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STUDIES ON THE NATURE OF DIFFERENT MOLECULAR FORMS OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE PURIFIED FROM HUMAN LEUKOCYTES

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Summary

Several molecular forms of human glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49) corresponding to different stages of post-synthetic modifications have been purified from human leukocytes.

The various enzyme forms were different in their specific activity, their kinetic properties and their isoelectrofusing pattern. The molecular weight of the subunits of the different forms was not modified. The changes in the electrofocusing pattern were not due to modifications of the N-terminal ends, the oxidation of thiol groups or the non-covalent fixation of an acid molecule upon the enzyme. Carboxypeptidase B cleaved a C-terminal lysine from the different enzyme forms and shifted the isoelectric point of the different enzyme active bands towards the acid pH.

The different enzyme forms studied here seemed to result from the action upon 'native glucose-6-phosphate dehydrogenase' of 'modifying factors' especially abundant in some leukemic granulocytes. The modifying factors did not seem to be consumed during the 'modification' of glucose-6-phosphate dehydrogenase. Moreover, the storage for one year of unmodified enzyme resulted in changes in its electrofocusing pattern similar to those quickly induced by the 'modifying factors'. Consequently it appears that the modifying factors are catalysts of the modification of special residues of glucose-6-phosphate dehydrogenase.

The hypothesis that this modification involves the deamination of asparagine or glutamine residues is put forward.

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We have recently demonstrated that, once synthesized, human glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49) undergoes modifications of its molecular specific activity, isoelectric point and kinetic properties [1]. These changes depend on both molecular aging and the type of cell synthesizing the glucose-6-phosphate dehydrogenase. They are due to active processes, being especially under the influence of factors of low molecular weight which we have partially characterized from leukemic granulocytic cells [2,3].

The aim of this work was to isolate and to characterize several non-interconvertible molecular forms of human glucose-6-phosphate dehydrogenase, to elucidate the nature of the changes undergone by the enzyme and to analyze the type of interaction existing between glucose-6-phosphate dehydrogenase and "modifying factors" [3].

Materials and Methods

Materials

The substrates of the enzymatic reactions were provided by Boehringer-Mannheim and by Sigma CC; the reagents used for the buffers came from Merck and Calbiochem. The ion exchangers (DEAE-Sephadex A-50, and Sephadex G-25) were furnished by Pharmacia. The column eluates were scanned at 280 nm in a Beckman DBG spectrophotometer. Acrylamide and bisacrylamide came from Eastman Kodak, the Ampholines from LKB, starch for gel electrophoresis from Connaught. The electrofocusing experiments on acrylamide-Ampholine columns were performed in a Bio-Rad 150 gel electrophoresis cell. The ultrafiltrations were performed through ultra thimbles UH 100 (Schleicher and Schüll laboratory). The enzymatic reactions were measured in a Zeiss PM QII spectrophotometer connected to a Servogor recorder.

Methods

Enzymatic assys, kinetic studies and enzyme purifications. Glucose-6-phosphate dehydrogenase activity was measured as previously reported [1]; the results were expressed in international units per mg of protein at 30°C. The purification and the special properties of human platelet glucose-6-phosphate dehydrogenase have been previously described [4].

Granulocytes from several patients with chronic myeloid leukemias (preparations A, B and D) and acute myeloid leukemia (preparation C) were isolated as previously described [5]. The granulocytes were extracted in 50 mM sodium phosphate buffer (pH 6.4) containing 1 mM ϵ-aminocaproic acid, 1 mM diisopropylfluorophosphate, 1 mM EDTA, 0.02 mM $NADP^+$ and 1 mM β-mercaptoethanol. The crude extracts of preparations B and C were then diluted to 2 mg of protein per ml, the pH was adjusted to 6.4 and both preparations were incubated for 24 h at 37°C. Preparations A and D were not submitted to this 24-h incubation.

Glucose-6-phosphate dehydrogenase was then purified to homogeneity according to the method of Kahn and Dreyfus [5]. The purity of the preparations was checked by sodium dodecyl sulphate polyacrylamide gel electro-

phoresis [6] and by double immunodiffusion [5]. The kinetic and immunological studies were performed according to methods previously published [1].

