EVIDENCE FOR STRUCTURAL DIFFERENCES BETWEEN HUMAN GLUCOSE-6-PHOSPHATE DEHYDROGENASE PURIFIED FROM LEUKOCYTES AND ERYTHROCYTES

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SUMMARY : The C-terminal end of pure glucose-6-phosphate dehydrogenase from human leukocytes and red cells has been determined by subjecting these enzymes to partial proteolysis by carboxypeptidase A and B. The C-termini of the leukocyte enzyme were Lysine-Leucine, while Glycine was found as the C-terminal residue of erythrocyte glucose-6-phosphate dehydrogenase. From the results reported in this paper and from data previously reported we may conclude that aging of glucose-6-phosphate dehydrogenase in the red cells is associated with partial proteolysis of the C-terminal end of the enzyme molecules.

Glucose-6-phosphate dehydrogenase [(Glc-6-PdHase), D-glucose-6-phosphate : NADP + 1-oxidoreductase, (EC 1.1.1.49)] is coded by a single gene in all the cells ; this structural gene is located on the X chromosome (1).

Nevertheless, we have previously demonstrated that, in man, Glc-6-PdHase existed under different molecular forms in different cells ; we have established that such a heterogeneity of the Glc-6-PdHase molecular forms could be ascribed to posttranslational changes, the intensity of which was markedly dependent on the cell type and on the average age of the enzyme molecules synthesized in those cells (2-3-4). For instance, the isoelectric point, the ratio of enzyme activity to Glc-6-PdHase-related antigen concentration and some kinetic properties differed for either leukocyte or erythrocyte Glc-6-PdHase (2). We demonstrate in this paper that Glc-6-PdHase purified from both types of cells are structurally different. These differences seem more

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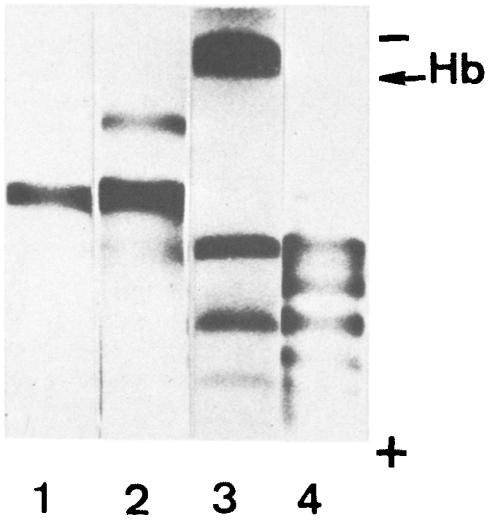

Figure 1 : Isoelectrofocusing pattern of leukocyte and red cell Glc-6-PdHase from whole extracts and pure preparations.

1 : leukocyte crude extract
2 : pure leukocyte Glc-6-PdHase
3 : hemolysate
4 : pure red cell Glc-6-PdHase

Hb : hemoglobin-Glc-6-PdHase was revealed on the gel by specific staining for the enzyme activity.

particularly to be due to a partial proteolysis of the C-terminal end of the native enzyme, responsible for the change from the leukocyte-type to the erythrocyte type Glc-6-PdHase.

EXPERIMENTAL : Glc-6-PdHase was totally purified from human red cells as previously described (5). Leukocytes were removed from patients suffering from hyperleukocytic chronic myeloid leukemia (4-5). Glc-6-PdHase was purified according to the technique previously published (4-5), slightly modified in order to prevent the in vitro alteration that leukocyte Glc-6-PdHase undergoes when purified strictly according to the usual method (5). An initial supplementary step was added, consisting of a CM Sephadex batchwise adsorption with elution by slight increase of pH (6 to 6.4) and addition of NADP+ to the elution buffer.

This modification allowed the purification of leukocyte Glc-6-PdHase

under a form unmodified when compared by isoelectrofocusing with enzyme from leukocyte crude extract : figure 1 shows the isoelectrofocusing pattern in acrylamide ampholine slab gel of erythrocyte and leukocyte Glc-6-PdHase from either whole extracts or purified preparations.

RESULTS AND DISCUSSION

The enzyme preparations were totally homogeneous as judged by sodium dodecyl-sulphate-acrylamide gel electrophoresis and double immunodiffusion.

The molecular weight of the subunits dissociated by sodium-dodecyl-sulphate was similar for both preparations.

No free N-terminal end could be detected , in any preparation, by the dansylchloride method (4-6), in agreement with the data of Yoshida (7).

The treatment of the pure leukocyte enzyme by carboxypeptidase B (Boehringer Mannheim) (figure 2) resulted,upon electrofocusing, in a progressive shift of the Glc-6-PdHase forms towards the acidic pHs. This decrease of isoelectric point was found again as the treated enzyme was dissociated in 8M urea, then focussed in gels containing 8M urea (figure 3). By contrast, carboxypeptidase B remained without any influence on the isoelectric point of erythrocyte Glc-6-PdHase.

