Biochimica et Biophysica Acta, 422 (1976) 241—246

BBA 67687

GLUCOSAMINEPHOSPHATE SYNTHASE OF HUMAN LIVER

HISAKO KIKUCHI and SHIGERU TSUIKI

Biochemistry Division, Research Institute for Tuberculosis, Leprosy and Cancer, Tohoku University, Sendai (Japan)

(Received July 7th, 1975)

Summary

Although human liver contains glucosaminephosphate synthase (glucosaminephosphate isomerase (glutamine-forming), EC 5.3.1.19), its activity is rapidly lost during the course of extraction. The inactivation, however, is largely prevented if the extraction medium contains isopropanol at 1% concentration; using these "stabilized" extracts, the glucosaminephosphate synthase activity of human liver has been shown to be similar to the activity previously reported in rat liver. The enzyme precipitated from these extracts by $(NH_4)_2SO_4$ is inhibited by UDP-*N*-acetylglucosamine, the concentration required to produce a half-maximal inhibition being 6 μM. These results seem to be sufficient to postulate that glucosaminephosphate synthase is important for UDP-*N*-acetylglucosamine synthesis in human liver. In contrast to the rat liver enzyme, the $(NH_4)_2SO_4$-precipitated human liver enzyme is resistant to trypsin and undergoes no conversion reaction when incubated with glucose 6-phosphate.

Introduction

In rat and mouse liver, the synthesis of UDP-N-acetylglucosamine (UDP-GlcNAc) is initiated by glucosaminephosphate synthase (glucosaminephosphate isomerase (glutamine-forming), EC 5.3.1.19) [1—5], which catalyzes the following reaction:

Fructose 6-phosphate + glutamine → glucosamine 6-phosphate + glutamate

However, Pogell and Gryder [1] have reported that the enzyme is absent from rabbit and pigeon liver and present only in traces in calf and beef liver. Rabbit liver was also known by us to be completely devoid of the enzyme activity (unpublished observation), although beef liver was recently shown by Winterburn and Phelps [6] to possess considerable activity. While these observations lead to a suggestion that all mammalian species may not utilize glucosamine-

phosphate synthase for hepatic synthesis of UDP-GlcNAc, so far no studies have been reported on the activity of this enzyme in human liver. This information, however, is necessary for evaluating the physiological importance of glucosaminephosphate synthase and will be presented in this communication.

Materials and Methods

Source of glucosaminephosphate synthase

Human livers were obtained at autopsy, frozen within 2 h, and stored at −70° until use. These appeared disease-free when examined histopathologically.

Extraction and partial purification of the enzyme

After thawing, the tissue was cleaned of fat, cut into small pieces, suspended in 2 vol. of 0.154 M KCl containing 1% (v/v) isopropanol (pH adjusted to 7.5), and homogenized for 1 min in a glass tube with a Teflon pestle. The homogenate was immediately centrifuged at 105 000 × *g* for 1 h and the resulting supernatant was used for enzyme assay or subjected to further fractionation.

The above supernatant was brought to 40% saturation by addition of saturated $(NH_4)_2SO_4$ solution and the mixture was centrifuged at 18 000 × *g* for 10 min. Glucosaminephosphate synthase was then precipitated by bringing the supernatant to 60% saturation. The precipitate was collected by centrifugation, dissolved in a minimum volume of 50 mM potassium phosphate (pH 7.5)/50 mM KCl and passed through a desalting column of Sephadex G-25. The protein eluates were collected and used for enzyme studies. All these operations were conducted at 0—4° C.

Assay of the enzyme

Glucosaminephosphate synthase was assayed by the method described in the preceding paper [7] with free glucosamine as standard. Although glucosamine 6-phosphate (GlcN-6-*P*) has a molecular extinction coefficient that is approx. 85% that of free glucosamine, no correction was made for this difference. One unit of enzyme was defined as the amount which catalyzed the formation of 1 nmol of GlcN-6-*P* per h. Protein concentrations were measured by the phenol reagent [8].

Chemicals

The sources of chemicals were described in the preceding paper [7].

