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Identification of different molecules leading to the formation of hyperanodic forms of human glucose-6-phosphate dehydrogenase.

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SUMMARY

Glucose-6-phosphate dehydrogenase undergoes in vitro a decrease of its isoelectric pH in the presence of its coenzyme $NADP^+$, and of either a NAD(P) glycohydrolase or an excess of its substrate, glucose-6-phosphate at acidic pHs.

The mechanism of in vitro production of hyperanodic bands of glucose-6-phosphate dehydrogenase has been studied. It consists in a covalent fixation of phosphoadenosine diphosphoribose or of a degradation product of NADPH. In the case of P-ADP-Rib, the reaction is stoichiometric, one molecule of ligand being bound to one subunit of enzyme. The bond between enzyme and P-ADP-Rib was characterized as a Schiff's base.

INTRODUCTION

Previous work from our laboratory has shown that the enzyme glucose-6-phosphate dehydrogenase (EC 1.1.1.49) can undergo post-translational modifications in vivo and in vitro. Major effects of these modifications are the appearance of hyperanodic bands of activity after isoelectric focusing (1) and a decreased stability towards heat, urea and proteolytic enzymes (2). Such hyperanodic forms are found in some leukemic cell extracts (3,4) and in eye lens fibers (5).

Abbreviations

$NADP^+$: nicotinamide adenine dinucleotide phosphate
$\varepsilon NADP^+$: IN^6 ethenoadenine dinucleotide phosphate
ADP-Rib : adenosine diphosphoribose
PADP-Rib : phosphoadenosine diphosphoribose
Glc-6-P : glucose-6-phosphate
2'-5'ADP : adenosine 2'-5' diphosphate

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We were able to reproduce in vitro a similar phenomenon using two models (2), the common feature of which being that they both required the presence of the coenzyme, $NADP^+$; no modification was found when the apoenzyme was completely stripped of its coenzyme. In a recent study (6) we have demonstrated that phosphoadenosine diphosphoribose, a product obtained by the action of an enzyme, $NAD(P)^+$ glycohydrolase (EC 3.2.2.6) on $NADP^+$, induces the modification of glucose-6-phosphate dehydrogenase while ADP-Rib and 2'5' ADP are inactive. Using a fluorescent analogue of $NADP^+$, $1N^6$ ethenoadenine dinucleotide phosphate ($\varepsilon NADP^+$), we obtained evidence that P-ADP-Rib binds covalently to the glucose-6-phosphate molecule. This evidence, however, was only qualitative.

In order to obtain quantitative data, radioactive $NADP^+$ was prepared. It was reacted with glucose-6-phosphate dehydrogenase under two experimental conditions which both lead to a change of the isoelectric point of the enzyme :

a) in the presence of $NAD(P)^+$ glycohydrolase (which generates P-ADP-Rib)

b) in the presence of glucose-6-phosphate (which generates NADPH), at acidic pH.

In addition, experiments were performed in order to elucidate the nature of binding of P-ADP-Rib to the enzyme molecule.

EXPERIMENTAL PROCEDURE

. Material

Human blood was obtained from blood banks. Special reagents : $NADP^+$, $\varepsilon NADP^+$, nicotinamide, NAD kinase were purchased from Sigma. NAD^+ labelled with ^{14}C in its nicotinamide moiety, or with ^3H in its adenine moiety, sodium borohydride ($NaBH_4$) labelled with ^3H, Protosol and Econofluor were obtained from NEN.

. Methods

Glucose-6-phosphate dehydrogenase was purified from human red cells and platelets as previously described (7). Glucose-6-phosphate dehydrogenase apoenzyme was prepared according to (2). Determinations of enzymatic activity and of $NADP^+$ level were performed according to Beutler (3). Electrofocusing of Glucose-6-phosphate dehydrogenase was run between pHs 3 and 10 according to Kahn et al (1)

Preparation of radioactive $NADP^+$. Radioactive $NADP^+$ (Adenine ^3H and Nicotinamide ^{14}C) was prepared by phosphorylation of a mixture of NAD^+ (Adenine ^3H) and NAD^+ (Nicotinamide ^{14}C) according to the reaction :

$NAD^+ + ATP \xrightarrow{\text{NAD Kinase}} NADP^+ + ADP$, as described by Chung (9).

