Biochimica et Biophysica Acta, 526 (1978) 318—327

BBA 68567

HYPERANODIC FORMS OF HUMAN GLUCOSE-6-PHOSPHATE DEHYDROGENASE

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(Received February 24th, 1978)

Summary

Pure glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49) is transformed into 'hyperanodic forms' when incubated at acidic pH and in the presence of $NADP^+$ with excess of glucose-6-phosphate or with some '$NADP^+$ modifying proteins' purified from the same cells.

The enzyme hyperanodic forms exhibit low isoelectric point, altered kinetic properties and high lability to heat, urea, and proteolysis. Differences between hyperanodic and native forms of glucose-6-phosphate dehydrogenase are also noted by microcomplement fixation analysis, ultraviolet absorbance difference spectrum and fluorescence emission spectrum.

Drastic denaturation of the enzyme by urea and acid treatment did not suppress the difference of isoelectric point between native and hyperanodic forms of glucose-6-phosphate dehydrogenase.

From our data we suggest that the conversion into hyperanodic forms could be due to the covalent binding on the enzyme of a degradation product of the pyridine nucleotide coenzyme. This modification could constitute a physiological transient step toward the definitive degradation of the enzyme.

We have previously shown that, once synthesized, glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49) underwent different types of posttranslational alterations [1], depending on the average age of the enzyme molecules and on the nature of the cells synthesizing these molecules [1,2]. Two types of modification have been distinguished: (1) a partial proteolysis of the C-terminal end of glucose-6-phosphate

dehydrogenase, occurring especially with maturation and aging of the erythroid cells [3]; this modification does not modify either activity of kinetics of the enzyme. (2) The transformation into the so-called 'hyperanodic forms' of glucose-6-phosphate dehydrogenase, associated with some changes of activity, kinetics and stability of the enzyme [2—4]. These forms exist in vivo in the lymphocytes and some leukemic cells [2,5] and appear in vitro during the purification of granulocyte glucose-6-phosphate dehydrogenase [1,3,4,6]. The aim of this work is to describe the various circumstances of appearance of these hyperanodic forms, their properties and their chemical nature.

Materials and Methods

Materials. Special chemicals and enzymes were obtained from Sigma Chemical Co. or Boehringer Mannheim. Common chemicals were standard reagent grade. Ion exchangers were supplied by Pharmacia; ampholines and agarose-acrylamide beads (Ultrogel) by LKB. The apparatus and the plates for polyacrylamide gradient gel electrophoresis were from Universal Scientific Limited (London).

Methods. Assays and enzyme purification were performed as described previously [4,6]. In order to purify granulocyte glucose-6-phosphate dehydrogenase with a focusing pattern identical with that of the enzyme from freshly isolated leukocyte extracts, the standard purification procedure [6] was modified by adding a preliminary step of CM-Sephadex chromatography [3].

Isoelectrofocusing [1], kinetic studies [1,4], double immunodiffusion [7], electroimmunodiffusion [8] and microcomplement fixation analysis [9,10] were performed according to the methods described in previous papers. We used, for the immunologic studies, antisera raised in rabbits against either red cell enzyme or native forms [3—6] of leucocyte glucose-6-phosphate dehydrogenase.

The molecular weight of the dissociated subunits was determined after dissociation by sodium dodecyl sulfate (SDS) and electrophoresis in SDS-polyacrylamide gel [11]. The molecular weight of the whole molecule was calculated by gel-filtration at various pH on Ultrogel AcA-34 or by electrophoresis in polyacrylamide gradient gels at pH 8 or 8.8. Human hemoglobin, catalase, aldolase and serum albumin were used as standards.

