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INTERCONVERTIBLE MICROHETEROGENEITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN RAT LIVER

KAZUHISA TAKETA AND AKIHARU WATANABE
The First Department of Internal Medicine, Okayama University Medical School, Okayama (Japan)

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SUMMARY

1. Glc-6-*P* dehydrogenase (D-glucose-6-phosphate:$NADP^+$ oxidoreductase, EC 1.1.1.49) specific to Glc-6-*P* and present in the soluble fraction of rat liver, was resolved into three major components by polyacrylamide gel disc electrophoresis. They were designated as I, II and III in order of decreasing mobility.

2. Fresh liver supernatants from normal rats had only II and III while those from carbon tetrachloride-injured rats revealed all three. Aged preparations showed more of the faster moving components, and a purified preparation of the enzyme was exclusively composed of I.

3. The three forms were found to be interconvertible without an appreciable change in activity. The conversion of slower into faster migrating forms was demonstrated by treatment with $HgCl_2$ and the reversal with β-mercaptoethanol.

4. Molecular weights of these forms were found similar, and their resolution on disc electrophoresis appeared to be mainly due to charge differences.

INTRODUCTION

A specific Glc-6-*P* dehydrogenase (D-glucose-6-phosphate:$NADP^+$ oxidoreductase, EC 1.1.1.49) in the soluble fraction of rat liver has been found to be induced in carbon tetrachloride injuries under conditions which differ in several aspects from those for the dietary induction of the dehydrogenase[1]. This led us to examine the presence of different molecular species of the enzyme in these experimental conditions of the animal. Several forms of Glc-6-*P* dehydrogenase in rat liver have been separated by means of starch[2] or polyacrylamide[3] gel electrophoresis, in which the major component of the specific dehydrogenase migrated as a single band.

In the present study the major component was further subfractionated into two or three bands by polyacrylamide gel disc electrophoresis. The present paper describes the natural occurrence, interconversion and molecular properties of the microheterogenous forms of Glc-6-*P* dehydrogenase in rat liver.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats maintained on Oriental Laboratory Chow MF and water *ad libitum* were used at body weights of 150–200 g. Dietary and other experimental conditions of the animals were as follows: "W", well fed; "S", starved for 48 h; "GC-24", starved for 48 h and refed for 24 h on glucose–casein (70:30, by wt.); "GC-72", refed similarly for 72 h; "CCl_4", starved for 48 h after a single intraperitoneal injection of 0.5 ml of 20% carbon tetrachloride in liquid paraffin per 100 g body weight; and "AH-130", maintained on the laboratory chow for 2–3 weeks after a subcutaneous transplantation of an ascites hepatoma, AH-130, until the tumor weight exceeded 10% of the body weight.

Enzyme preparations

Liver, skeletal muscle and kidney homogenates were prepared in 4 vol. of a suspending medium (0.154 M KCl, 0.32 mM $KHCO_3$ and 4 mM EDTA, pH 7.5) per g of tissue in a glass homogenizer with a Teflon pestle. Supernatants were separated by centrifugation of the homogenates for 60 min at either 25 000 or 105 000 $\times$ *g* with comparable results. Hemolysates were similarly prepared by freezing and thawing of washed red cells.

Aliquots of the liver supernatants were dialyzed against 5 mM Tris–HCl, pH 8.0, containing 5% (v/v) glycerol, 2 mM β-mercaptoethanol and 0.5 mM EDTA. Glc-6-*P* dehydrogenase in the dialyzed supernatants was fractionated into three peaks, A, B and C, on DEAE-cellulose, and the enzyme in the main Peak B fraction was further purified by CM-cellulose column chromatography with a specific substrate elution[4,5] to nearly homogeneous enzyme preparation. Protein contents were determined by the method of LOWRY *et al.*[6].

Enzyme assays

The activity of Glc-6-*P* dehydrogenase was determined spectrophotometrically at 37° by following the rate of NADPH formation at 340 nm with a Gilford recording spectrophotometer, Model 240. The incubation system consisted of 50 mM Tris–HCl, pH 7.5, 0.15 mM $NADP^+$, 2.5 mM Glc-6-*P*(omitted for blank), 20 mM $MgCl_2$ and appropriate amounts of the enzyme in a final volume of 1.0 ml. The activity was expressed in standard units (μmoles of NADPH formed per min).

