Characterization of Rat Hepatoma Glucosamine 6-Phosphate Synthase and Its Relation to Liver and Fetal Forms

Taeko Miyagi and Shigeru Tsuiki

Biochemistry Laboratory, Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai 980, Japan

ABSTRACT

Glucosamine 6-phosphate synthase (EC 5.3.1.19) purified from various rat tissues by a procedure involving chromatography on diethylaminoethyl Sephadex and hydroxylapatite were characterized by means of isoelectric focusing. The nonhepatic isozyme, previously reported to be present in Yoshida sarcoma, has a pI of 4.1 and is distinguished from the hepatic isozyme, with a pI of 5.0. The pI 4.1 form is the major one in all of the fast-growing, transplantable hepatomas studied. Although not detectable in 19-day fetal liver or normal adult liver, the pI 4.1 form has been observed in the whole 12-day fetus and adult brain as almost the sole form of glucosamine 6-phosphate synthase.

INTRODUCTION

We have previously reported that liver neoplasia is associated with alterations of GlcN-6-P synthase [glucosamine phosphate isomerase (glutamine-forming), EC 5.3.1.19; formerly L-glutamine: D-fructose 6-phosphate amidotransferase, EC 2.6.1.16], a major regulatory enzyme for the synthesis of UDP-N-acetylg glucosamine (5, 9). Although the enzyme partially purified from a rat ascites hepatoma [Yoshida sarcoma (4)] was different from the enzyme from rat liver in physical, kinetic, and immunological properties (9), the nature and significance of this molecular alteration remained obscure, since no other tissues were included in the study.

In the present study, GlcN-6-P synthase was purified from a variety of cancerous and noncancerous tissues of the rat, and their isozyme patterns were investigated by means of isoelectric focusing. We found that, although absent from late-fetal liver, the nonhepatic isozyme described above is present in the whole 12-day fetus as well as in adult brain. Preliminary accounts of this work have been presented (13).

MATERIALS AND METHODS

Animals and Tumors. Male Donryu rats weighing 150 to 200 g and fed ad libitum were used. Of the 4 strains of transplantable rat hepatomas, one (AH-109A) was inoculated s.c., and others (Yoshida sarcoma, AH-66F, and AH-130) were inoculated i.p. It should be noted that Yoshida sarcoma is now known to be a hepatoparenchymal carcinoma (4). These tumors were harvested 5 (ascites tumors) or 12 (solid AH-109A) days after inoculation. Pregnant female rats were dated from the time of fertilization, and the fetuses were delivered by cesarian section.

Purification of GlcN-6-P Synthase. All the preparative experiments described below were conducted at 0–4°C, and all the phosphate buffers used in these experiments had a pH of 7.5. Extracts were prepared from liver, AH-109A, fetus, and brain by homogenization in 2 volumes of 0.25 M sucrose-25 mM KCl-10 mM glucose-6-phosphate-10 mM potassium phosphate, followed by centrifugation at 10,000 × g (10 min) and at 105,000 × g (1 hr). Ascites hepatoma cells packed in the homogenizing medium above were homogenized in an equal volume of water. The homogenate was then made isotonic by addition of 0.5 M sucrose-50 mM KCl-20 mM glucose-6-phosphate-20 mM potassium phosphate and centrifuged as described above.

For purification of GlcN-6-P synthase, these extracts were fractionated with (NH4)2SO4, and the fraction precipitating between 40 and 60% of saturation was collected by centrifugation. It was then dissolved in 50 mM potassium phosphate-0.1 M KCl-1 mM EDTA, desalted by flow through a Sephadex G-25 column, and applied to a DEAE-Sephadex column (1.5 x 7 cm) previously equilibrated with the same buffer. The column was washed with the equilibrating buffer, and GlcN-6-P synthase was eluted with 50 mM potassium phosphate-0.3 M KCl-1 mM EDTA. The active fraction was then applied to a hydroxyapatite column (1.5 × 5 cm) previously equilibrated with 50 mM potassium phosphate-1 mM EDTA, and the column was washed successively with the equilibrating buffer, 0.15 M potassium phosphate-1 mM EDTA, and 0.35 M KCl-1 mM EDTA. GlcN-6-P synthase eluted with the last buffer. This procedure gave 40 to 50% yields of enzyme purified approximately 70-fold. The final eluate was concentrated by dialysis in a collodion bag, desalted by flow through a Sephadex G-25 column equilibrated with 10 mM potassium phosphate, and analyzed by isoelectric focusing.

We previously reported that GlcN-6-P synthase of Yoshida sarcoma, but not that of normal adult liver, requires glutamine and GlcN-6-P for stability (9). GlcN-6-P synthases from adult brain, whole 12-day fetus and AH hepatomas also exhibit this characteristic (Table 1). Therefore, chromatography of these enzymes on DEAE-Sephadex and hydroxyapatite was performed in the presence of 20 mM glutamine and 0.05 mM GlcN-6-P.