$NADP^+$ was then isolated and purified on a Dowex 1-X2 (formate) column. The column was eluted with a gradient of formic acid : H_2O/4 N formic acid (10). The specific activity of the product, $NADP^+$, expressed in CPM par nanomole, was 24000 for ^3H and 17500 for ^{14}C.

Incubations. The incubations of Apo glucose-6-phosphate dehydrogenase, with Glc-6-P or $NAD(P)^+$ glycohydrolase from red cells membranes were carried out in a total volume of 100 µl at 37°C, in the following conditions : 50 mM phosphate buffer (pH 6.4), 0.1 mM EDTA, 0.1 mM/β mercaptoethanol, 0.1 mM/εaminocaproïc acid, 0.1 mM diisopropylfluorophosphate, 0.02 % sodium azide, 0.1 mM labelled $NADP^+$.

To induce the modification, Glc-6-P (1 mM) or membranes (1 mg/ml) were added to the incubation. Anodic bands appeared after 6 hours but the incubation lasted routinely 16 hours in order to insure complete transformation.

$NAD(P)^+$ glycohydrolase. In the experiments involving $NAD(P)^+$ glycohydrolase activity , we used red cell membranes, prepared and purified according to Fairbanks et al (11). The activity of glycohydrolase preparations was assayed using the sensitive fluorometric technique (12), with $\varepsilon NADP^+$ as a substrate. Cleavage of the nicotinamide moiety results in an increase in fluorescence. Using this test, a constant amount of enzyme, in terms of glycohydrolase activity, was added.

NADPH derivatives. NADPH (10^{-3}M) was treated according to Yoshida and Dave (13) in the following way :

A. NADPH incubated in 0.01 N HCl for 3 minutes at 25° and neutralized
B. NADPH incubated in 0.01 N HCl for 2 hours at 25° and neutralized
C. NADPH incubated in 0.05 M phosphate buffer pH 6.4 for 5 hours at 37°
D. NADPH incubated in 0.5 M phosphate buffer pH 6.4 for 5 hours at 37°.

In agreement with Yoshida and Dave, we found with all four products a disappearance of the specific absorption band of NADPH at 340 nm and a variable increase in absorption in the region 280-300 nm. Each product was neutralized and incubated with glucose-6-phosphate dehydrogenase. The enzyme was then submitted to electrofocusing.

Isolation of labelled glucose-6-phosphate dehydrogenase. Since the radioactivity of reacted glucose-6-phosphate dehydrogenase was relatively low, the methods usually employed for the study of incorporation of adenosine-diphosphoribose gave unsatisfactory results : cold trichloracetic acid preparation gave too high blank values (14), which made interpretation difficult ; hot trichloracetic acid freed most of the radioactivity. We, therefore, used an original technique which has been applied previously by Marie and Kahn (15) to pyruvate kinase.

10 µl to 30 µl of the incubation mixtures (the amount of glucose-6-phosphate dehydrogenase being known by protein determination) were spotted onto a Whatman 3 MM filter paper (8 x 9 cm) previously treated with a 2 % (W/V) trichloracetic acid solution containing cold 1 mM $NADP^+$. Several samples could be spotted on a single paper, which was dried and then gently stirred in a 10 % (W/V) TCA solution containing cold 1 mM $NADP^+$, at 4°C for 20 minutes. Non covalently bound radioactivity was eliminated by electrophoresis in a special apparatus designed for destaining polyacrylamide slab gels. The electrolyte solution was : methanol 5 % + acetic acid 7.5 %. After electrophoresis, the paper was rinsed with water, alcohol, acetone and then dried. Small pieces containing the spotted samples were cut out and counted for radioactivity. Radioactivity was measured in 3 % (v/v) Protosol in Econofluor in a LKB counter.