Results

In preliminary studies, post-mortem human livers were homogenized in 0.154 M KCl/1 mM EDTA/12 mM glucose 6-phosphate (Glc-6-*P*) (adjusted to pH 7.5). As soon as the homogenization was complete, the homogenate was centrifuged at 2000 × *g* for 10 min and the supernatant was further centrifuged at 105 000 × *g* for 1 h. When each supernatant was assayed for glucosaminephosphate synthase immediately after preparation, the second supernatant was found to contain only less than 10% of the activity present in the first super-

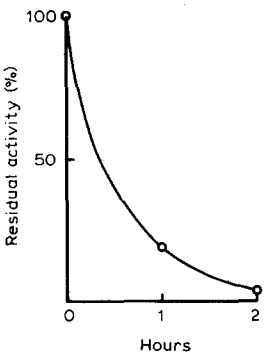


Fig. 1. Effect of aging of human liver extract at 0°C on glucosaminephosphate synthase activity. The tissue was homogenized in 0.154 M KCl/1 mM EDTA/12 mM Glc-6-*P* (pH 7.5). 2000 × *g* supernatant of the homogenate was then allowed to stand at 0°C.

natant (Table I). Since no enzyme activity was associated with the 105 000 × *g* pellet, the enzyme must have been inactivated rapidly in the course of the second centrifugation. This was substantiated by aging the 2000 × *g* supernatant at 0°C: as shown in Fig. 1, almost all activity was lost within 2 h.

The extent to which glucosaminephosphate synthase was recovered in the second supernatant thus offered a useful index for finding the agents that would prevent the enzyme from inactivation. In the experiment shown in Table I, Glc-6-*P* and fructose 6-phosphate (Fru-6-*P*), effective stabilizing agents for the rat liver enzyme [1,5,7,9—11], failed to protect the human liver enzyme; dithiothreitol was slightly effective, but much better protection was attained by isopropanol (10%, v/v). The latter was originally employed as the solvent for phenylmethane sulfonyl fluoride, a synthetic inhibitor for serine protease [12]. The inhibitor per se produced no protection.

TABLE I

EFFECT OF VARIOUS COMPOUNDS ON EXTRACTION OF GLUCOSAMINEPHOSPHATE SYNTHASE FROM HUMAN LIVER

Human liver was homogenized in different media indicated below but otherwise as described in the text. The homogenate was centrifuged at 2000 × *g* for 10 min and the supernatant was further centrifuged at 105 000 × *g* for 1 h. The two supernatants were assayed for glucosaminephosphate synthase in the absence of dithiothreitol immediately after preparation.

	105 000 × *g* sup. * / 2 000 × *g* sup.
0.154 M KCl + 1 mM EDTA + 12 mM Glc-6-*P*	0.06
+ 5 mM dithiothreitol	0.29
+ 10 mM Glc-6-*P*	0.23
+ 10 mM Fru-6-*P*	0.11
+ 15 mM glutamine	0.21
+ 10 % (v/v) isopropanol	0.67
+10% isopropanol	0.65

* The ratio of activity in 105 000 × *g* and 2000 × *g* supernatants.

TABLE II

EXTRACTION AND FRACTIONATION OF GLUCOSAMINEPHOSPHATE SYNTHASE FROM HUMAN LIVER

Details of the procedure were given in the text. The activities listed were those in the presence of dithiothreitol.

	Total activity (units)	Specific activity		Recovery (%)	—dithiothreitol * / + dithiothreitol
		(units/mg protein)	(units/g liver)		
105 000 × *g* supernatant	6264	34.8	3140	100	0.83
$(NH_4)_2SO_4$ precipitate					
0—40% saturation	396	10.2		6.3	
40—60%	1687	22.5		27	0.78
60—80%	58	1.1		0.9	

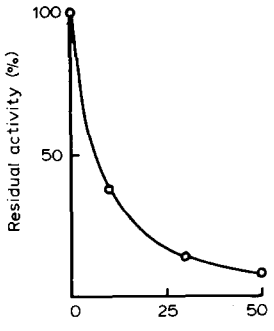
* The ratio of activity measured with and without dithiothreitol.

Different concentrations of isopropanol were then tested. It was found that the highest recovery of enzyme activity in the second supernatant (almost 90%) was seen at 1% (v/v). Many other alcohols were screened at the same concentration for their effect on glucosaminephosphate synthase. Only *n*-amyl-alcohol was found to be as effective as isopropanol in stabilizing the enzyme.