Isoelectrofocusing. The isoelectrofocusing experiments were performed according to a method modified from that of Drysdale et al. [1,7]. The pH gradient of the polyacrylamide ampholine gel was also measured according to the method of Drysdale et al. [7].

After dissociation for 30 min at 4°C in a 10 mM Tris/chloride buffer, pH 8, containing 8 M urea and 2% (v/v) β-mercaptoethanol, the various purified glucose-6-phosphate dehydrogenase preparations were focused in polyacrylamide ampholine gels [1] containing 8 M urea. In one experiment the purified preparations were dissociated first in a 10 M guanidine hydrochloride/2% (v/v) β-mercaptoethanol solution (2 h at 25°C); then the extracts were extensively dialysed against the 8 M urea solution described above and focused in 8 M urea polyacrylamide ampholine gels. The reduced and carboxymethylated enzymes [8] were also focused.

When the polyacrylamide ampholine gels were to be stained for proteins, they were fixed for 24 h in 15% (w/v) trichloroacetic acid, with numerous solution changes, then stained with Coomassie Blue according to the method of Righetti and Drysdale [9].

NH_2 end-group determination. Dansylation of lyophilized glucose-6-phosphate dehydrogenase was conducted according to the method of Weiner et al. [10]. Dansylamino acids were identified in polyamide plates with the solvent system used by Hartley [11,12]. A better resolution of the basic dansylamino acids, if any, was obtained by using 3 M ammonia in the place of the solvent II described by Woods and Wang [12].

Analysis of the COOH end-groups. The influence of three carboxypeptidases (A, B and C) on the entire glucose-6-phosphate dehydrogenase molecule was appraised by incubating for various times at 37°C 10 μg of purified enzyme in 50 μl of the following carboxypeptidase solutions: 50 mM Tris/Cl buffer, pH 8, containing 1 M NaCl and 0.1 mg/ml carboxypeptidase A; 50 mM Tris/Cl buffer, pH 8, containing 100 mM NaCl and 0.5 mg/ml carboxypeptidase B; 100 mM sodium citrate buffer, pH 6, containing 0.25 mg/ml carboxypeptidase C. In addition, all these solutions contained 1 mM EDTA, 0.1 mM $NADP^+$, 1 mM β-mercaptoethanol and 2 mM diisopropylfluorophosphate. The modifications of the enzymatic activity and of the electrofocusing pattern of the active bands were analyzed in these various samples.

The influence of carboxypeptidase B was determined by analysis of the amino acid specifically hydrolysed from the purified enzymes (preparations C and D) by carboxypeptidase B, according to the following method: Native glucose-6-phosphate dehydrogenase was dialyzed against 0.2 M phosphate buffer pH 8.6 containing 0.2 mM $MgCl_2$. Carboxypeptidase B was added to the enzyme solution (50 I.U./μmol) and digestion left to proceed for five hours. The product was lyophilized and dansylated by adding suitable amounts of water and dansyl chloride in acetone (2.5 mg/ml) and incubating at 37°C for 45 min; the dansylated amino acids were then identified by chromatography in polyamide plates.

Studies on the nature of the interactions between glucose-6-phosphate dehydrogenase and modifying factors. Whether or not the 'modifying factors'

were consumed during incubation in the presence of glucose-6-phosphate dehydrogenase was appraised in the following way: The quantity of 'modifying factors' (boiled and ultrafiltered leukemic extracts described elsewhere [3]) just necessary to modify the isoelectric point of $10 \cdot 10^{-3}$ I.U. of purified enzyme was determined by incubating glucose-6-phosphate dehydrogenase with increasing dilutions of the leukemic extracts. Then this dilution was added to 2 mg of purified glucose-6-phosphate dehydrogenase (preparation A) dissolved in 200 µl of 50 mM sodium phosphate buffer (pH 6.4) containing the usual protectors; the mixture of modifying factors and concentrated enzyme was placed inside a dialysis tube outside which were 200 µl of the 50 mM phosphate buffer, pH 6.4, containing a 'tracer dose' of glucose-6-phosphate dehydrogenase ($10 \cdot 10^{-3}$ I.U.). The ability of the 'modifying factors' to change the isoelectric point of the outside enzyme was appraised by incubating the system described above for 24 h at 37°C and by focusing the outside extract in polyacrylamide ampholine gel: if the modifying factors had been consumed by the inside enzyme in excess, then they should not be able to 'modify' the outside enzyme through the dialysis membrane.