Disc electrophoresis

Disc electrophoresis in polyacrylamide gels was performed as described by DAVIS[7]. Gel columns were prepared in glass tubes (0.5 cm $\times$ 10.0 cm) using 7% acrylamide monomer, unless otherwise stated, for separation gel. Sample-gel mixtures (0.2 ml per tube) contained a marker bovine hemoglobin (0.1 mg) and $NADP^+$ (15 nmoles). $NADP^+$ (0.015 mM) was also included in the cathodal buffer solution, which was prepared fresh in each run. Electrophoresis was carried out at 4° for 2 h and 15 min with a constant current of 4 mA per tube. Glc-6-*P* dehydrogenase was stained for the activity by incubating the gels at 37° in the dark each in 3 ml of the above incubation medium containing, in addition, 0.06 mg of phenazine methosulfate

and 0.6 mg of nitro blue tetrazolium. The stained formazan discs (or bands) were identified by their R_{Hb} values.

Treatments of Glc-6-*P* dehydrogenase preparations with $HgCl_2$ or β-mercaptoethanol were run at 25° for 15 min immediately before application on columns for disc electrophoresis.

Chemicals

The following chemicals were obtained from the respective commercial sources: $NADP^+$ (monosodium salt), Glc-6-*P* (disodium salt) and nitro blue tetrazolium, Sigma Chemical Co.; bovine hemoglobin (recryst.), Nutritional Biochemical Corp. Other chemicals were commercially available reagent-grade products.

RESULTS AND DISCUSSION

Natural occurrence

Polyacrylamide gel disc electrophoresis of rat liver supernatants revealed nine discernible formazan bands of Glc-6-*P* dehydrogenase (Fig. 1). Seven of them were specific to Glc-6-*P* and designated as follows*: I, II and III (in order of decreasing mobility), for a group of major and most anodal bands; I′, II′ and III′, similarly for a group of slower moving, faint but discrete bands; and IV for an undefined broad band with an intermediate mobility. Two or three of the major bands were invariably present in all the tissue extracts examined. Other minor bands seemed to appear in limited cases, *e.g.* when larger amounts of the enzyme in aged extracts were applied.

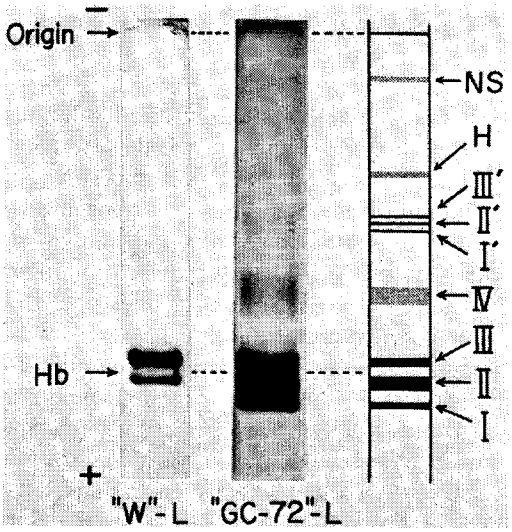

Fig. 1. Disc electrophoretic patterns of Glc-6-*P* dehydrogenase and their diagrammatical illustration. "W"-L, a fresh liver supernatant from "W". "GC-72"-L, a dialyzed liver supernatant from "GC-72". Specific activities (units/mg protein) of the enzyme in "W"-L and "GC-72"-L were 0.048 and 0.409, respectively. Hb, the migrated position of the main marker hemoglobin.

* The designation of bands also refers to that of components or forms of Glc-6-*P* dehydrogenase.

The relative intensities of I′, II′ and III′ exactly followed those of major bands. Band H was stained with Gal-6-*P* (2.5 mM) as intense as with Glc-6-*P*, whereas the other bands were practically negative with Gal-6-*P* as substrate. None of these bands could be demonstrated with 6-*P*-gluconate (2.5 mM). Omission of substrate gave only the slowest moving, nonspecific band, NS. Band H, therefore, was regarded as representing hexose-6-phosphate dehydrogenase[8] and other Glc-6-*P* specific bands were taken as microheterogenous forms (*cf.* ref. 9) of the specific Glc-6-*P* dehydrogenase.

On re-electrophoresis of I, II and III Glc-6-*P* dehydrogenases, each produced a single band with its original mobility, thus eliminating the possibility of an artificial resolution deriving from the disc electrophoresis.

The higher resolution achieved by the present technique was mainly due to the reduction in the amount of enzyme to be applied and the presence of $NADP^+$ in the cathodal buffer solution.

