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Phospho ADP ribosylation of human glucose 6 phosphate dehydrogenase : probable mechanism of the occurrence of hyperanodic forms

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SUMMARY

1) Membrane preparations from human red cells are able to lower in vitro the isoelectric pH of the enzyme glucose-6-phosphate dehydrogenase in the presence of the coenzyme $NADP^+$. This action can be obtained through a dialysis bag, and, therefore, involves a dialysable product of $NADP^+$ degradation.

2) This product could be identified as phosphoadenosine diphosphoribose by chromatographic isolation. The split product of $NADP^+$ was able to provoke anodisation of the enzyme, as was also an authentic sample of phospho-ADP ribose. ADP ribose and 2'-5' adenosine diphosphate were inactive.

3) Experiments performed with a fluorescent analogue of $NADP^+$ enabled us to deduce that phospho ADP ribose binds strongly to glucose 6 phosphate dehydrogenase.

We conclude that a reaction of phospho ADP ribosylation of glucose 6 phosphate dehydrogenase takes place under in vitro conditions and may play a role in the in vivo appearance of hyperanodic bands of the enzyme in cells.

INTRODUCTION

We have shown previously that the enzyme glucose 6 phosphate dehydrogenase (G6PD, EC 1.1.1.49) can undergo in vivo and in vitro posttranslational modifications. The major effect of these modifications is the appearance of hyperanodic bands of activity during isoelectric focusing (1). Such hyperanodic forms are found in vivo in normal lymphocytes (2), in some leukemic cells (2,3) and in eye lens fibers (14).

We were able to reproduce in vitro a similar phenomenon on purified G6PD using three different models, whose common feature was that they all required the presence of the coenzyme, $NADP^+$: a) a peptide prepared from some leukemic

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$NADP^+$: Nicotinamide adenine dinucleotide phosphate
2'-5' ADP : 2'-5' adenosine diphosphate
ADP ribose : Adenosine diphospho ribose
PADP ribose : Phospho adenosine diphospho ribose
$\varepsilon NADP^+$: 1 N^6 ethenoadenine dinuletotide phosphate

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leukocytes (3), b) an excess of glucose-6-phosphate at acidic pH (5) and c) protein fractions capable of hydrolyzing $NADP^+$ (5). No modification was found when the apoenzyme was completely stripped of its coenzyme. From these results, it appeared that some reagents resulting from a modification of the coenzyme, $NADP^+$ were able to induce changes in the behaviour of the enzyme. The spectral properties of the denaturated hyperanodic forms seemed to indicate that they corresponded to molecules with covalently bound nucleotides (5).

In the present work, phosphoadenosine diphosphoribose has been identified as one of the $NADP^+$ degradation products, and evidence is given that this compound binds to the enzyme in a non-enzymatic reaction. Phosphoadenosine diphosphoribose is the product of hydrolysis of $NADP^+$ by an enzyme, NAD^+ (P) glucohydrolase, which is present in a variety of cells (6,7).

MATERIAL AND METHODS

Material

Human blood was obtained from blood banks. Special reagents : $NADP^+$, 2'-5' ADP, ADPRibose, Phospho ADP Ribose, $\varepsilon NADP^+$, nicotinamide were purchased from Sigma. Other reagents were obtained from Merck. PEI plates were obtained from Macherey-Nagel.

Methods

Glucose 6 phosphate dehydrogenase (G6PD) was purified from human red blood cells and platelets according to previously described methods (8,9). G6PD apoenzyme was prepared according to (5). Determinations of G6PD activity and of $NADP^+$ were performed according to Beutler (10).

In vitro incubation of G6PD for demonstrating the modifying activity was made as described (5). Isoelectric focusing was run according to (1). $NADP^+$ glucohydrolase activity was assayed by following $NADP^+$ concentrations after various times of incubation. In some experiments NADPase was assayed with a fluorimetric technique, using ε $NADP^+$ as the substrate (11,12).

The chromatographic separation of the products of $NADP^+$ catabolism on Dowex AG1-X2 was made by a modification of Schmitz's technique (14) proposed by de Flora et al (15). Thin layer chromatography was performed on PEI-cellulose plates with molar LiCl as solvent (16). Preparation of red cell membranes was carried out according to Fairbanks et al (17).