Influence of storage on the electrofocusing pattern of purified platelet glucose-6-phosphate dehydrogenase. The purification and the initial focusing pattern (a single predominant active band) of human platelet glucose-6-phosphate dehydrogenase have been previously described [4]. The influence of storage of this enzyme for 1 year, as a precipitate in a 65% saturated ammonium sulfate solution containing 50 mM Tris/Cl buffer (pH 8) and the usual protectors, was appraised by electrofocusing of the active forms in acrylamide ampholine gels, as described above.

Results

(1) Purity of the preparation

All the preparations were homogenous when tested by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Fig. 1) and by immunodiffusion. Moreover the relative migration rate in sodium dodecyl sulfate polyacrylamide gel was the same for all preparations.

(2) Comparison between incubated and non-incubated preparations

(a) Specific activity of the various enzyme preparations. Table I shows that those enzymes purified from previously incubated leukocyte extracts had a lower specific activities (respectively 145 and 121 I.U./mg of protein for the preparation B and C) than the enzymes purified from fresh extracts (182 and 180 I.U./mg of protein for the preparations A and D).

(b) Kinetic properties (Table I). K_m of the enzymes B and C (61 ± 4 µM and 65 ± 6 µM) was significantly higher than that of the enzymes A and D (39.5 ± 2 µM and 45 µM). The utilization of deamino $NADP^+$ was also significantly lower for the 'modified' enzymes B and C (52.4 ± 3.4% and 50 ± 5%) than for the enzymes A and D (66 ± 6% and 72%). The other characteristics studied did not differ from one preparation to the other.

(c) Electrophoretic properties. The electrofocusing patterns demonstrated by either specific staining for glucose-6-phosphate dehydrogenase or by protein

staining using Coomassie Blue were closely related for a given preparation (Fig. 2).

The slight differences between the electrofocusing pattern as the gels were stained either for enzyme activity or for protein could be due to the very different quantities of glucose-6-phosphate dehydrogenase deposited on the gels: about 0.03 μg in the former cases, 20 μg in the latter ones. In any case, the close relation between the active bands and the protein bands of glucose-6-phosphate dehydrogenase seems to indicate that there are not, in the various preparations, any totally inactive enzyme forms.

The focusing pattern of the enzymes purified from fresh leukocyte extracts (A and D) was composed of major forms with isoelectric points of approx. 6.70 to 6.86, and of minor forms with lowered isoelectric points, down to 6.2 (the "hyperanodic forms", refs 1 to 4). By contrast the most anodic forms were predominant in the enzyme preparations purified from preincubated leukocyte extracts (B and C, Fig. 3). Differences in isoelectric point were found again for the subunits of the various enzyme forms dissociated in 8 M urea and 2% (v/v) β-mercaptoethanol, carboxymethylated or not, then focused