Isoelectric Focusing. Isoelectric focusing was carried out according to the method of Vesterberg and Svensson (14) using the LKB 8101 column with a volume of 110 ml and Amphoteric carrier ampholytes, pH 3.5 to 5.0. A 0 to 40% sucrose density gradient containing 1% Amphoteric and 1 mM dithiothreitol was formed, and 2 to 4 ml of sample were applied. The voltage was set initially at 300 V and was increased to 800 V after 12 hr. The focusing was then conducted for 15 to 20 hr while the column was kept at 2°C. Fractions of 1.5 ml were...
Table 1

Effect of glutamine and GlcN-6-P on the purification of GlcN-6-P synthase from various rat tissues

GlcN-6-P synthase purified from rat tissues by fractionation with (NH₄)₂SO₄ (40 to 60 mg protein) was applied to a DEAE-Sephadex column (1.5 x 18 cm) previously equilibrated with 50 mM potassium phosphate (pH 7.2)-50 mM KCl-1 mM EDTA. Then a gradient of KCl from 0.05 to 0.5 M was applied, fractions of 2 ml were collected, and GlcN-6-P synthase was assayed in each fraction. In Experiment B, all the buffers used for the purification contained 20 mM glutamine and 0.05 mM GlcN-6-P. Values are given in percentage of recovery from the chromatographic step.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Liver (19-day)</th>
<th>Yoshida sarcoma</th>
<th>Whole fetus (12-day)</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. None</td>
<td>85</td>
<td>82</td>
<td>43</td>
<td>32</td>
</tr>
<tr>
<td>B. Glutamine + GlcN-6-P</td>
<td>90</td>
<td>78</td>
<td>76</td>
<td>80</td>
</tr>
</tbody>
</table>

collected, and pH (20°) and enzyme were assayed in each fraction.

**Assay of GlcN-6-P Synthase.** GlcN-6 synthase was assayed by measuring the formation of GlcN-6-P as described previously (8). The standard assay mixture contained 70 mM potassium phosphate (pH 7.5), 16 mM glutamine, 10 mM fructose 6-phosphate, 4 mM dithiothreitol, and enzyme in 1 ml. When assaying fractions obtained by isoelectric focusing, however, 200 mM potassium phosphate was used to adjust the pH to 7.5.

**Preparation of Antibody to Rat Liver GlcN-6-P Synthase.** For use in antibody production, adult liver GlcN-6-P synthase purified more than 200-fold by the procedure described previously (8) was further purified by filtration through a Sephadex G-200 column (1.5 x 40 cm) that had been equilibrated with 20 mM potassium phosphate (pH 7.5). The active fraction was concentrated by dialysis, and an adult albino rabbit was immunized by 5 s.c. injections of the concentrated enzyme (5 mg protein for each) at weekly intervals. Prior to injection, the enzyme was mixed with an equal volume of Freund’s complete adjuvant. Two weeks after the last injection, a booster injection was given; 1 week later, the rabbit was bled. γ-Globulin was precipitated from the antiserum between 20 and 33% saturation of (NH₄)₂SO₄, and purified further by elution from a DEAE-Sephadex column (1).

**Chemicals.** Fructose 6-phosphate was purchased from Boehringer, Mannheim, W. Germany, and GlcN-6-P and UDP-N-acetylglucosamine were from Sigma Chemical Co., Saint Louis, Mo. Carrier ampholytes were obtained from LKB Corp., Bromma, Sweden, and Freund’s complete adjuvant was from Iatron Laboratories, Tokyo, Japan.

**RESULTS**

**GlcN-6-P Synthase of Hepatomas as Compared with the Enzyme of Normal Adult Liver.** In the course of investigating GlcN-6-P synthase of normal adult liver and a hepatoma (Yoshida sarcoma), we found that, when glutamine and GlcN-6-P were absent, the hepatoma enzyme but not the liver enzyme was inactivated by purification (9). The subsequent studies demonstrated that the 2 enzymes also differ in kinetic, chromatographic, and immunological properties (9). It became possible to identify each form more easily by the use of isoelectric focusing, provided that the enzyme had been purified sufficiently.

Assay of the GlcN-6-P synthases from liver and 4 hepatomas by this technique gave the patterns shown in Chart 1. The hepatoma enzyme patterns (Chart 1, Curves B to E) are all alike, and the major peak, with a pl (isoelectric point) of 4.1, is clearly distinguished from the liver enzyme (Chart 1, Curve A), with a pl of 5.0. The data of Chart 1 indicate that the hepatoma enzymes are not as isozymically homogeneous as the liver enzyme. A minor peak seen at pH 5.0 is evidently due to the residual liver form. Although these data suggest that the physical forms of the tumor have little relation to the isozymic expression of GlcN-6-P synthase, it is also noteworthy that only the enzyme from solid AH-109A has a definite peak around 4.5.