Accordingly, human livers were homogenized in 0.154 M KCl/1% (v/v) isopropanol (adjusted to pH 7.5) and the homogenates were centrifuged at 105 000 × *g* for 1 h. Assay of the resulting supernatants consistently gave glucosaminephosphate synthase activity of 26—35 units/mg of protein, or rather, 2200—3200 units/g of liver (see Table II). These values are not very far from the corresponding values for rat liver (2000—2300 units/g*). Omission of Fru-6-*P* caused a complete cessation of GlcN-6-*P* production, while if glutamine were omitted, the activity was reduced by 89%. NH_4Cl would not substitute for glutamine as amino donor. These results make it clear that the GlcN-6-*P* formation described above was exclusively due to glucosamine-phosphate synthase.

The most characteristic feature of glucosamine-phosphate synthase from rat liver is that it is inhibited by low concentrations of UDP-GlcNAc [3,5,11, 13,14]. In order to eliminate UDP-GlcNAc 2′-epimerase which might interfere with the inhibition [5], the human liver supernatant was fractionated by increasing concentrations of $(NH_4)_2SO_4$. As was the case with rat liver extracts [5,14], the 2′-epimerase was precipitated at 40% of saturated $(NH_4)_2SO_4$ and most of glucosaminephosphate synthase activity was recovered between 40 and 60% (Table II). As shown in Fig. 2, the precipitated glucosaminephosphate synthase was highly inhibited by UDP-GlcNAc and its concentration required to produce a half-maximal inhibition was 6 μM. This value is much lower than previously reported values for the rat liver enzyme (about 50 μM, ref. 5,14). The recovery of enzyme activity with $(NH_4)_2SO_4$ precipitation was remark-

* This value was obtained from the data given in Ref. 5 knowing that 1 g (wet weight) of liver yields approx. 90 mg of 105 000 × *g* supernatant protein.



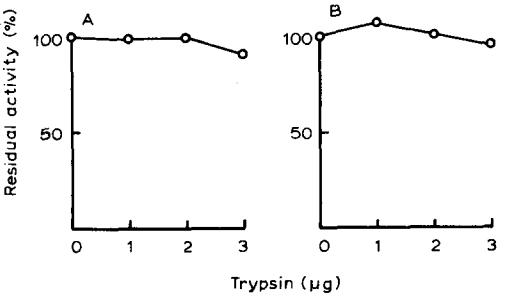


Fig. 2. Inhibition of glucosaminephosphate synthase by UDP-GlcNAc. The $(NH_4)_2SO_4$-precipitated enzyme fraction was assayed in the presence of varying concentrations of UDP-GlcNAc.

Fig. 3. Sensitivity of glucosaminephosphate synthase to digestion by trypsin. The $(NH_4)_2SO_4$-precipitated enzyme fraction was treated with varying amounts of trypsin either directly (A) or after incubation with 10 mM Glc-6-*P* at 37°C for 20 min (B). After the addition of soybean trypsin inhibitor, the activities remaining were determined. Details of the procedure were described elsewhere [7].

ably low (Table II), but once precipitated, the enzyme was rather stable and had lost only 15% of its activity after a month's storage at −25°.

We have reported in the preceding paper that glucosaminephosphate synthase prepared from rat liver by $(NH_4)_2SO_4$ precipitation is susceptible to digestion by trypsin, but becomes insusceptible upon incubation with Glc-6-*P* [7]. Also, during the incubation, the enzyme activity changed in such a manner that the ratio of activity measured without and with dithiothreitol increased to approach unity [7]. The $(NH_4)_2SO_4$-precipitated enzyme from human liver, however, differs from the rat liver enzyme in that the above activity ratio was 0.78 (Table II) (in contrast to 0.39 for the rat liver enzyme [7]) and that the activity was highly resistant to trypsin (Fig. 3). The enzyme exhibited no changes in activity (data not shown) and tryptic susceptibility (Fig. 3) upon incubation with Glc-6-*P*.