Glucose-6-phosphate dehydrogenase apoenzyme was prepared by precipitating pure enzyme three-times in a 70% saturated $(NH_4)_2SO_4$ solution (pH 8 with solid Tris), then dialyzing it for 48—72 h against 500 vols. 50 mM Tris-HCl buffer (pH 8)/2 mM β-mercaptoethanol/1 mM EDTA/5% (v/v) glycerol/2 mg/ml Norit. The dialysis buffer was changed at least 5 times. The presence of tightly bound $NADP^+$ or NADPH was checked by fluorometry, according to De Flora et al. [12] after hydrolysis of the enzyme sample by papain [2] (ratio papain : glucose-6-phosphate dehydrogenase = 1 : 50, w/w) for 2 h at 50°C.

Unless otherwise noted, the experiments of in vitro incubation of the enzyme were performed as described in ref. 2, for about 18 h at 37°C in 50 mM sodium phosphate buffer (pH 6.3—6.4)/0.1 mM β-mercaptoethanol/0.1 mM EDTA/2 mM diisopropylfluorophosphate/5 mM ϵ-aminocaproic acid/1 mg/ml albumin/0.02 mM $NADP^+$.

Stability to proteolysis of pure enzyme preparations was achieved using trypsin and pronase (ratio proteolytic enzyme : total proteins ≈ 1 : 100, w/w). The enzymes were incubated in a 50 mM phosphate buffer (pH 8 for trypsin and pH 7.4 for pronase)/1 mM EDTA/1 mM β-mercaptoethanol/0.2 mM $NADP^+$/1.25 mg/ml bovine serum albumin. Proteolysis was stopped after different incubation times by adding 2 mM diisopropylfluorophosphate (for trypsin) and cooling at 0°C; then residual enzyme activity was measured.

Ultraviolet absorption difference spectra were performed using an Acta III Beckman spectrophotometer and fluorescence emission spectra of the enzymes excited at 290 nm using an Aminco Bowman fluorometer.

Results

Incubation with excess of glucose-6-phosphate at acidic pH (Fig. 1).

Incubation of pure glucose-6-phosphate dehydrogenase with excess of glucose-6-phosphate (i.e. a ratio of glucose-6-phosphate:$NADP^+$ > 2) caused the total transformation of the native enzyme (p*I* 6.76) into hyperanodic forms (p*I* 6.21—6.3). At pH 6.4 and 37°C this transformation began after 6 h of incubation, and was complete after 12—18 h. As previously noted with another system [2], the transition native band a → hyperanodic forms was drastically pH dependent; it took place rapidly at acidic pH (below 6.4), slowly at neutral pH (about 7.0) and not at all above pH 8. There was no means of reversing the