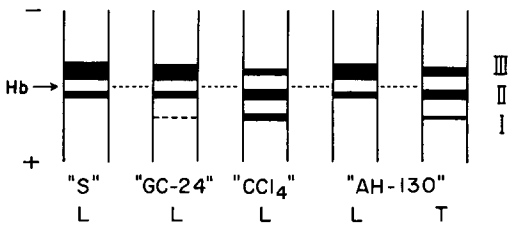

Fig. 2. Major bands of Glc-6-*P* dehydrogenase in fresh tissue extracts on disc electrophoresis. L and T refer to supernatants of liver and tumor tissues, respectively. Specific activities of the enzyme in the extracts were: "S"-L, 0.039; "GC-24"-L, 0.265; "CCl_4"-L, 0.216; "AH-130"-L, 0.059; and "AH-130"-T, 0.072.

Freshly prepared liver supernatants from well-fed rats exhibited only Bands II and III among the major components of the enzyme (Fig. 1). Typical patterns of the major bands in fresh preparations made from rats under different experimental conditions are shown in Fig. 2. Liver supernatants from starved rats gave a pattern similar to that of well-fed rats, those from refed rats showed, in addition, weak Band I, and those from carbon tetrachloride-poisoned rats yielded a more prominent Band I as well as Band II with a concomitant decrease in Band III intensity. Extracts from hepatoma tissues of AH-130, particularly those from apparently necrotic tissues, gave a fairly intense Band I, although the pattern of the host livers was indistinguishable from that of normal rat livers. It is also evident from the results shown in Fig. 2 that the increases in total Glc-6-*P* dehydrogenase activity (see the legend to Fig. 2) in the refed and carbon tetrachloride-intoxicated rat livers are not only due to the appearance of Band I activity but also due to the increases in activity of Band II and III components. Major band patterns of skeletal muscle, kidney and erythrocyte Glc-6-*P* dehydrogenases were identical to that of normal rat livers and not modified by the above experimental conditions.

Interconversion

When liver supernatants from well-fed rats were kept at 4° for 3 days, an

altered band pattern similar to that of the carbon tetrachloride-treated rats was obtained, *i.e.* faster moving components of the enzyme increased during the storage. Erythrocyte Glc-6-*P* dehydrogenase underwent similar changes at 4° after hemolysis, giving rise to a pattern with a predominant Band I. Those changes in band pattern during the storage occurred without any appreciable change in activity. Liver supernatants from well-fed rats could be stored at −25° for several weeks without marked changes in the band pattern, although each band became a little blurred. However, liver supernatants from carbon tetrachloride-poisoned rats and partially purified preparations (Peak A, B and C) revealed a gradual increase in faster moving components and a concomitant loss of activity even at −25°. The loss of activity caused by heating or treatment with laurate[10] did not alter the relative intensity among the three major bands.

Treatments of a liver supernatant with increasing amounts of $HgCl_2$ from 15 to 100 nmoles prior to disc electrophoresis resulted in a similar sequential alteration in band pattern without any significant change in activity. This process was not affected by the presence of $NADP^+$ or the marker hemoglobin but appeared to be influenced by the total protein content in the sample, more $HgCl_2$ being required with larger amounts of protein. When the supernatant was first subjected to disc electrophoresis and then the resulting gels were treated before staining, each with 3 ml of 0, 0.02 or 0.5 mM $HgCl_2$, all the gels showed identical bands, indicating that the altered distribution of band intensity was not caused by the activation of a faster moving, inactive species of the enzyme but by the successive increase in mobility of the active species.

An attempt to demonstrate the sequential conversions of Component III to II and II to I by treatment with $HgCl_2$ was not successful because the dehydrogenase components extracted from the gel after electrophoresis were shown not to be susceptible to the $HgCl_2$ treatment in terms of forming faster moving molecular species.

The transformation of slower into faster migrating components of the enzyme in fresh liver supernatants kept at 4° was partially prevented by the presence of 15 mM β-mercaptoethanol. $NADP^+$ (0.15 mM) or EDTA (2 mM) had no such preventive effect. β-Mercaptoethanol was also effective in reversal of the transformation without altering the activity. An altered band pattern caused by treatment of 10 μl of a liver supernatant (protein content, 16.8 mg/ml) from well-fed rats with 2 nmoles of $HgCl_2$

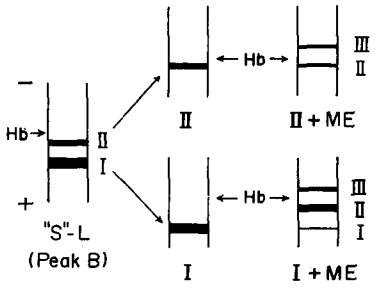

Fig. 3. Effect of β-mercaptoethanol on separated I and II components of Glc-6-*P* dehydrogenase. "S"-L (Peak B), a Peak B fraction prepared from a liver supernatant from "S". Extracts from Disc I and II of "S"-L (Peak B) were each incubated with (I + ME and II + ME) or without (I and II) 15 mM β-mercaptoethanol.