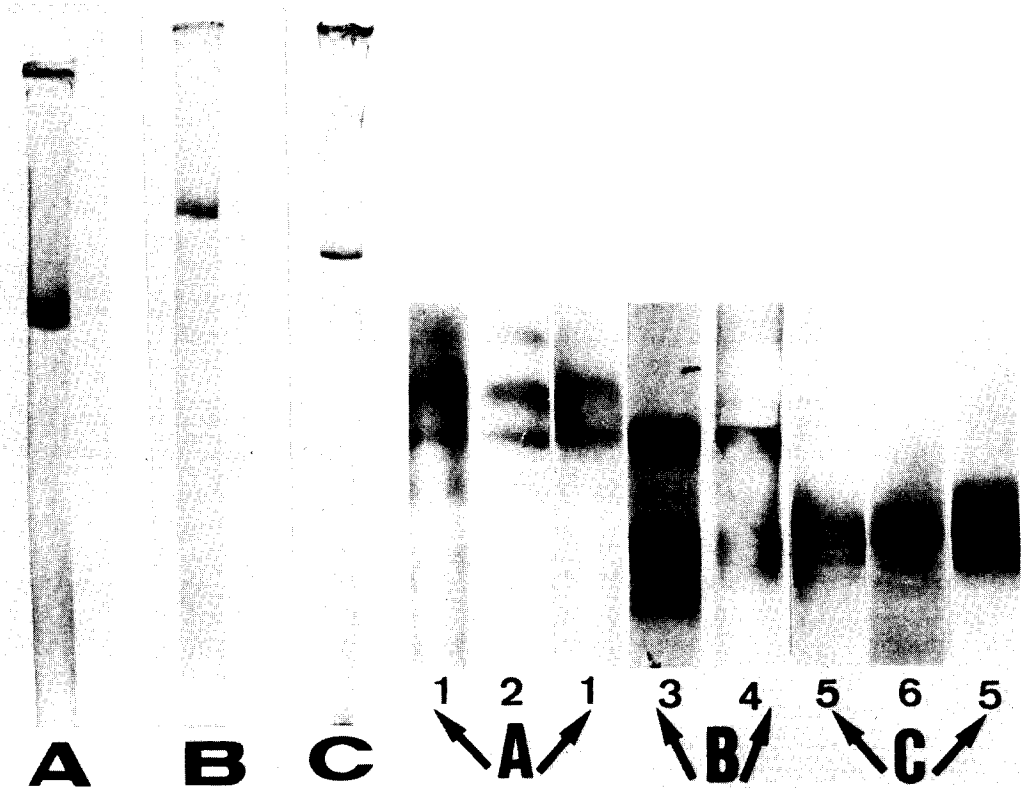

Fig. 1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of three purified leukocyte glucose-6-phosphate dehydrogenase preparations (A, B and C). The relative migrations (i.e. the ratio of the mobility of the enzyme to the mobility of the Bromophenol Blue) of the three enzymes were identical.

Fig. 2. Electrofocusing pattern of the preparations A, B and C. 2, 3, 5: staining for enzymatic activity. Dilutions with activity of about $5 \cdot 10^{-3}$ I.U. were deposited on the gel. 1, 4, 6: staining with Coomassie Blue; 20 μg of proteins were deposited on the gel.

TABLE I

CHARACTERISTICS OF THE VARIOUS GLUCOSE-6-PHOSPHATE DEHYDROGENASE FORMS PURIFIED FROM HUMAN LEUKOCYTES

A, B and D: enzyme purified from leukocytes of patients with chronic granulocytic leukemia. C: enzyme purified from leukemic myeloblasts. The crude extracts B and C were incubated before the purification for 24 h at 37°C in a 50 mM sodium phosphate buffer, pH 6.4 (see Methods). The measurements were done in 100 mM Tris/Cl buffer, pH 8, at 30°C, except for K_m ($NADP^+$), K_i (NADPH) and inhibition by ATP which were measured in 50 mM Tris/Cl buffer, pH 7.3, containing 100 mM KCl and 4 mM $MgCl_2$ at 37°C. K_m ($NADP^+$) and inhibition by ATP were also determined by fluorimetry. For the determination of K_m (glucose 6-phosphate), $NADP^+$ was 0.2 mM; for the determination of K_m ($NADP^+$) and K_i (NADPH), glucose 6-phosphate was 0.6 mM. The inhibition by ATP was measured in the presence of glucose 6-phosphate, 30 μM; $NADP^+$, 5 μM and ATP, 1.5 mM. The utilization of the substrate analogs appreciated with 0.6 mM 2-deoxyglucose 6-phosphate, 0.6 mM galactose 6-phosphate or 0.2 mM deamino $NADP^+$, i.e. with concentrations of analogs identical to those of the true substrates. Some kinetic characteristics (e.g. K_m ($NADP^+$), K_i (NADPH), inhibition by ATP) were not determined for all the preparations because previous studies showed that those characteristics do not seem to differ for the various preparations.