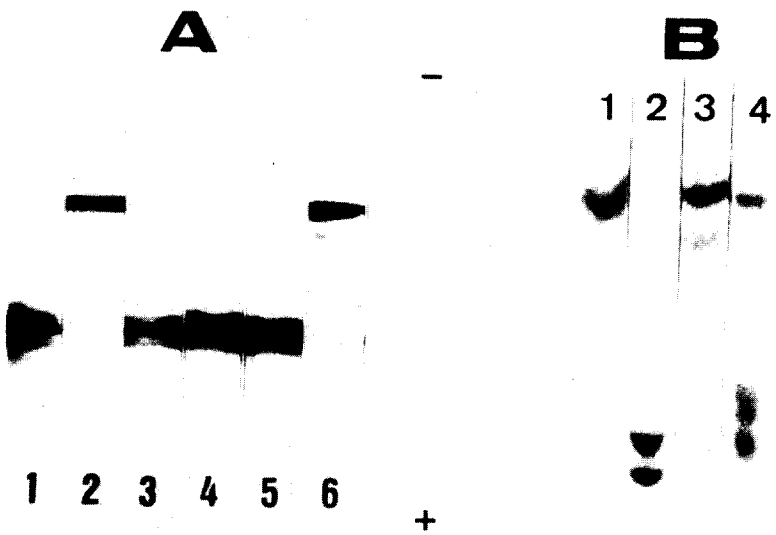

Fig. 1. Isoelectrofocusing pattern of native and hyperanodic forms of glucose-6-phosphate dehydrogenase. (A) Undissociated enzyme, purified from human leukocytes. Staining for enzyme activity. 1 and 3, enzyme treated with excess of glucose-6-phosphate at pH 6.4; 2, untreated native enzyme; 4 and 5, enzyme treated with the $NADP^+$ modifying proteins; 6, native enzyme incubated under the same conditions as in 1 and 3, but without glucose-6-phosphate. (B) Isoelectrofocusing in 8 M urea of pure leukocyte enzymes dissociated by urea. Staining by Coomassie Blue [3,4]. 1 and 3; untreated native enzyme; 2, enzyme treated with glucose-6-phosphate; 4, enzyme purified as a mixture of native and hyperanodic forms [4] because of the absence of the first step of CM-Sephadex chromatography, batchwise. In all cases focusing was performed in 5% (w/v) polyacrylamide gel containing 2% (v/v) ampholines of pH range 3—9.5. For further conditions see Materials and Methods.

glucose-6-phosphate-dependent transformation into hyperanodic forms: especially, the difference of isoelectric point between native enzyme and hyperanodic forms persisted after dissociation and focusing in 8 M urea of either untreated preparations (Fig. 1) or enzyme previously dialysed for 48 h against 0.5% formic acid.

The glucose-6-phosphate-dependent modification of glucose-6-phosphate dehydrogenase was totally inhibited by an NADPH-consuming system, namely a mixture of oxidized glutathione (3 mM) and glutathione reductase (10 I.U./ml).

Finally, the incubation of apoenzyme with glucose-6-phosphate, but without $NADP^+$, did not cause transformation into hyperanodic forms.

Purification of a macromolecular fraction able to induce the conversion of glucose-6-phosphate dehydrogenase into its hyperanodic forms. The fraction not bound on CM-Sephadex (in 10 mM phosphate, pH 6) at the time of the first step of leukocyte glucose-6-phosphate dehydrogenase purification [3], was first fractionated by ammonium sulphate, then further purified by DEAE-Sephadex chromatography using exactly the same conditions as for glucose-6-phosphate dehydrogenase [6]. The preparation obtained was not homogeneous as judged by polyacrylamide gel electrophoresis. By contrast with the 'FX-$NADP^+$-binding protein' described by Morelli et al. [13] the active products did not bind to 2′5′ ADP-Sepharose [13,14]; upon gel filtration in Sephadex G-50 it was found in the void volume.

Interaction between glucose-6-phosphate dehydrogenase and the active molecular fraction. Incubation in the usual way of pure glucose-6-phosphate dehydrogenase with 10 μg of the fraction described above caused the total transformation into 'hyperanodic forms' whose isoelectric pattern was indistinguishable of that of the glucose-6-phosphate-treated enzyme. The pH dependence of this reaction was similar to that described for the glucose-6-phosphate-dependent reaction. Heating the 'active fraction' at 56°C for 15 min (or boiling for a few seconds) inactivated its ability to convert native enzyme into hyperanodic forms. The transformation into hyperanodic forms under the influence of this fraction was not blocked by the mixture oxidized glutathione/glutathione reductase used in the glucose-6-phosphate-dependent system. The activity of the macromolecular fraction persisted when it was placed inside a dialysis bag, glucose-6-phosphate dehydrogenase being deposited in the outside buffer. By contrast in the absence of $NADP^+$, apo-glucose-6-phosphate dehydrogenase was insensitive to the action of this fraction.

Interaction between $NADP^+$ and the 'active macromolecular fraction'. As mentioned before, $NADP^+$ was indispensable to the action of this active fraction. High $NADP^+$ concentration (>0.5 mM), however, inhibited the conversion into hyperanodic forms. The 'active fraction' incubated at 37°C with 0.2 mM $NADP^+$ in phosphate buffer (pH 6.4) induced a progressive consumption of the coenzyme; after a 18 h incubation only 1 : 10 to 1 : 3 of the initial $NADP^+$ could be assayed by its reduction into NADPH. The nature of the modification of $NADP^+$ is as yet unknown; the product of this reaction was not fluorescent.