The nature of the C-terminal residues of both these Glc-6-PdHase preparations was studied by analyzing the aminoacids specifically cleaved by carboxypeptidases A and B. The released residues were either identified by dansylation, as previously described (4), or, for leukocyte Glc-6-PdHase, quantitatively assayed with a Liquimat-F-Labotron aminoacid analyzer. The results of these experiments are summarized in table I : Carboxypeptidase A removed a single residue, L-leucine, from leukocyte enzyme, even after a long digestion time. By contrast, glycine and L-alanine were hydrolyzed from red cell Glc-6-PdHase. Since this result is in total agreement with studies previously published by Yoshida (8) we did not attempt to confirm this finding by quantification of the hydrolyzed residues.

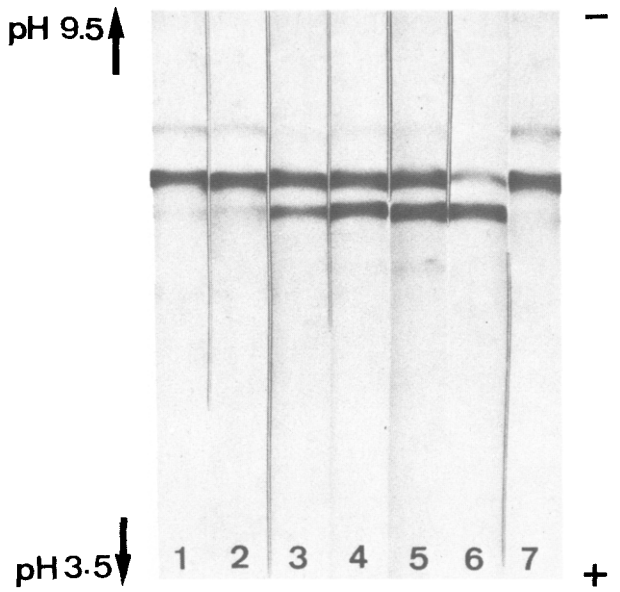

Figure 2 : Influence of carboxypeptidase B on the isoelectric point of pure leukocyte Glc-6-PdHase.

20 µg of Glc-6-PdHase were incubated at 37° for 0 min. (gel n°1), 10 min. (gel n°2), 30 min. (gel n°3), 60 min. (gel n°4), 120 min. (gel n°5) and 18 hours (gel n°6) in 50 µl of a 100 mM tris-Cl buffer pH 7.5 containing 100 mM NaCl, 2 mM diisopropylfluorophosphate, 1 mM EDTA, 0.02 mM NADP+ and 2 µg carboxypeptidase B. The gel n°7 shows Glc-6-PdHase incubated for 18 h in the buffer described above, without carboxypeptidase B. Staining for Glc-6-PdHase activity.

The commercial preparations of Carboxypeptidase B (containing always carboxypeptidase A as a contaminant) were able to slowly liberate L-leucine and L-lysine from leukocyte Glc-6-PdHase and minute amounts of glycine and L-alanine from red cell Glc-6-PdHase.

When digestion was sequentially performed with carboxypeptidase A, then carboxypeptidase B, 0.84 nmole of L-leucine and 0.52 nmole of L-lysine were cleaved from 1 nmole of leukocyte Glc-6-PdHase subunit (MW : 56,000). This treatment hydrolyzed glycine and L-alanine from red cell Glc-6-PdHase. All the above results were obtained with three different preparations of leukocyte and of red cell Glc-6-PdHase.

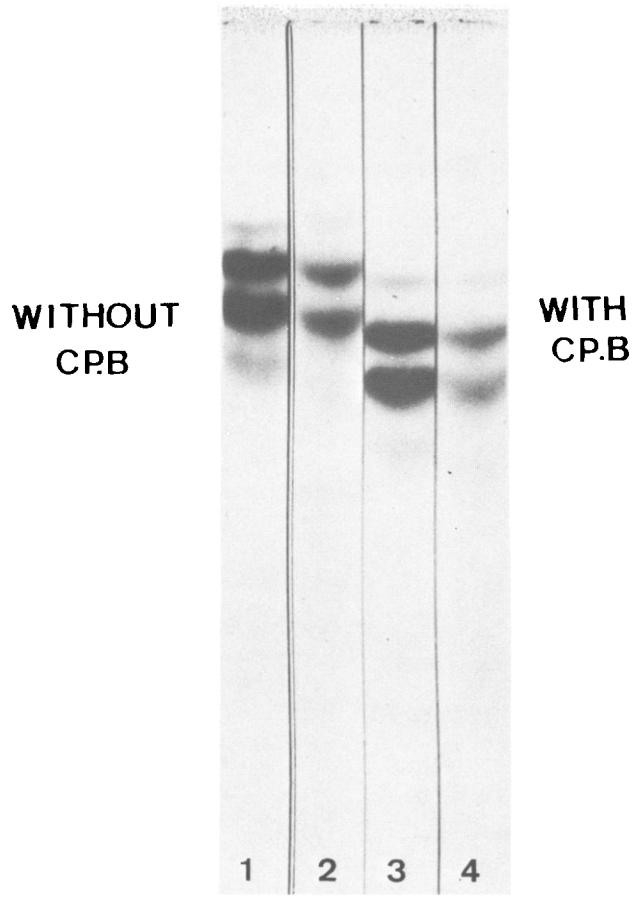

Figure 3 : Influence of carboxypeptidase B on the isoelectric point of the dissociated subunits of leukocyte Glc-6-PdHase.

