Biochimica et Biophysica Acta, 422 (1976) 231—240

BBA 67686

STABILIZATION OF GLUCOSAMINEPHOSPHATE SYNTHASE FROM RAT LIVER BY HEXOSE 6-PHOSPHATES

PROPERTIES AND INTERCONVERSION OF TWO MOLECULAR FORMS

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(Received July 7th, 1975)

Summary

Glucosaminephosphate synthase (glucosaminephosphate isomerase (glutamine-forming), EC 5.3.1.19) prepared from rat liver by extraction in the presence of glucose 6-phosphate (Glc-6-*P*) followed by precipitation with $(NH_4)_2SO_4$ is susceptible to digestion by trypsin. This enzyme, designated form A, can be converted to tryptic-insusceptible form B upon incubation with Glc-6-*P* or fructose 6-phosphate (Fru-6-*P*) at 37°C. The two forms also differ in the degree of activation by dithiothreitol, the degree of inhibition by methylglyoxal and the behavior on DEAE-Sephadex and Sephadex G-200 column chromatography.

During purification with DEAE-Sephadex followed by hydroxyapatite, form B is converted to form A if Fru-6-*P* is absent and form A to form B if Fru-6-*P* is present. The two forms are therefore interconvertible. Under the conditions of purification, form B is more stable than form A, since the purity and yield of the final product are greater with form B than with form A.

These findings suggest that the two forms of glucosaminephosphate synthase differ conformationally and that the equilibrium position depends on the concentration of Fru-6-*P*. Glc-6-*P* is effective only when it gives rise to Fru-6-*P* by mediation of glucose-phosphate isomerase.

Introduction

Glusosaminephosphate synthase (glucosaminephosphate isomerase (glutamine-forming), EC 5.3.1.19; formerly EC 2.6.1.16) catalyzes the formation of glucosamine 6-phosphate (GlcN-6-*P*) from fructose 6-phosphate (Fru-6-*P*) and glutamine, the first step in the pathway specific for the formation of UDP-*N*-acetylglucosamine (UDP-GlcNAc). The enzyme is involved in the control of

the pathway by feedback inhibition, since it is inhibited by low concentrations of UDP-GlcNAc [1,2]. It has recently been shown that the enzyme is particularly rich in neoplastic and fetal tissues [3—6], suggesting a possible participation in cell replication.

In spite of important physiological functions assigned to this enzyme, little is known as yet regarding its detailed structure and properties. The enzyme is extremely labile and undergoes a variety of inactivation- and alteration-reactions during extraction and purification [2,3,7]. The extent to which the enzyme is activated by dithiothreitol also varies considerably according to the tissue source and purification procedure [3,6].

Pogell and Gryder [8,9] were the first to show that glucose 6-phosphate (Glc-6-*P*) confers stability on glucosaminephosphate synthase from rat liver and most of the subsequent studies employed this sugar phosphate as stabilizing agent [2,3,5—7,10]. Winterburn and Phelps [11,12] on the other hand reported that the enzyme purified from rat liver with Fru-6-*P* is more stable than that without Fru-6-*P*. The nature of these stabilizing effects, however, is virtually unknown.

In the course of investigating glucosaminephosphate synthase from rat liver, it was observed that certain properties of the enzyme were altered when it was incubated with Glc-6-*P*. Studies that followed have revealed that the enzyme can exist in two interconvertible forms, the most note-worthy difference between them being susceptibility to proteolytic digestion. These findings are reported in detail in the present communication.

Materials and Methods

Preparation of glucosaminephosphate synthase

Livers from male Donryu rats (180—250 g) were homogenized for 1 min with 2 vol. of 0.154 M KCl/1 mM EDTA/12 mM Glc-6-*P* adjusted to pH 7.5. The homogenate was centrifuged at 105 000 × *g* for 1 h and from this supernatant, glucosaminephosphate synthase was precipitated between 40 and 60% of saturated $(NH_4)_2SO_4$. The precipitate was 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. All these operations were conducted at 0—4° C. Details of the procedure were given previously [7].