RESULTS

1) Action of red cell membrane preparations

Action of membrane preparations could be shown either with a suspension of membranes or with membranes extracted with 0.5 % Triton X 100. Fig. 1 shows the results of an incubation of G6PD (0.02 units) with 10 or 20 µg of

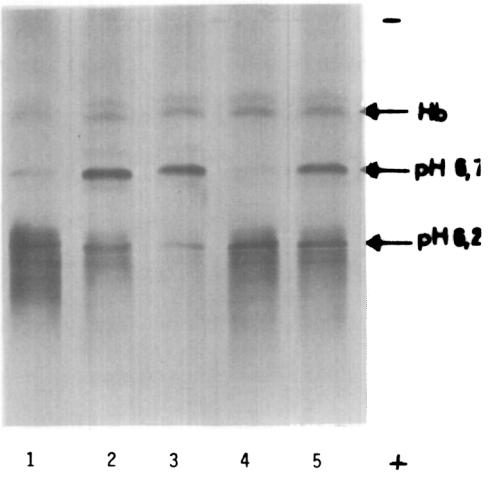

Fig. 1. Electrofocalisation (pH 3-10) on polyacrylamide gel of Glucose 6 phosphate deshydrogenase incubated at 37° overnight in the presence of :

1,4 $NADP^+$ + membrane enzyme
2,5 Membrane enzyme
3 Control
1,2 Membrane enzyme and G6PD were separated by a dialysis bag.

Glucose 6 phosphate dehydrogenase was stained specifically.
Traces of hemoglobin were added as reference.

solubilized membranes and 10^{-5} M $NADP^+$. Native bands have disappeared completely and have been replaced by bands with a lower isoelectric pH. From the results obtained previously (5) it was calculated that this change in pHi corresponds to the addition of three to four negative charges per subunit. The membrane enzymes were not active on apoG6PD when absolutely no $NADP^+$ was present. The residual $NADP^+$ remaining on G6PD even after dialysis was sufficient for anodic bands to appear when the enzyme was incubated with the membranes, without exogenous $NADP^+$ added.

Table 1 shows the effects of 300 µg of solubilized membranes on a 5.10^{-4}M $NADP^+$ solution. The decrease of $NADP^+$ slowed down progressively with time, due to the appearance of the reaction products and to a progressive inactivation of the $NADP^+$ hydrolyzing enzyme. A direct action of the membrane enzyme on glucose 6 phosphate dehydrogenase was not necessary. When G6PD was placed inside a dialysis bag, and membranes were placed outside in presence of $NADP^+$, the modification of G6PD took place inside the bag. A dialysable

TABLE I

Disappearance of $NADP^+$ by incubating 5.10^{-4} M $NADP^+$ with 300 µg of membrane proteins.

Time incubation (hours)	$NADP^+$ nM
0	500
2	400
4	300
6	250
22	60

product of $NADP^+$ degradation, therefore, was able to react directly with glucose 6 phosphate dehydrogenase.

$NADP^+$ and native G6PD were protected by 10^{-3} M nicotinamide as already observed by several authors for reactions involving NAD^+ (18,19). By contrast, no reversion could be obtained with nicotinamide, in the presence or absence of the membrane enzyme, as opposed to the results described in the case of NAD^+ and diphtheria toxin (19).

2) Identification of the product of the reaction

$NADP^+$ (10^{-4} M) was incubated overnight with a membrane preparation. The suspension was then heated to 100° C for 5 min., centrifuged at 10000 g for 10 min., and the supernatant was placed on a Dowex X2 column (0.5 cm x 18cm) equilibrated with water. Elution was performed in three steps : a) formic acid 0.2 M, b) formic acid 6 M, c) formic acid 6 M containing 0.8 M ammonium formiate (for details, see legend of fig. 2). The elution profile is shown in fig. 2. Each peak was concentrated by evaporation and taken up in a small volume of water. Peak 3 contained salt : the product was adsorbed on norite, and desorbed with 50 % ethylalcohol, containing 1 % ammonium hydroxyde, then concentrated again. The three peaks could be identified : peak 1 as nicotinamide by its migration on PEI and the yellow color of the spot when treated with cyanogen bromide and p-aminobenzoic acid (20) ; peak 2 as $NADP^+$ by migration on PEI plate and specific assay for $NADP^+$according to (10) ; peak 3 migrated like