	Glucose-6-phosphate dehydrogenase preparation			
	A	B	C	D
Specific activity (I.U./mg protein	182	145	121	180
K_m glucose 6-phosphate (μM)	39.5 ± 2 (n = 6)	61 ± 4 (n = 6)	65 ± 6 (n = 5)	45
K_m $NADP^+$ (μM)	10.1	10.2	11	—
K_i NADPH (μM)	20	23	—	—
Inhibition by ATP (%)	40	30	30	—
Utilization of substrate analogs (%)				
2-deoxyglucose 6-phosphate	<4	<4	<4	<4
galactose 6-phosphate	<4	<4	<4	<4
deamino $NADP^+$	66 ± 6 (n = 6)	52.4 ± 3.4 (n = 6)	50 ± 5 (n = 6)	72
pH curve form and pH_{opt}	Truncate, 8.5	Truncate, 8.5	Truncate, 8.5	Truncate, 8.5

in polyacrylamide ampholine gel containing 8 M urea (Fig. 4). The same result was obtained when the foregoing dissociation of the subunits was performed in 10 M guanidine hydrochloride/2% β-mercaptoethanol solution.

(3) End-group analysis

NH_2 end-group. By dansylation of the various glucose-6-phosphate dehydrogenase forms with an overnight hydrolysis time we obtained a fluorescent spot, migrating poorly in solvents I and II, that disappeared with prolonged hydrolysis; with prolonged hydrolysis we did not obtain other dansylated products than ε-dansyllysine.

COOH end-group. Carboxypeptidase A induced neither change in the electrofocusing pattern, nor decrease in the enzymatic activity of any glucose-6-phosphate dehydrogenase forms. By contrast carboxypeptidase B induced a shift towards the acidic pH of all the active forms of the glucose-6-phosphate dehydrogenase preparations (Fig. 5), without any diminution of the enzyme activity or any change of the kinetic properties.

Carboxypeptidase C also induced an acidification of the active enzyme forms (Fig. 5), associated with a progressive inactivation of the enzyme. The focusing

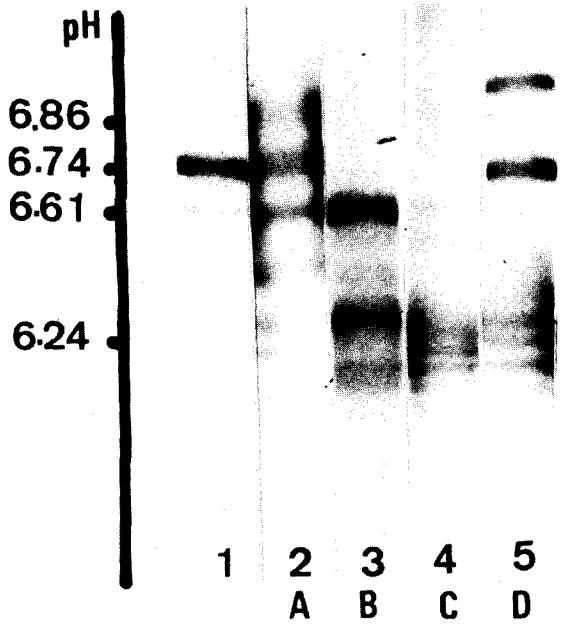

Fig. 3. Electrofocusing pattern of the active bands of various purified glucose-6-phosphate dehydrogenase preparations. 1, platelet enzyme; 2, leukocyte enzyme, preparation A; 3, leukocyte enzyme, preparation B; 4, leukocyte enzyme, preparation C; 5, leukocyte enzyme, preparation D.