These interactions with $NADP^+$ prompted us to designate the active macromolecular fraction by the general term of '$NADP^+$ modifying proteins' and this designation will be used from now on in this paper.

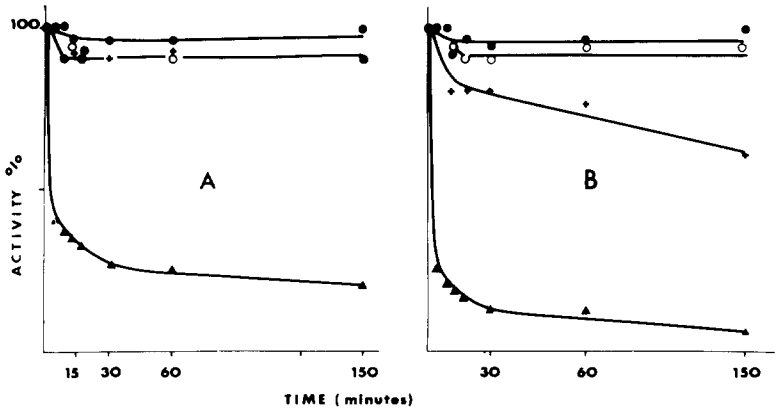

Fig. 2. Stability to proteolysis of native and hyperanodic forms of platelet enzyme. A, trypsin hydrolysis; B, pronase hydrolysis. Native enzyme was incubated with or without glucose-6-phosphate as described in Materials and Methods. The focusing pattern of the glucose-6-phosphate-treated and untreated enzyme was verified, and both preparations were extensively dialysed together against a 50 mM Tris-HCl buffer (pH 8) containing 0.02 mM $NADP^+$, 1 mM EDTA and 1 mM β-mercaptoethanol. The digestion experiment was performed by incubating at 37°C 1 I.U. enzyme in 1 ml of a 50 mM sodium phosphate buffer (pH 8 for trypsin and 7.4 for pronase) containing 1 mM EDTA, 1 mM β-mercaptoethanol, 0.2 mM $NADP^+$, 1.25 mg/ml bovine albumin and 12 μg trypsin (in A) or pronase (in B). In the control experiments the enzymes were incubated under the same conditions, but without protease. ○———○, native enzyme incubated without protease; ●———●, hyperanodic forms incubated without protease; +———+, native enzyme + protease; ▲———▲, hyperanodic forms + protease.

Kinetics and stability properties of the hyperanodic forms. All the properties of the glucose-6-phosphate dehydrogenase hyperanodic forms were similar whether they had been produced by incubation of leukocyte crude extracts, or by the treatment at acidic pH of pure native forms by glucose-6-phosphate or $NADP^+$ modifying proteins.

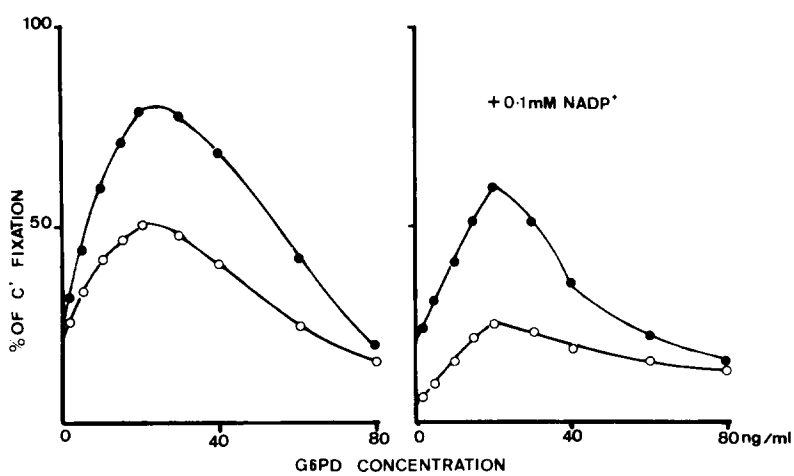

Fig. 3. Microcomplement fixation analysis of native and hyperanodic forms of pure leukocyte enzyme. ○———○, native enzyme; ●———●, hyperanodic forms obtained as described in the legend of Fig. 2, then dialysed against the buffer used for the complement fixation (C′ fixation) [9]. Left, microcomplement fixation analysis was performed in the presence of 0.1 mM $NADP^+$. Anti-leukocyte glucose-6-phosphate-dehydrogenase (G6PD) was raised in rabbits against native enzyme. In these experiments it was diluted 7200-fold.