Borohydride reduction of the P-ADP-Rib adduct . Native and modified glucose-6-phosphate dehydrogenase (1 nM) were incubated at 0° for 1 hour un-

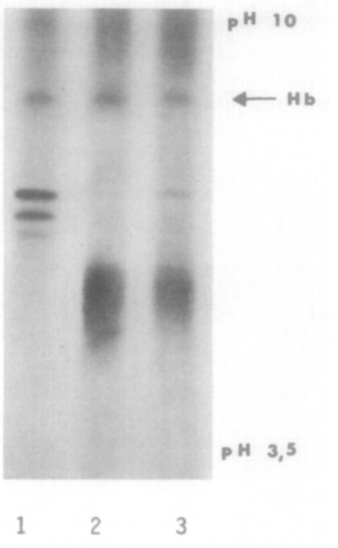

Fig. 1. Electrofusing of G6PD

Staining for G6PD activity. Hemoglobin (Hb) was used as marker.

1 : Native G6PD

2 : G6PD modified by NAD(P) hydrolase

3 : G6PD modified by G6P

der a nitrogen atmosphere with 10 nM of tritiated $NaBH_4$ (20 μ curies). The incorporation of radioactivity was tested by the method described above.

RESULTS AND DISCUSSION

1) Evidence for modification of glucose-6-phosphate dehydrogenase

Purified Apo glucose-6-phosphate dehydrogenase was incubated for 18 hours at 37° in the presence of double labelled $NADP^+$ with a red cell membranes preparation possessing $NAD(P)^+$ hydrolase activity or with Glc-6-P, and then submitted to isoelectric focusing. Figure 1 shows that the isoelectric point is lowered under both conditions. The transformation from native to hyperanodic forms is complete in the membrane system, while a weak remaining native band can be observed after incubation with glucose-6-phosphate

TABLE I

LABELLING OF GLUCOSE 6 PHOSPHATE DEHYDROGENASE IN THE PRESENCE OF RADIO-ACTIVE $NADP^+$ AND :

1. Red-cells Membranes
2. Glucose-6-phosphate

Results are expressed as cpm above background (20 cpm) and as picomoles of bound ligand.

	CPM		Picomoles		$^3H/^{14}C$
	^3H	^{14}C	^3H	^{14}C	
Double labelled $NADP^+$ 10 pmol	240	175			1.37
Membranes - Blank	5	5	0	0	
Glucose-6-phosphate dehydrogenase.10 pmol Blank	5	5	0	0	
Glucose-6-phosphate dehydrogenase.10 pmol+ membranes	220	0	9.2	0	
Glucose-6-phosphate dehydrogenase.10 pmol + Glc-6-P	140	50	5.8	2.8	2.8

Experimental conditions

. Each incubation was carried out with 1 nmol of Glucose-6-phosphate dehydrogenase and 8 nmol of $NADP^+$.

. Aliquots of 10, 20 and 30 p.mol of glucose-6-phosphate dehydrogenase were treated and counted for radioactivity in triplicate. Only results corresponding to 10 pmol are presented.

. Other aliquots were used for protein determination according to Lowry.

