric form) were calculated on the basis of actual specific activities of the homogeneous enzyme preparations, which were found to range from 156 to 198 I.U./mg, and assuming a molecular weight of 52,000 for the monomer (14).

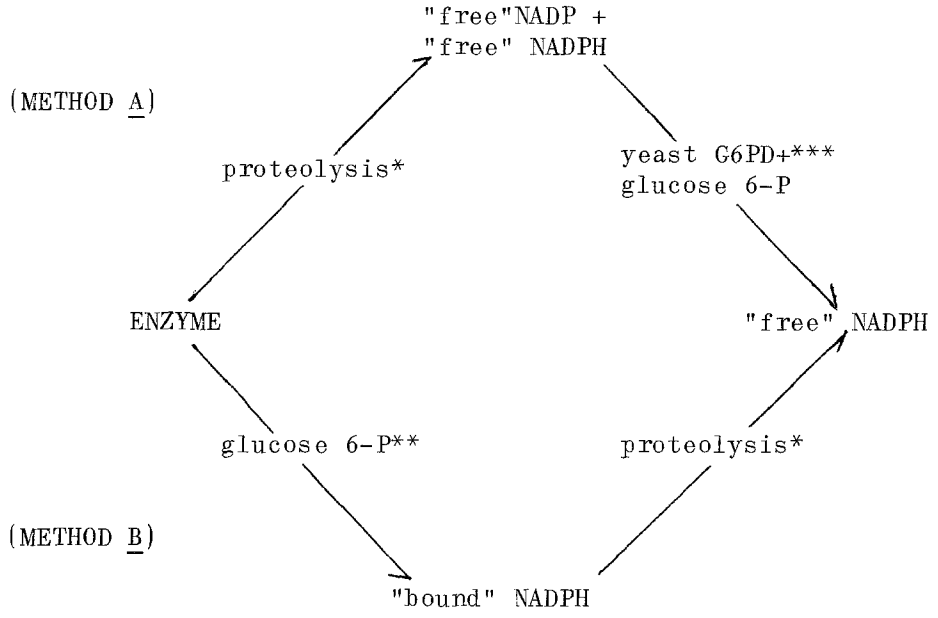
Sedimentation equilibrium experiments were carried out according to the high speed method of Yphantis (15), using Rayleigh interferometric optics and maintaining exactly the same environmental conditions as for spectrofluorimetric analyses.

Fluorescence spectra were determined by means of an Aminco Bowman spectrofluorimeter. Measurements of NADPH were carried out both on the native protein and after its short incubation with 1 mM glucose 6-P, which produces extensive reduction of the apoenzyme-bound NADP (16). The enhanced fluorescence emission of bound NADPH as compared with that of free NADPH (16) prompted us to hydrolyze G6PD in order to determine release of the bound coenzyme from the protein. Two alternative procedures were used, which are shown in Fig. 1. Method A allows to measure in two separate steps both the native NADPH and that formed by reduction of native NADP. Conversely, method B does not discriminate between NADP and NADPH, therefore giving the sum of the two forms of the coenzyme.

RESULTS

Removal of the loosely bound fraction of NADP from G6PD was obtained by submitting the purified enzyme preparations to either of the following treatments: a) dialysis for 24 hours at 2°C

Fig. 1. Methods for analysis of apoprotein-bound NADP and NADPH.



* The incubation mixture (0.55 ml) contained 0.045 M Tris-HCl, pH 9.0, 0.67 mg 2x crystallized trypsin (Worthington) and variable amounts (0.2 to 1.8 mg) of homogeneous G6PD, preliminarily processed through dialysis against Na acetate containing Norit or gel chromatography on Sephadex G-25 (see "Results"). Control experiments were also incubated in parallel, which contained corresponding amounts of NADP and NADPH instead of G6PD. After incubation for 2 hours at 25°C, 0.1 ml aliquots were transferred to spectrofluorimetric cuvettes: the content of "free" NADPH was then determined by recording the intensity of fluorescence emission at 450 nm with constant excitation at 350 nm and using freshly prepared NADPH solutions as standards. The very limited contribution of trypsin to fluorescence was completely eliminated by subtracting the values obtained with proper blanks of trypsin.

** The final concentration of glucose 6-P was 1 mM

*** Completion of the reaction was checked by further additions of 5 µl of yeast G6PD directly into the spectrofluorimetric cuvettes.

against 1,000 volumes of 0.075M Na acetate, pH 6.0, containing 1 mM EDTA, 0.2% (vol/vol) β-mercaptoethanol, 5% glycerol (Buffer A) and to which 2 mg Norit A per ml were added, b) chromatography on Sephadex G-25 columns (40 x 0.7 cm), using Buffer A as eluant. Analysis of the eluate from Sephadex G-25 revealed two clearly resolved peaks of u.v.-absorbing material, the first of which was coincident with the profile of catalytic activity and showed appreciable amounts of NADP (detected spectrofluorimetrically after addition of glucose 6-P, as described in the legend to Fig. 1). The low molecular weight material corresponding to the second peak was identified with free NADP on the basis of its absorption spectrum and of its rapid conversion to NADPH in presence of both yeast G6PD and glucose 6-P.