was completely reversed to the original pattern by immediate incubation of the $HgCl_2$-treated supernatant with 3 μmoles of β-mercaptoethanol. The effect of β-mercaptoethanol on individual components of the enzyme is shown in Fig. 3. Component I produced Bands I, II and III, and Component II yielded Bands II and III. The highly purified preparation having sole Component I gave Bands I and II by incubating with 15 mM β-mercaptoethanol prior to the electrophoresis. These results apparently indicate that the change in band pattern produced by β-mercaptoethanol is not due to the activation or inactivation of respective species of the enzyme but due to sequential conversions of faster into slower moving components.

The only known polymorphism of Glc-6-*P* dehydrogenase is related to a subunit interaction[10–12]. The re-association of subunits demonstrated in the presence of β-mercaptoethanol and $NADP^+$ in those reports has no relevance to the β-mercaptoethanol-dependent conversion of I to II and III forms in the present study. Apparently the treatment with laurate, which is known to lead to disaggregation of the enzyme[10], was not associated with the change in band pattern.

Molecular properties

Sucrose density gradient centrifugation of a partially purified Peak B fraction having main I and II components and a trace of III also gave a single peak of the activity, which revealed predominant I and faint II bands (Fig. 4). The results could be best explained by the possible transformation of slower into faster moving components in the course of centrifugation because the total recovery of the activity was 93%. Similar transformation was observed during filtration of the enzyme on Sephadex G-200. No evidence of different molecular weights among I, II and III components was obtained in gel filtration and ultracentrifugal analysis.

Since the above techniques were found not suited for studies of the molecular properties of the enzyme, which underwent considerable changes during the sepa-

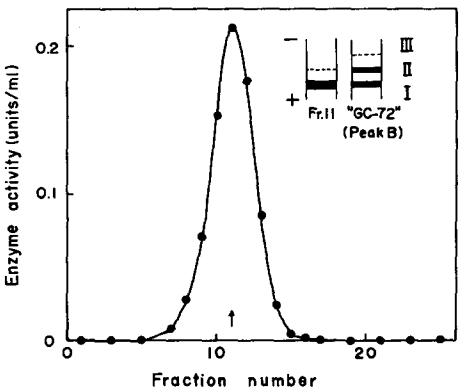

Fig. 4. Disc electrophoretic patterns of Glc-6-*P* dehydrogenase following sucrose density gradient centrifugation. Glc-6-*P* dehydrogenase of a Peak B fraction prepared from "GC-72" was centrifuged for 15 h at 3° in a Hitachi 40 P centrifuge with RPS-40 rotor set at 39 000 rev./min. 0.164 unit of the enzyme (0.097 mg protein) in 40 μl was layered on 4.6 ml of a 5–20% sucrose linear gradient containing 5 mM Tris–HCl, pH 8.2, and 0.015 mM $NADP^+$. The fraction size was 0.2 ml. The fraction taken for disc electrophoresis is indicated by the arrow. The apparent molecular weight was calculated to be 104 000.

ration process, a disc electrophoresis with varying concentrations of acrylamide monomer was employed in order to examine the size and charge differences among the dehydrogenase components while the enzymes were migrating through the gel. Fig. 5 represents R_F values of the dehydrogenase components plotted on logarithmic scale against concentrations of the monomer. Straight lines were obtained for all the enzyme components and for the reference hemoglobin. The lines for I, II and III were almost parallel to each other with a tendency of less marked differences in R_F as the monomer concentration was reduced, but the differences were still present at the