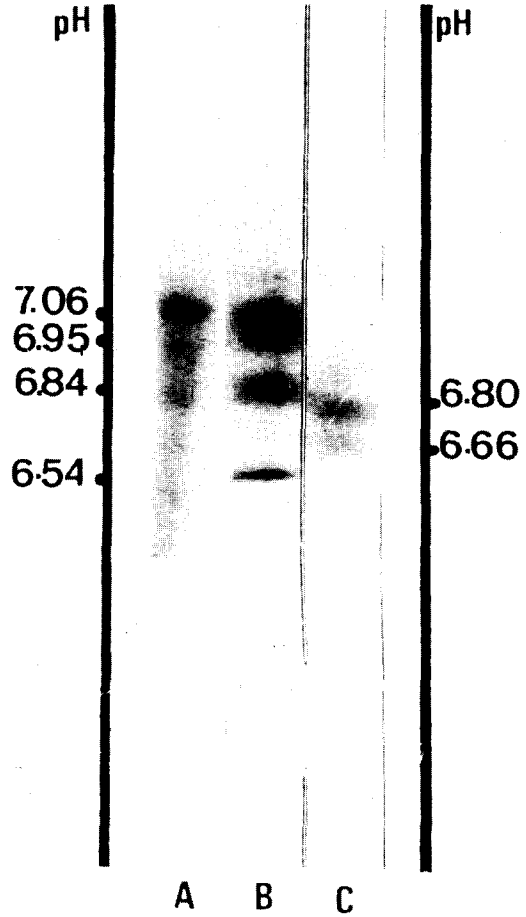

Fig. 4. Focusing in 8 M urea of the enzymes A, B and C previously dissociated in 8 M urea and 2% β-mercaptoethanol.

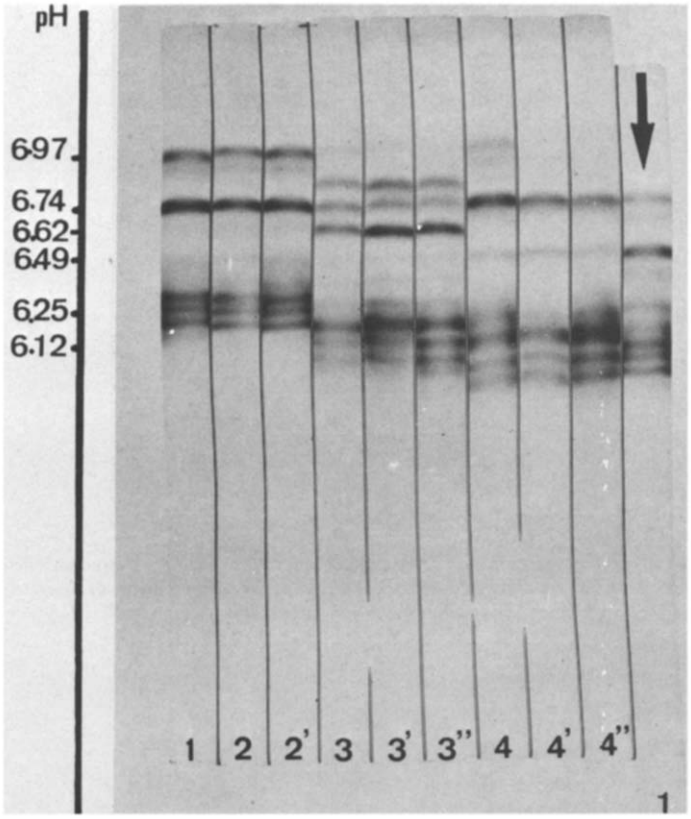

Fig. 5. Influence of carboxypeptidases A, B and C upon the focusing pattern of the active bands of the preparation D. 1, untreated enzyme; 2, 30 min incubation with carboxypeptidase A; 2′, 2 hours incubation with carboxypeptidase A; 3, 10 min incubation with carboxypeptidase B; 3′, 30 min incubation with carboxypeptidase B; 3″, 2 hours incubation with carboxypeptidase B; 4, 10 min incubation with carboxypeptidase C; 4′, 30 min incubation with carboxypeptidase C; 4″, 2 hours incubation with carboxypeptidase C. Right, a gel with untreated enzyme was arranged in such a manner that the shift of the active bands produced by carboxypeptidase C is obvious. Control incubation of the enzyme with the buffers without the carboxypeptidases did not result in change of the isoelectric point.

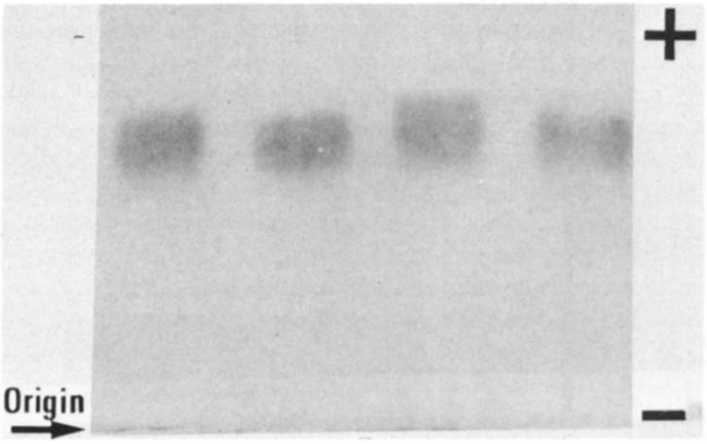

Fig. 6. Starch gel electrophoresis pattern of leukocyte glucose-6-phosphate dehydrogenase (preparation D) treated with carboxypeptidase B and untreated. From left to right, alternatively, treated and untreated enzyme.