**GlcN-6-P Syntheses of Fetal Tissues.** The hepatoma-type isozyme of aldolase (12), pyruvate kinase (3), and phosphorylase (10) are all reported to be present in late-fetal liver. In order to obtain further insights into the nature of the hepatoma-type isozyme of GlcN-6-P synthase, the enzyme purified from 19-day fetal liver was analyzed by isoelectric focusing. As shown in Chart 2A, the enzyme focused at pH’s of 4.7 and 5.0, and no peak could be observed at pH 4.1.

The failure to locate the hepatoma-type isozyme in late-fetal liver prompted attempts at searching for this form of enzyme in the earlier fetal tissues. The isoelectric focusing pattern of the enzyme purified from whole 12-day fetus (Chart 2B) points to the presence of only one form, which is similar to the hepatoma enzyme, as indicated by its pl of 4.1. The fetal enzyme was also similar to the hepatoma enzyme in that its stability was markedly increased by glutamine and GlcN-6-P (Table 1).
sues rich in GlcN-6-P synthase, such as brain, submaxillary gland, and testis, were studied, only the enzyme from brain was found to require glutamine and GlcN-6-P for stability (Table 1). The isoelectric focusing pattern of the purified brain enzyme (Chart 2C) demonstrates that the predominant form of GlcN-6-P synthase in this organ has a pI of 4.1.

The identity of the brain enzyme with the hepatoma enzyme and its nonidentity with the liver enzyme were further substantiated by kinetic and immunological data. Chart 3 shows that the concentration of UDP-N-acetylglucosamine required for 50% inhibition is 18 \( \mu \)M for the brain enzyme and 50 \( \mu \)M for the liver enzyme. Values of 20 and 50 \( \mu \)M were previously reported for the hepatoma and liver enzymes, respectively (9).

The brain enzyme, like the hepatoma enzyme (9), is distinguishable from the liver enzyme immunologically. As shown in Chart 4, an antibody to the liver enzyme, prepared as described under "Materials and Methods," effectively neutralized the liver enzyme, but there was little effect on the brain enzyme. Attempts have been made to prepare antibody to the hepatoma-type isozyme. The results, however, have been unsuccessful to date, presumably because of its lability.

**DISCUSSION**

Despite its important function in regulation of amino sugar biosynthesis, GlcN-6-P synthase has been characterized only inadequately because of its extremely low tissue content and stability (5, 8, 9). Under these circumstances, isoelectric focusing has been used in this laboratory to separate GlcN-6-P synthase into different forms. When subjected to this analysis, the liver enzyme focused at a pH of 5.0. Although we previously reported the pI of Yoshida sarcoma enzyme to be 4.5 (9), the present study clearly demonstrated that all the rat hepatomas studied, including Yoshida sarcoma, possess a form with a pI of 4.1 as the major component of GlcN-6-P synthase.

Protein precipitation, a major difficulty encountered with isoelectric focusing, has been overcome in the present study by using more extensively purified GlcN-6-P synthases for analysis. The yields of the purified enzymes were generally 40 to 50%, thereby excluding the possibility that only particular forms of the enzyme had been purified. Furthermore, at least for the liver and hepatoma enzymes, the data obtained by isoelectric focusing are in good accord with the chromatographic data that the hepatoma enzyme is a more acidic protein than the liver enzyme (9).

It is becoming increasingly apparent that liver neoplasia is associated with profound alterations of enzymes, in which isozymes characteristic of liver are replaced by nonhepatic isozymes (11, 15). Also noteworthy is the finding that the nonhepatic isozymes appearing anew in hepatomas are those that are expressed in the fetus but are repressed in adult liver (11, 15). These observations have been interpreted as indicating that neoplasia is a disease in which differentiation is severely affected.

As to GlcN-6-P synthase, the results of the present study extend previous data (9) and provide further support for the presence in rat hepatomas of an isozyme differing from the adult liver form. Although not detectable in 19-day fetal liver, this isozyme was found to be the sole form in whole 12-day fetus. The appearance of this pI 4.1 form in hepatomas is therefore considered to be an example of the fetal protein expression characteristic of neoplasia.
The carcinofetal isozyme of GlcN-6-P synthase is also present as the major form in adult brain. Similar observations have been reported by other investigators for the carcinofetal isozymes of aldolase (12), phosphorylase (10), and branched-chain amino acid transaminase (2). Because the brain develops much faster than other organs, one might expect the carcinofetal isozyme present in 12-day fetus to originate solely from the brain. However, the failure to locate any other form of GlcN-6-P synthase in the fetus argues against such a view. Furthermore, the level of GlcN-6-P synthase is much higher in the 12-day fetus than in adult brain.

Because GlcN-6-P synthase is a major regulatory enzyme for amino sugar biosynthesis (7), any marked alteration in this enzyme might result in alterations in cellular amino sugar-containing macromolecules such as glycoproteins, glycolipids, and mucopolysaccharides. Attention may therefore be directed to the possibility that the results of the present as well as previous studies (9) may provide reasonable explanations for at least some of the various alterations known to occur in these molecules upon carcinogenesis. Further investigations are necessary to clarify this point.

REFERENCES

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