In 3 and 5, Glc-6-PdHase was treated for 18 h by carboxypeptidase B under the conditions described in fig. 2. In 1 and 2 enzyme was incubated without carboxypeptidase B. After treatment, Glc-6-PdHase was dissociated in a solution of 8 M urea containing 1% β-mercaptoethanol, then focused in gels of acrylamide ampholine containing 8 M urea, too.

The amounts of protein deposited at the top of the gels were 40 µg in 1 and 3, and 10 µg in 2 and 4.

Staining with Coomassie blue (11) after fixation of proteins in 50% methanol.

Table I : Study of the aminoacids specifically hydrolyzed from leukocyte and erythrocyte Glc-6-PdHase by carboxypeptidases A and B.

		AMINOACIDS	
Carboxypeptidase	Glc-6-PdHase source	identified by dansylation	Assayed with aminoacid analyzer (nmoles liberated/nmole of Glc-6-PdHase subunit)
A	Leukocytes	leucine	0.84 nmole leucine
	Red cells	glycine, alanine	not tested
B	Leukocytes	after 18 h incubation, leucine lysine	not tested
	Red cells	after 18h incubation, glycine, alanine	not tested
A + B	Leukocytes	leucine, lysine	0.84nmole leucine 0.52 nmole lysine
	Red cells	glycine, alanine	not tested

- The carboxypeptidase digestion was performed in 200 mM sodium phosphate buffer pH 8.6 containing 2mM $MgCl_2$ and 1.5 mM diisopropylfluorophosphate. Glc-6-PdHase concentration was measured immediately before starting the carboxypeptidase digestion experiments according to the method of Böhlen et al. (10) modified in that orthophtaladelyde instead of fluorescamine was used as fluorogenic reagent.
- The ratio carboxypeptidase/Glc-6-PdHase was, on a molecular basis, 1 : 50 for both carboxypeptidase A and carboxypeptidase B.
- Digestion was left to proceed for 5, 10, 30, 60 and 120 minutes for carboxypeptidase A, and for 5 and 18 hours for carboxypeptidase B. The quantitative assay of the hydrolyzed aminoacids was performed with preparations incubated for 2 hours with carboxypeptidase A. In the experiments studying the sequential influence of carboxypeptidase A and carboxypeptidase B, carboxypeptidase A was inactivated by freezing-thawing, then carboxypeptidase B was added, and the incubation left to proceed for 18 hours.

From these results it appeared that pure leukocyte and erythrocyte Glc-6-PdHase differed by their C-terminal ends. The C-terminal residues of the red cell enzyme are Alanine-Glycine as previously reported by Yoshida (8), whereas the C-terminal end of leukocyte Glc-6-PdHase is : Lysine-Leucine.

We have recently proved that human platelet Glc-6-PdHase had the same C-terminal end as leukocyte enzyme (i.e. Lysine, Leucine, Bertrand et al., unpublished data).

Structural alterations in vitro of our preparations seem to be rather unlikely since the electrofocusing and electrophoretic patterns, the kinetic properties and the ratio of enzyme activity to Glc-6-PdHase-related antigen concentration were not, or only slightly, modified during the purification procedure of either leukocyte or red cell Glc-6-PdHase. Consequently we assume that the structural differences between these enzymes exist in vivo and do not result from in vitro alterations occuring in the course of the purification procedure. We have previously demonstrated that reticulocyte Glc-6-PdHase showed an active band identical with that of leukocyte or platelet enzyme, the anodic bands characteristic of the mature red cells appearing with aging of these cells (2-9). These data suggest that the enzyme found in leukocytes (or in platelets) would be the native Glc-6-PdHase form. The red cell enzyme would arise from this native form by partial proteolysis of Leucine and Lysine. The number, however, of the residues split from the carboxy-terminus must be small since no difference in size can be found between red and white cell enzyme by sodium dodecyl sulphate acrylamide gel electrophoresis. The fact that all the Glc-6-PdHase molecules purified from red cells seem to have a modified C-terminal end could be due to the use, as a starting material for the purification procedure, of old, outdated, blood samples.

Partial proteolysis is only one of the posttranslational events that, once synthesized, Glc-6-PdHase undergoes. We have previously described the change of all the Glc-6-PdHase forms into "hyperanodic forms", and we have

established that the mechanism of that change did not involve a partial proteolysis of the enzyme molecules (3-4). In the same way the heterogeneity of the whole molecules (fig. 1) or of the dissociated subunits (fig. 3) of pure Glc-6-PdHase did not result from a partial proteolysis, since a single C-terminal end, and no free N-terminal, were detected in leukocyte as well as in red cell enzyme.

Such a dependance on the cell type of the post-synthetic modification phenomena is most likely not restricted to Glc-6-PdHase. This could be responsible, in some cases, for functional modifications of enzymes. In some tissues, such alterations could be more dramatic than in the case of Glc-6-PdHase.

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