pattern obtained after treatment of the enzyme with carboxypeptidase C was slightly different from that obtained with carboxypeptidase B (Fig. 5). The anodisation of the enzyme forms induced by carboxypeptidase B was confirmed by starch gel electrophoresis (Fig. 6).

The aminoacid liberated by carboxypeptidase B was identified by dansylation to be lysine. Such a result was found with all three enzyme preparations tested (B, C and D).

(4) Mode of interaction between 'modifying factors' and glucose-6-phosphate dehydrogenase

The modifying factors (granulocyte boiled and ultrafiltered extracts) when incubated in a dialysis bag in the presence of an excess of purified glucose-6-phosphate dehydrogenase and dialyzed against a small amount of enzyme placed outside the membrane, were able to dialyze and to modify the isoelectric point of the external glucose-6-phosphate dehydrogenase. This experiment shows that during incubation in vitro with glucose-6-phosphate dehydro-

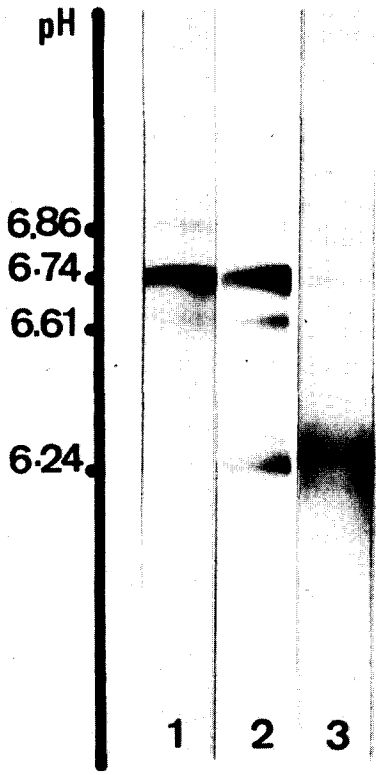

Fig. 7. Influence of storage upon the electrofocusing pattern of purified platelet glucose-6-phosphate dehydrogenase. 1, freshly purified enzyme. 2, enzyme stored for 1 year in the form of a precipitate in 65% saturated ammonium sulfate solution buffered with 50 mM Tris/Cl, pH 8. 3, enzyme incubated for 18 h at 37°C at pH 6.4 [1,3] with boiled and ultrafiltered leukemic extracts.

genase, the modifying factors did not seem to be either fixed or destroyed.

Furthermore the protracted storage of highly purified platelet glucose-6-phosphate dehydrogenase resulted in minor changes in the isoelectrofocusing pattern, of the same type as the changes quickly induced by the 'modifying factors' (Fig. 7).

Discussion

The enzymes purified from fresh extracts (preparations A and D) showed an electrofocusing pattern made of major cathodic bands [1,2] associated to minor 'hyperanodic forms', which were not found in the freshly extracted leukocytes. Their existence seems to be due to the presence of 'modifying factors' partially altering the enzyme during the purification procedure. Support for this conclusion is given by the fact that this phenomenon is not observed when the factors are not present or are rapidly removed from the extract. Platelets have been shown to be poor in these modifying factors, which explains how an unmodified purified platelet glucose-6-phosphate dehydrogenase could be obtained [1,2,3,4] (Fig. 3). Moreover chromatography of the crude leukocyte extract on Sephadex G-25, which allows to separate the modifying factors ($M_r < 4000$) [3] suppresses any further alteration of the focusing pattern during the purification of glucose-6-phosphate dehydrogenase.

The changes of specific activity and of kinetic properties of the enzymes purified under a 'modified form' confirm results previously reported [1,2].