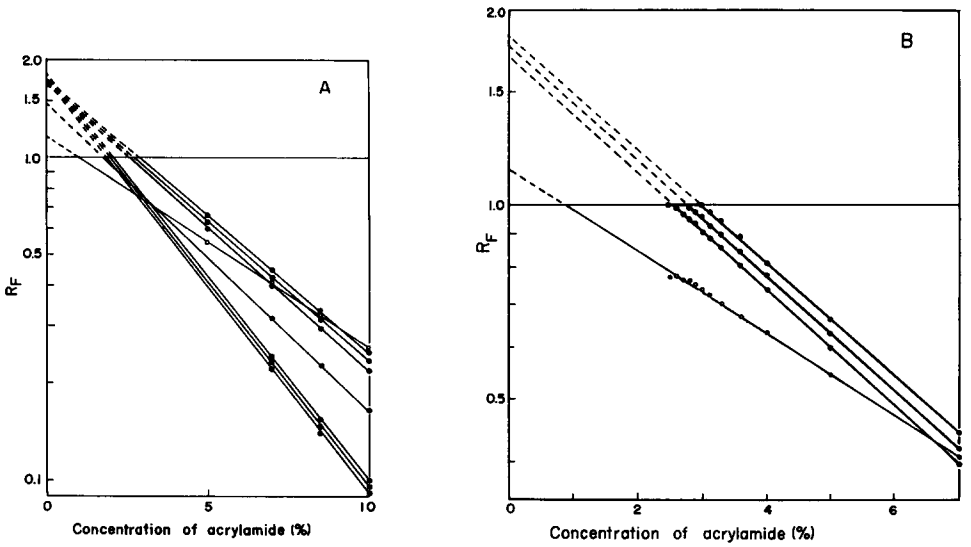

Fig. 5. Effect of the concentration of acrylamide monomer on R_F values of Glc-6-*P* dehydrogenase components at higher (A) and lower (B) monomer concentrations. A. ●—●, Glc-6-*P* dehydrogenase of "GC-72"-L in Fig. 1; from top to bottom: I, II, III, IV, I′, II′ and III′. ○—○,bovine hemoglobin. R_F values were calculated from R_{Hb} values of the enzyme and R_F values of hemoglobin obtained by extrapolation of the values at lower monomer concentrations (B). The distance of Schlieren boundary from the origin was taken as that of front migration in calculation of R_F. B. ●—●, Glc-6-*P* dehydrogenase of a $HgCl_2$-treated fresh liver supernatant from "W"; from top to bottom; I, II and III. ○—○, bovine hemoglobin. The concentration of *N,N′*-methylenebisacrylamide was kept constant, and twice the standard amount of *N,N,N′,N′*-tetramethylethylenediamine was present in the separation gel. Electrophoretic period of time was reduced to 70 min with no effect on R_F [7]. Accurate measurements of migrated distances were made on enlarged photographs of the gels.

extrapolated zero concentration. This is strong evidence for the presence of small but definite differences in net charge among the three components. Although the differences in slope among them are minute as compared with the difference between those and the hemoglobin lines, this might indicate that the three enzyme forms have small but recognizable differences in molecular size. The group of lines for I, II and III and that for I′, II′ and III′ appeared to meet at the zero concentration of the monomer, indicating that the latter components have a considerably larger molecular size than the former and both are similar in charge. Since the line for IV fell on the ordinate between the points for the dehydrogenase and hemoglobin, Component IV

would have a less negative charge than the other dehydrogenases and an intermediate molecular size of the major and minor enzyme species.

It may be also noted in Fig. 5B that I, II and III components gave a single band with an R_F of 1.0 at a monomer concentration of 2.5% and that this was not due to an ineffective molecular sieving by the gel at the reduced monomer concentration but due to a stacking of all three components into the Schlieren boundary.

Based on all the evidences obtained in the present study, I, II and III forms of Glc-6-*P* dehydrogenase in rat liver and other tissues would be best placed in the category of the microheterogeneous forms introduced after translation[9].

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REFERENCES

1 K. TAKETA, A. TANAKA AND K. KOSAKA, *J. Japan. Biochem. Soc.*, 41 (1969) 379.
2 C. R. SHAW AND A. L. KOEN, *Ann. N.Y. Acad. Sci.*, 151 (1968) 149.
3 S. H. HORI AND S. MATSUI, *J. Histochem. Cytochem.*, 15 (1967) 530.
4 M. G. SARNGADHARAN, A. WATANABE AND B. M. POGELL, *J. Biol. Chem.*, 245 (1970) 1926.
5 T. MATSUDA AND Y. YUGARI, *J. Biochem. Tokyo*, 61 (1967) 535.
6 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
7 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
8 S. OHNO, H. W. PAYNE, M. MORRISON AND E. BEUTLER, *Science*, 153 (1966) 1015.
9 C. J. EPSTEIN AND A. N. SCHECHTER, *Ann. N.Y. Acad. Sci.*, 151 (1968) 85.
10 Y. YUGARI AND T. MATSUDA, *J. Biochem. Tokyo*, 61 (1967) 541.
11 H. N. KIRKMAN AND E. M. HENDRICKSON, *J. Biol. Chem.*, 237 (1962) 2371.
12 H. R. LEVY, R. R. RAINERI AND B. H. NEVOLDINE, *J. Biol. Chem.*, 241 (1966) 2181.