The kinetics of hyperanodic forms have been previously reported [4].

In addition, hyperanodic forms were highly sensitive to heat [1,4], to urea, and, as shown in Fig. 2, to proteolytic degradation.

Immunologic properties. Native and hyperanodic forms of glucose-6-phosphate dehydrogenase were immunologically identical when studied by double immunodiffusion and reacted identically with various lots of anti-glucose-6-phosphate dehydrogenase antiserum when studied by electroimmunodiffusion [8]. Clear differences, however, could be proven by microcomplement fixation analysis (Fig. 3). The maximum complement fixation was obtained for the same antigen concentration, but the maximum amount of complement fixed was far higher for the hyperanodic forms than for native glucose-6-phosphate dehydrogenase. $NADP^+$ (at the final concentration of 0.1 mM) decreased in a parallel fashion the complement fixation for both native and hyperanodic forms.

Quaternary structure

The pH-dependent equilibrium between the dimeric and tetrameric forms [15] was not modified in the hyperanodic forms with respect to native enzyme. In polyacrylamide gradient electrophoresis at pH 8 (in 10 mM Tris-HCl buffer (pH 8)/0.02 mM $NADP^+$/1 mM EDTA/1 mM β-mercaptoethanol) dimers predominated at low enzyme concentration (5 μg/ml; staining for enzyme activity) while tetramers predominated at a higher enzyme concentra-

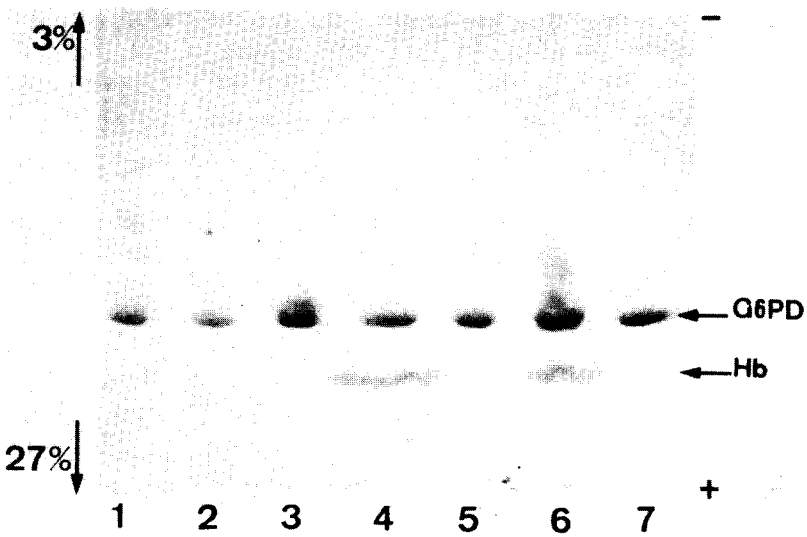

Fig. 4. Electrophoresis in polyacrylamide gradient gel, staining for enzyme activity. Gradient was from 3 to 27% acrylamide (w/v). The plates were preequilibrated with the electrophoresis buffer: 20 mM Tris/glycine (pH 8.8) containing 0.1 mM β-mercaptoethanol, 0.02 mM $NADP^+$ and 1 mM EDTA. The electrophoresis ran for 18 h at 2°C with 30 mA/plate (10 × 9 × 3 mm). Total enzyme activity of each enzyme sample was approx. 10 mI.U. 1, pure leukocyte enzyme, native forms; 2, pure red cell enzyme; 3, leukocyte crude extract; 4 and 6, crude hemolysate; 5, pure leukocyte enzyme hyperanodic forms; 7, pure platelet enzyme, hyperanodic forms. The hyperanodic forms were obtained in the glucose-6-phosphate-dependent system in 5 and in the protein-dependent system in 7. G6PD, glucose-6-phosphate dehydrogenase; Hb, hemoglobin.