The molecular weight of the enzyme eluted from Sephadex G-25 or dialyzed against Buffer A containing Norit, was determined by sedimentation equilibrium experiments and was constantly found to be 212,000±3,000, therefore indicating a homogeneous tetrameric population, as expected on the basis of low pH and ionic strength of the buffer (5,7). This finding accounts for the high stability of G6PD activity throughout gel chromatography and extensive dialysis.

The enzyme processed through gel filtration contains in most cases a fluorophore which can be identified with a reduced pyridine nucleotide. This was shown by two facts: a) the fluorescence excitation spectrum with constant emission at 450 nm revealed two activation peaks at 350 nm and 290 nm; b) on exciting the protein at 350 nm, a significant though variable peak of fluorescence emission was recorded at 440 nm. This peak was found to disappear progressively when the tryptic hydrolysates of G6PD (Fig. 1, method A) were incubated with either glutathione reductase (Boehringer) and GSSG, or glutamate dehydrogenase (Boehringer), α-ketoglutarate and NH_3. Although these systems are active with both NADH and NADPH, the reduced dinucleotide bound to G6PD can be most probably identified with NADPH, in view of the high coenzyme specificity of the human erythrocyte enzyme (2,7,16).

Table 1. NADP and NADPH content of Glucose 6-phosphate dehydrogenase from human erythrocytes.

Experiment number [a]	Treatment	Method employed [b]	G6PD concentration [c] (μM)	NADP+NADPH / monomer	NADPH / monomer	NADP / monomer
1a	Sephadex	A	12	-	0.10	0.92
1b	Sephadex	B	12	1.10	-	-
2	Sephadex	A	8	-	0.36	0.95
3	Sephadex	A	18	-	0.46	1.07
4a	Sephadex	A	19	-	0.29	1.03
4b	Sephadex	B	19	1.25	-	-
5a	Sephadex	A	7.5	-	0.39	1.1
5b	Sephadex	B	7.5	1.46	-	-
6a	Dialysis	A	12.5	-	0.01	1.04
6b	Dialysis	B	12.5	1.09	-	-
7	Dialysis	A	62	-	undetectable	1.0
8	Dialysis	A	62	-	undetectable	0.97

a) The experiments indicated under the same number refer to the same enzyme preparation processed through both Methods, A and B

b) see Fig. 1

c) referred to the monomeric species (see "Materials and Methods")

At variance with these observations, no NADPH was found to be combined with G6PD when the same enzyme preparations were submitted to dialysis against Buffer A containing Norit. This difference indicates that the latter treatment is more effective than gel chromatography in removing NADPH from G6PD and that NADPH itself is not a basic structural component of the enzyme.

As far as the oxidized NADP is concerned, this was quantitated according to Method A (as shown in Fig. 1). Table I summarizes the content of apoprotein-bound NADP, both in oxidized and reduced forms. With some preparations, both Methods A and B were used, in order to provide an internal control for presence or absence of NADPH. The results, extrapolated to the monomeric species, demonstrate that one polypeptide chain is associated with one equivalent of structural NADP, irrespective of the treatment used to preliminarily remove the loosely bound NADP. On the other hand, the content of bound NADPH ranged between 0.10 to 0.46 moles dinucleotide per mole of monomer in the enzyme chromatographed on Sephadex G-25, and became undetectable in extensively dialyzed enzyme preparations.

DISCUSSION

The finding of four moles of structural NADP per mole of tetramer (Table I) is higher than those reported previously (2,5,8, 9). We believe however that this figure corresponds to the real content of tightly bound NADP, since, 1) it was highly reproducible, whichever procedure was used to remove the loosely bound NADP, 2) our determinations were carried out in carefully defined experimental conditions as regards the type of G6PD population (homogeneously tetrameric) and its processing prior to spectrofluorimetric analyses, 3) these measurements were completely unaffected by pitfalls inherent to different fluorescence or absorbance intensities of bound and unbound NADPH, because of preliminary resolution of the coenzyme as a result of extensive digestion of the protein.

Presence of NADPH associated to human G6PD had previously been reported on the basis of spectrofluorimetric experiments and more recently postulated to be of physiological significance on the basis of thermostability studies (17). The results presented in this paper, however, show that NADPH is not essential in creating the basic structural requirement for display of catalytic activity, which is on the contrary satisfied by the tightly bound NADP. Our findings demonstrate that NADPH is more tightly bound to the tetrameric apoprotein than the "catalytic" NADP, yet not as tightly as the "structural" NADP. Moreover, they indicate that binding of this fraction of NADPH occurs at sites which are different from those occupied by the structural NADP (Table I). Experiments are in progress in our laboratory in order to establish whether such NADPH-binding sites are specific for the reduced form of this dinucleotide, or rather they are identical with the "catalytic" sites for NADP (in which case, their affinity for NADPH would be consistently higher than for NADP).

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