From the results reported herein it can be concluded that the various molecular forms of glucose-6-phosphate dehydrogenase differ by stable structural changes involving the enzyme subunit itself. These changes do not appreciably modify the molecular weight of either the subunits (since the migration in sodium dodecyl sulphate gel was identical for all the glucose-6-phosphate dehydrogenase forms), or the entire molecule [1].

The fact that neither the high concentration of β-mercaptoethanol nor the carboxymethylation suppressed the differences of electrofocusing pattern between the different enzyme forms proves that the cysteine residues are not involved in this molecular heterogeneity. The potent dissociating agents (8 M urea or 10 M guanidine hydrochloride) did not suppress this heterogeneity, which indicates that the non-covalent binding of an acid molecule is not responsible for the anodisation of the 'modified' enzyme forms.

We have previously eliminated the hypothesis of the fixation of sialyl residues [1]; a phenomenon of phosphorylation of a serine residue seems to be unlikely, since the modification in vitro of the isoelectric point of purified glucose-6-phosphate dehydrogenase did not require any phosphorylated compound.

No free N-terminal residue could be detected, in any enzyme forms. Such a fact was described by Yoshida who proved that pyroglutamic acid is the N-terminal amino acid of erythrocyte glucose-6-phosphate dehydrogenase [13]. The fluorescent spot obtained after normal hydrolysis time was interpreted as a non-cleaved dipeptide containing a dansyl-ϵ-lysine.

Carboxypeptidase B is an enzyme with a narrow specificity towards the basic

C-terminal residues, lysine and arginine [14]; it was able to shift the isoelectric points of the active bands of leukocyte glucose-6-phosphate dehydrogenase towards acid pH values, and to liberate lysine from this purified enzyme.

These data seem to indicate that a C-terminal lysine exists in the 'unmodified' as well as in the 'modified' forms of leukocyte glucose-6-phosphate dehydrogenase. Consequently the transformation of the native enzyme (predominant band a, refs. 1—4) into "hyperanodic forms" [1—3], for instance under the influence of the leukemic "modifying factors", does not seem to be associated with a change of the C-terminal end.

The C-terminal end of erythrocyte glucose-6-phosphate dehydrogenase determined by Yoshida et al. did not include lysine, but was Ala-Ala-Gly [15]. Preliminary results obtained in our laboratory show that, indeed, carboxypeptidase B has no influence upon the erythrocyte enzyme and does not liberate lysine from a purified preparation of this enzyme. Therefore, it appears that glucose-6-phosphate dehydrogenase from erythrocytes and from leukocytes could differ in their C-terminus. If confirmed this could signify that post-translational events might involve a limited change of the C-termini of glucose-6-phosphate dehydrogenase in various tissues. Carboxypeptidase C is also able to hydrolyse the basic residues of the C-terminal end, as well as the neutral and the acid residues [16]. The hydrolysis of the C-terminal lysine could explain the anodisation, and further sequential hydrolysis the enzyme inactivation induced by carboxypeptidase C. We cannot explain, however, the different electrofocusing pattern obtained under the influence of both these carboxypeptidases.

The 'modifying factors' do not seem to be consumed during their incubation in vitro with glucose-6-phosphate dehydrogenase; moreover prolonged storage of glucose-6-phosphate dehydrogenase purified in the form of homogeneous cathodic bands (i.e., platelet enzyme, ref. 4) results by itself in the appearance of 'hyperanodic forms'. It appears therefore that the modifying factors are catalysts of changes involving some residues of the enzyme. These changes modify neither the N nor C-terminal ends, nor the SH groups. Consequently the hypothesis of the deamidation of glutamine or asparagine residues, as already described for several proteins [17—20] can be put forward. If this hypothesis were confirmed, it would be the first example so far reported of the catalytic deamidation of proteins.

In conclusion, glucose-6-phosphate dehydrogenase seems to undergo post-synthetic modifications altering its specific activity, its isoelectric point and its kinetic properties. Some of these modifications might be catalysed by 'modifying factors' abundant in some leukemic granulocytes and involve a phenomenon of deamidation of asparagine or (and) glutamine residues. Such a mechanism could be responsible for the various non-interconvertible forms of glucose-6-phosphate dehydrogenase purified from different preparations of human leukemic leukocytes.

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