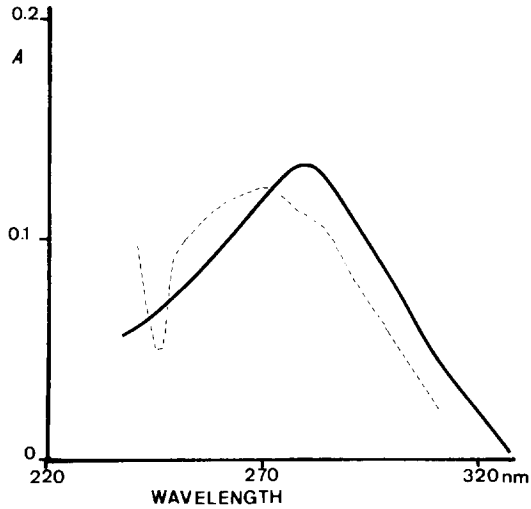

Fig. 5. Ultraviolet absorbance difference spectrum of hyperanodic forms with native enzymes as reference. Native enzyme and hyperanodic forms were obtained as described in the legend of Fig. 2, then both preparations were treated, as reported in Materials and Methods, in order to prepare the apoenzymes. Tightly bound $NADP^+$ or NADPH were undetectable in either enzyme (see Materials and Methods). Protein concentration was 0.44 mg/ml and optical length was 1 cm. ———, experimental spectrum; - - - - -, expected spectrum for an enzyme binding 1 modified NADPH molecule per protomer (mol. wt. = 56 000). This theoretical curve was plotted from the observed spectrum of $NADP^+$ incubated for 18 h at 37°C and pH 6.4 in the presence of excess glucose-6-phosphate and minute amounts of glucose-6-phosphate dehydrogenase.

tion (1 mg/ml; staining for proteins). In 20 mM Tris/glycine buffer (pH 8.8) dimers were the only form found. Their molecular weight, similar for pure enzyme or homogenate enzymes, was 110 000 ± 1800 (Fig. 4). Molecular weight of the SDS dissociated subunits was also identical for native and hyperanodic pure enzymes.

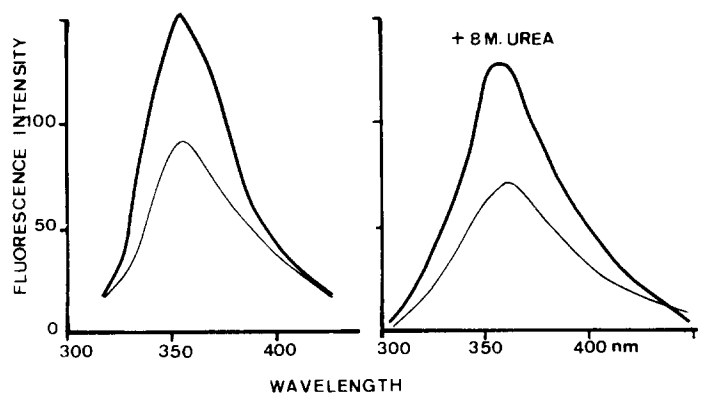

Fig. 6. Fluorescence emission spectra of native (———) and hyperanodic forms (———). Excitation wavelength, 290 nm. The apoenzymes were obtained as described in the legend of Fig. 5, and the spectra were studied in the buffer used to prepare these apoenzymes. Enzyme concentration, 0.2 mg/ml. Right, the enzyme preparations were dialysed for 18 h against the buffer containing 8 M urea before studying the spectra. The addition of 0.1 mM $NADP^+$ or NADPH to the enzyme preparations did not change their spectra.