Discussion

It has well been established that in rat liver, the active synthesis of serum glycoproteins is dependent on the activity of glucosaminephosphate synthase [5]. The present studies on glucosaminephosphate synthase of human liver have yielded values ranging from 2200 to 3200 units/g for the tissue level of the enzyme. While these values are comparable with those previously reported for rat liver [5], the actual level of the enzyme in human liver may be still higher, since in the present work, the possibility of deterioration of the enzyme before, during and following autopsy cannot be excluded. These findings strongly indicate that in human liver, glucosaminephosphate synthase plays an important role in the biosynthesis of UDP-GlcNAc and therefore of serum glycoproteins. Also consistent with this view is the finding that the enzyme from human liver is inhibited by very low concentrations of UDP-GlcNAc. Thus

in human liver as well as in rat liver, UDP-GlcNAc controls its own rate of synthesis by feedback inhibition on the first enzyme of the pathway.

The glucosaminephosphate synthase of human liver, however, differs from the rat liver enzyme in its stability. Although the use of isopropanol markedly improved the stability of the human liver enzyme during extraction, the rat liver enzyme was sufficiently stable without this alcohol. Moreover, in contrast to the rat liver enzyme, the recovery of the human liver enzyme with $(NH_4)_2SO_4$ precipitation was strikingly low (Table II). Here isopropanol afforded little or no protection.

Another noteworthy difference between human liver and rat liver glucosaminephosphate synthase is the behavior of the $(NH_4)_2SO_4$-precipitated enzyme towards trypsin: although the rat liver enzyme became tryptic-insusceptible only after preincubation with Glc-6-*P* [7], the enzyme from human liver was resistant to trypsin already prior to preincubation (Fig. 3). The preceding work on rat liver glucosaminephosphate synthase has revealed that $(NH_4)_2SO_4$ precipitation readily converts the enzyme from the tryptic-insusceptible "form B" to the tryptic-susceptible "form A", which can be reconverted to form B by incubation with Glc-6-*P* [7].

It is as yet unknown how isopropanol stabilizes glucosaminephosphate synthase of human liver. One possible explanation is that isopropanol counteracted some inactivating agent released from the tissue during homogenization, since the effect of isopropanol appears to be confined to homogenization and extraction steps. This point is currently under investigation.

Acknowledgements

The authors wish to thank Dr. Shun Hosoda, Laboratory of Pathology, Aichi Cancer Center Institute, Nagoya, Japan, for the supply of human liver materials. The present work was supported by scientific research funds from the Ministry of Welfare of Japan.

References

1 Pogell, B.M. and Gryder, R.M. (1957) J. Biol. Chem. 228, 701—711
2 Ghosh, S., Blumenthal, H.J., Davidson, E. and Roseman, S. (1960) J. Biol. Chem. 235, 1265—1273
3 Kornfeld, S., Kornfeld, R., Neufeld, E.F. and O'Brien, P.J. (1964) Proc. Natl. Acad. Sci. U.S. 52, 371—379
4 Bates, C.J. and Handschumacher, R.S. (1969) Adv. Enzyme Regul. 7, 183—204
5 Kikuchi H., Kobayashi, Y. and Tsuiki, S. (1971) Biochim. Biophys. Acta 237, 412—421
6 Winterburn, P.J. and Phelps, C.F. (1973) in The Enzyme of Glutamine Metabolism (Prusiner, S. and Stadtman, E.R., eds.), pp. 343—363, Academic Press, New York
7 Kikuchi, H. and Tsuiki, S. (1976) Biochim. Biophys. Acta 422, 000—000
8 Lowry, O.H., Rosebrough, H.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
9 Gryder, R.M. and Pogell, B.M. (1960) J. Biol. Chem. 235, 558—562
10 Richards, T.C. and Greengard, O. (1973) Biochim. Biophys. Acta 304, 842—850
11 Winterburn, P.J. and Phelps, C.F. (1971) Biochem. J. 121, 701—710
12 Fahrney, D.E. and Gold, A.M. (1963) J. Am. Chem. Soc. 85, 997—1000
13 Kornfeld, R. (1967) J. Biol. Chem. 242, 3135—3142
14 Miyagi, T. and Tsuiki, S. (1971) Biochim. Biophys. Acta 250, 51—62