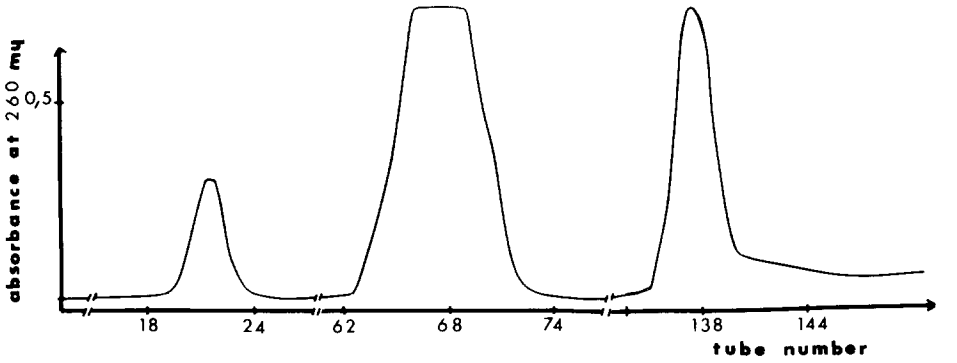

Fig. 2. Elution profile on Dowex of $NADP^+$ treated by membrane enzyme.
The gradient are composed as following :
tube number 0 to 37 : H_2O to HCOOH 0,2 N
38 to 120: HCOOH 0,2 N to HCOOH 6 N
121 to 150: HCOOH 6 N to HCOOH 6 N + NH_4^+ 0,8 N
Three peaks were eluted :
peak I : tube number 28-24
II : tube number 62-74
III : tube number 132-140.

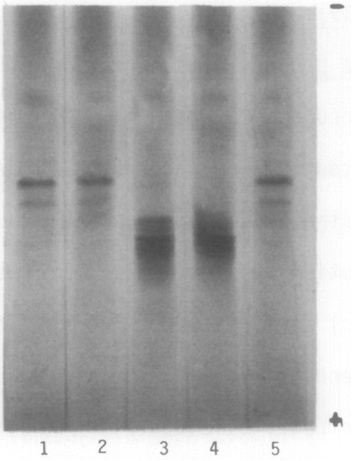

Fig. 3. Electrofocalisation (pH 3-10) on polyacrylamide gel of glucose 6 phosphate dehydrogenase incubated at 37° C overnight with the following compounds :
1 : ADP Ribose
2 : 2',5' ADP Ribose
3 : PADP Ribose
4 : Peak 3 of the elution on Dowex of $NADP^+$ treated by membrane enzyme.
5 : Control.

an authentic sample of phospho adenosine diphosphoribose on PEI plate. Each peak was incubated with apo G6PD. Only peak 3 was able to induce anodisation of G6PD (fig. 3).

3) Specificity of $NADP^+$

Attempts were made to determine whether $NADP^+$ could be replaced by NAD^+, since it is assumed that the hydrolase can act on NAD^+ and $NADP^+$ (21). This could only be attempted with apo G6PD since traces of $NADP^+$ were enough to bring about the phenomenon. An additional difficulty was that apo G6PD is very labile whenever it lacks the protective action of its $NADP^+$ coenzyme, which cannot be replaced by $NADP^+$. The difficulties could be circumvented by first incubating NAD^+ with the membranes, then adding the reaction product to the apo G6PD. No effect was observed. The reaction, then, is specific for $NADP^+$.

4) Comparison with derivatives of NAD^+ and $NADP^+$ (fig. 3).

Three derivatives of the coenzymes NAD^+ and $NADP^+$ were used : a) 2',5' adenosine diphosphate, which is used in the rapid purification technique of G6PD on account of its affinity towards the enzyme (17) ; b) ADP ribose, a product of the reaction of NAD glucohydrolase on NAD^+ and c) phospho ADP ribose, a product of the action of the same enzyme on $NADP^+$.

Each of these compounds was incubated with 0.02 UI of pure glucose 6 phosphate dehydrogenase at 10^{-5} to 10^{-3} M concentrations for 5 minutes to 15 hours. Only phospho-ADP ribose was able to modify the isoelectric focusing pattern in the same way as did the membrane enzyme in the presence of $NADP^+$. This effect was observed equally well with the native G6PD as with the stripped apoenzyme.

No effect on G6PD was observed with ADP ribose and 2',5' ADP.

The time course of the reaction between glucose 6 phosphate dehydrogenase and P-ADP ribose was studied using a concentration of 10^{-4} M, it tooks 5 minutes at 37° to obtain a visible effect (while after 15 hours at + 4° C no change was observed). The reaction was complete within two hours. $NADP^+$ was inhibitory when used at a concentration ten times that of P-ADP ribose, nicotinamide (0.1 M) was not inhibitory.