Spectral properties of the apoenzymes

The ultraviolet absorption difference spectrum of hyperanodic forms with native enzyme as reference was a typical addition spectrum, with a maximum of absorption at 278 nm (Fig. 5). ϵ^{1cm}_{280nm} of native apoenzyme was about 1.0 while that of the hyperanodic forms was 1.26.

The fluorescence emission spectra of both enzymes excited at 290 nm showed, for the modified enzyme, an important increase of the main fluorescence peak at 350 nm (Fig. 6).

This increase in intensity persisted when the spectra were studied with enzymes dissociated in 8 M urea. Proteolysis of both enzymes by papain, however, led to the disappearance of this difference in fluorescence intensity.

Discussion

The results reported above establish clearly that:

(1) $NADP^+$ is indispensable to the transformation of glucose-6-phosphate dehydrogenase into hyperanodic forms in both systems studied in this paper; apoenzyme is totally insensitive.

(2) The glucose-6-phosphate-dependent reaction involves the transformation of $NADP^+$ into NADPH, and is inhibited by an NADPH-consuming system.

(3) By contrast, NADPH is not an intermediate of the reaction induced by the $NADP^+$ modifying proteins. Another degradation product of $NADP^+$, nevertheless, is most likely implicated in this reaction since, provided that $NADP^+$ is present in the medium, these proteins are active on glucose-6-phosphate dehydrogenase through a dialysis membrane.

(4) Hyperanodic forms are due to an irreversible, probably covalent, modification of the enzyme subunits. The difference of isoelectric point between native enzyme and hyperanodic forms (about 0.8 pH unit in urea) indicates that these latter forms have several additional negative charges (or lost positive charges) [16]. Since no intermediate forms could be found between native and hyperanodic forms, these negative charges are acquired (or these positive charges are lost) as a whole. These data strongly suggest that the additional negative charges fixed on the hyperanodic forms could arise from a degradation product of $NADP^+$ itself. This hypothesis is further supported by the spectral properties of hyperanodic forms; the covalent fixation of a degraded $NADP^+$ molecule could account for the increase in ultraviolet absorption between 260 and 290 nm. We have shown, in Fig. 5, the theoretical ultraviolet difference spectrum of apoenzyme binding one modified pyridine nucleotide molecule per protomer. This curve was plotted from the spectrum of $NADP^+$ incubated at acidic pH (18 h at 37°C) with minute amounts of glucose-6-phosphate dehydrogenase (10 mI.U./ml) and excess glucose-6-phosphate. It should be noted that the actual difference spectrum exhibits a maximum of absorbance at 278 nm instead of 270 nm for the theoretical curve; the patterns and the amplitude of both spectra were, nevertheless, similar.

The increase of the main peak of fluorescence of the hyperanodic forms excited at 290 nm is not due only to conformational changes since it persists after dissociation in 8 M urea; neither is the binding of a fluorescent compound on the enzyme involved, since the differences of fluorescence emission spec-

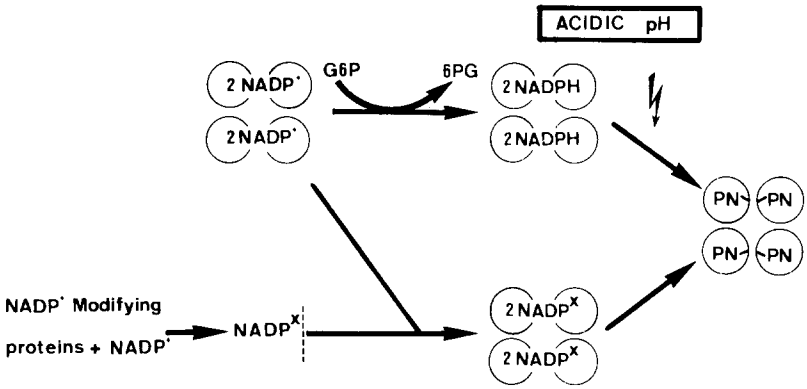

Fig. 7. Hypothetical mechanisms of the conversion of native enzyme into hyperanodic forms. $NADP^X$ = $NADP^+$ modified by the $NADP^+$ modifying proteins. PN, pyridine nucleotide residue covalently linked to the enzyme; dashed, verticle line, dialysing membrane. It should be noted that we have represented tetrameric species, because they are the predominant ones at acidic pH [15].