2) Incorporation of radioactivity in glucose-6-phosphate dehydrogenase after incubation with radioactive $NADP^+$ (table I).

a) Control studies showed no significant radioactivity above background when glucose-6-phosphate dehydrogenase alone or red cell membranes alone were incubated with radioactive $NADP^+$.

b) In the presence of $NAD(P)^+$ hydrolase, only ^3H was incorporated. One mole of ligand was incorporated per mole of enzyme subunit. No ^{14}C was found on the enzyme. This result confirms that the nicotinamide moiety has been split off by $NAD(P)^+$ glycohydrolase, and that the bound ligand is PADP ribose as already demonstrated (6).

c) In the presence of Glc-6-P, both ^{3}H and ^{14}C were incorporated. Less than one mole of ^{3}H was found per enzyme subunit (0.58). In addition, the ratio ^{14}C to ^{3}H was lower than in the initial $NADP^{+}$, since only 0.28 M of ^{14}C was bound per mole of enzyme subunit. The Glc-6-P system has been demonstrated to act through a degradation product of NADPH since it is inhibited by a $NADP^{+}$ reconstituting system (oxidized glutathione plus glutathione reductase as shown previously (2). It acts only below pH 7, where NADPH is unstable. In order to identify this degradation product of NADPH we submitted NADPH to the conditions described in "Methods". All resulting products induce some modification of Glucose-6-phosphate dehydrogenase, but the transformation is complete with product B only (after prolonged acidic treatment).

The overall results of Glc-6-P system experiments can be summarized as follows : there is a binding of a degradation product of NADPH to glucose-6-phosphate dehydrogenase. The lower incorporation of ^{14}C (corroborated by three additional experiments) could be interpreted by the fact that degradation of NADPH is heterogenous, some molecules losing their nicotinamide moiety while others keep it.

3) Nature of the binding of P-ADP-Rib to glucose-6-phosphate dehydrogenase

After completion of this binding, two experiments were performed in order to identify the nature of the chemical bound between P-ADP-Rib and glucose-6-phosphate dehydrogenase

a) action of neutral hydroxylamine was tested according to (17). No reversion from hyperanodic to native forms could be observed. This result excluded the hypothesis of ester-like bond.

b) the formation of a Schiff's base was tested by the borohydride technique (16). The results are expressed in table II.

TABLE II

TREATMENT OF NATIVE AND MODIFIED G6PD WITH RADIOACTIVE $NAHB_4$: BOUND RADIOACTIVITY

Apo G6PD	Radioactivity of Native G6PD (cpm)	Radioactivity of modified G6PD (cpm)	Difference due to the adduct (cpm)
0.125 nmole	444	2114	1670
0.25 nmole	998	4260	3262
0.5 nmole	1707	8160	6453

We can see that modified glucose-6-phosphate dehydrogenase incorporates about 5 times more radioactivity than native glucose-6-phosphate dehydrogenase, the difference in ^3H incorporation being the measure of Schiff's base formation. Like Kun et al, we observe some incorporation in the native molecule, probably due to the exchange of H atoms with ^3H atoms coming from the extremely unstable $NaBH_4$.

CONCLUSIONS

A previous work established the binding of P-ADP-Rib to glucose-6-phosphate dehydrogenase.

The present work describes the covalent binding to glucose-6-phosphate dehydrogenase of two types of conversion products of $NADP^+$.

a) phosphoadenosine diphosphoribose

b) degradation products of NADPH

The reactions are non-enzymatic and require no external energy.

In the present work most of the evidence relies on experiments made with radioactive $NADP^+$. It adds to previous results obtained with a fluorescent analogue of $NADP^+$ (6) and to the finding that the UV absorption difference spectrum of hyperanodic versus native forms of glucose-6-phosphate dehydrogenase is an addition spectrum with a maximum at 280 nm (2).

So we may consider it to be proved that P-ADP-Rib and a NADPH derivative can bind covalently to glucose-6-phosphate dehydrogenase, inducing

a modification of its isoelectric point. There is likely to be a Schiff's base formation between P-ADP-Rib and glucose-6-phosphate dehydrogenase.

In contrast to the many described ADP ribosylation reactions, this seems to be the first PADP ribosylation that has been observed and also the first example of the addition of a non-nicotinamide split product of NADPH. Further work will aim at elucidating the eventual physiological importance of the phenomenon.

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