5) Nature of the product bound to the hyperanodic forms of glucose-6-phosphate dehydrogenase

An attempt was made to demonstrate binding of a $NADP^+$ derivative to glucose 6 phosphate dehydrogenase. We used the fluorescent analogue of $NADP^+$: $\varepsilon NADP^+$ and a red cell membrane preparation.

a) εNADP incubated with membranes in the same conditions as that described above for $NADP^+$, gave the same chromatographic profile (fig. 3) on the Dowex X2 column. Peak 3 was fluorescent, and its incubation with G6PD led to the anodization of the enzyme. We conclude that the membranes acted on $\varepsilon NADP^+$ by hydrolysing the molecule into nicotinamide and εPADP Ribose.

b) Apo G6PD (200 µg) was incubated with a) $\varepsilon NADP^+$ 10^{-4} M alone, as control ; b) $\varepsilon NADP^+$ 10^{-4} and 50 µl of suspended membranes in order to hydrolyse $\varepsilon NADP^+$ into εPADP Ribose and nicotinamide and c) $\varepsilon NADP^+$ 10^{-4} M and non fluorescent PADP Ribose 10^{-3} M. The pHi of the last two samples was modified by εPADP Ribose and PADP Ribose.

After the incubation, glucose 6 phosphate dehydrogenase was submitted to electrophoresis on cellulose acetate in 6 M urea at pH 8.0 for four hours in the cold, at a voltage of 135 V. Each strip was cut in two parts : one half was stained for proteins ; the other half was eluted in a tris buffer pH 8 and the fluorescence of the eluate was measured in an Aminco Bowman spectrofluorimeter at 300 nm (excitation) and 410 nm (emission). We found that the fluorescence of glucose 6 phosphate dehydrogenase modified by εPADP Ribose was increased four times, compared to the fluorescence of the native enzyme. By contrast, no increase in fluorescence was found in the sample incubated with fluorescent $\varepsilon NADP^+$ and non fluorescent PADP ribose.

The fact that fluorescence persisted after treatment of glucose 6 phosphate dehydrogenase with 6 M urea appears as strong evidence for a direct binding of phospho ADP ribose to the enzyme.

DISCUSSION

The present work demonstrates that under the influence of a $NADP^+$ (NAD^+) glycohydrolase, the coenzyme $NADP^+$ is transformed into a product which is able to modify the isoelectric pH of the enzyme, glucose 6 phosphate dehydrogenase. The active product was identified as phospho-adenosine diphospho-ribose, resulting from the splitting of nicotinamide from $NADP^+$. Identification rested on column and thin layer chromatographic evidence, as well as on the action of the isolated split product, and of an authentic sample (Sigma). The corresponding derivative from NAD^+, ADP Ribose, which is known to react with many proteins (22) had no effect. Finally, 2',5' adenosine diphosphate, was also inefficient, despite its affinity towards G6PD, which has been demonstrated by de Flora et al (23). Affinity, therefore, is not enough to induce the anodic modification of the enzyme.

Phospho ADP ribosylation, up to now, could not be reversed, neither by conventional ways (reducing agents) nor by enzymatic reversion with NAD(P) glycohydrolase in the presence of 0.1 M nicotinamide.

It is not yet possible to state whether the in vitro product obtained by the action of P-ADPR on G6PD is identical to the hyperanodic bands of G6PD which can be found in vivo. We have previously obtained evidence that hyperanodic bands of G6PD have a lower specific activity (1,5) and that they are less stable towards heat and proteolytic agents (5) than the native enzyme. The lack of reversibility and decrease in stability of hyperanodic forms make them good candidates as possible intermediate step in the degradative process of the enzyme (24).

Other authors have studied P-ADP ribose. Results recalling ours have been obtained by Ajmar et al (25). They have demonstrated that a glycohydrolase, in the presence of $NADP^+$, affects the other enzyme of the hexosemonophosphate shunt, 6 phosphogluconate dehydrogenase. This effect is inhibitory, but, in addition, the electrophoretic mobility of the enzyme towards the anode on starch gel is decreased. Since, however, there seems to be an increase in the positive

charge, it is unlikely that a binding takes place between the 6 phosphogluconate dehydrogenase and this very negatively charged reagent. It seems, therefore, that P-ADP Ribose could act on the two enzymes by different mechanisms.

In conclusion, we believe we have shown a binding between P-ADP Ribose and the enzyme, G6PD, which endows the enzyme with new charge and stability properties.

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