trum between native and hyperanodic forms of glucose-6-phosphate dehydrogenase disappear after digestion by papain. The most probable hypothesis is, therefore, that of the excitation of some tryptophan residues by the binding onto the enzyme of the modified coenzyme degradation product.

Some recent data from our laboratory, and others, strongly support the interpretation proposed above; first, we had recently the opportunity to use a fluorescent $NADP^+$ analogue ($\epsilon NADP^+$ [12,13]) and to show that the hyperanodic forms obtained with this compound remained intensively fluorescent, even after several days of dialysis, treatment by 8 M urea and proteolysis by trypsin (Skala et al., in preparation). Second, Schellenberg [17] provided direct evidence that NADH could covalently bind at acidic pH with tryptophanyl residues of various NAD/NADH-dependent enzymes.

The hypothesis of the fixation of a modified molecule of the coenzyme as being responsible for the conversion of native into hyperanodic forms is summarized in Fig. 7. Glucose-6-phosphate transforms the bound $NADP^+$ into NADPH. NADPH is highly unstable at acidic pH [18], and it is speculated that one of its degradation product could covalently bind to a special residue of the protein. The so-called $NADP^+$ modifying proteins would transform $NADP^+$ into modified molecules bound by glucose-6-phosphate dehydrogenase. At acidic pH these molecules could result in the same reaction as NADPH. This sequential mechanism is further supported by the ability of the $NADP^+$-modifying proteins to act through a dialysis membrane, provided that $NADP^+$ is present in the medium.

The conversion of glucose-6-phosphate dehydrogenase into hyperanodic forms has various consequences on its properties. As expected, molecular weight of the subunits is not modified, neither is there a change of the pH-dependent equilibrium between dimeric and tetrameric forms. Kinetic properties are slightly altered; above all stability is drastically decreased. Microcomplement fixation is able to detect conformational changes associated with the conversion into hyperanodic forms: the higher complement fixation by modified glucose-6-phosphate dehydrogenase seems to indicate that more antigenic determinants are accessible on these forms than on native enzyme. It is

a striking finding that this result was obtained although antisera were raised against native forms of enzyme. It could be that native human glucose-6-phosphate dehydrogenase is readily transformed into hyperanodic forms in the animals before stimulating the antibody production.

In conclusion we give in this paper some evidence that, in vivo and in vitro, glucose-6-phosphate dehydrogenase could react with some degradation product of the coenzyme $NADP^+$/NADPH and yield a stable complex between the enzyme and this product. These complexes, called hyperanodic forms, are characterized by a high lability to physical agents and proteolysis, such that they could constitute a physiological step toward the definitive degradation of the enzyme. A finding supporting this assumption is that the in vivo formation of hyperanodic forms is especially important and rapid in cells synthesizing unstable mutant glucose-6-phosphate dehydrogenase variants [19—21]. In vivo, the transformation into hyperanodic forms could be especially regulated by some proteins active on $NADP^+$ and facilitating the formation of the enzyme-modified coenzyme complex. Such an interaction between two proteins through the modifications of a common ligand could have some implications in the post-synthetic regulation of activity of several enzymes. This phenomenon could be compared to inactivation of phosphofructokinase by fructose 1,6 bisphosphase [22,23].

Acknowledgements

We thank Mss F. Carrouget and Mrs. M. Urbanek for the typing of the manuscript. This work was supported by a grant from INSERM (ATP 14-75-37) and CRL 78-5-125.

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