Incubation with Glc-6-P

The desalted enzyme solution (15—25 mg protein per ml) was added to 100 mM Glc-6-*P* in the ratio 1 : 9 (v/v) and the mixture was incubated at 37° C. At the times indicated, 0.1 ml aliquots were removed for assay.

Digestion with trypsin

0.1 ml of the glucosaminephosphate synthase sample was incubated with 0.7 ml of a solution containing 50 μmol of sodium phosphate (pH 7.5), 1 μmol of EDTA and various amounts of trypsin for 10 min at 37° C followed by soybean trypsin inhibitor (10 μg) and the remaining activity was assayed.

Assay

The standard assay mixture contained the following (μmol) in a final volume of 1 ml: sodium phosphate (pH 7.5), 50; glutamine, 15; Fru-6-*P*, 10; EDTA, 1; dithiothreitol (when indicated), 5; and enzyme. After incubation for 1 h at 37°C, the mixture was heated for 2 min at 100°C and centrifuged; GlcN-6-*P* was determined in the supernatant by the method of Ghosh et al. [13]. 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 [14].

Purification

All operations were performed at 0—4°C unless otherwise stated.

Form B: 18 ml of the $(NH_4)_2SO_4$-precipitated enzyme fraction prepared as above were divided into two equal parts. 1 ml of 100 mM Glc-6-*P* was added to one part, which was incubated for 20 min at 37°C and applied to a column of DEAE-Sephadex (10 ml) previously equilibrated with 50 mM potassium phosphate (pH 7.5)/50 mM KCl/2 mM Fru-6-*P*. The column was first washed with 80 ml of the same buffer and then with 40 ml of 150 mM KCl and 30 ml of 250 mM KCl, each in 50 mM potassium phosphate (pH 7.5)/2 mM Fru-6-*P*. The enzyme activity was eluted by a 250 mM KCl buffer solution and the solution was applied to a column of hydroxyapatite (5 ml) previously equilibrated with 50 mM potassium phosphate (pH 7.5)/50 mM KCl/2 mM Fru-6-*P*. After being washed with 10 ml of the same buffer, the column was washed with 20 ml of 150 mM potassium phosphate (pH 7.5) and 10 ml of 350 mM potassium phosphate (pH 7.5) each in 50 mM KCl-2 mM Fru-6-*P*. The enzyme activity emerged from the column with the last wash.

Form A: the remaining part of the $(NH_4)_2SO_4$-precipitated enzyme fraction was directly applied to a column of DEAE-Sephadex (10 ml) previously equilibrated with 50 mM potassium phosphate (pH 7.5)/50 mM KCl. The column was first washed with the same buffer and then with 100 and 300 mM KCl successively, each in 50 mM potassium phosphate (pH 7.5). The 300 mM KCl fraction, which contained enzyme activity, was applied to a column of hydroxyapatite. The procedure was the same as that for form A but Fru-6-*P* was omitted from the buffers. The enzyme activity was eluted by the last wash.

Chemicals and commercial enzymes

Glc-6-*P*, Fru-6-*P*, glucose 1-phosphate (Glc-1-*P*), fructose 1,6-diphosphate (Fru-1,6-P_2) and 6-phosphogluconate (6-*P*-gluconate), urease (soybean), catalase (beef liver) and lactate dehydrogenase (rabbit muscle) were the products of Boehringer. UDP-GlcNAc and soybean trypsin inhibitor were obtained from Sigma. Trypsin was purchased from Novo industri, Denmark, and methylglyoxal from Nakarai Chemicals, Kyoto.

Results

In previous papers from this laboratory [3,4], the glucosaminephosphate synthase of rat liver was shown to be prepared by extraction with Glc-6-*P* followed by fractionation with $(NH_4)_2SO_4$; the activity of this $(NH_4)_2SO_4$-

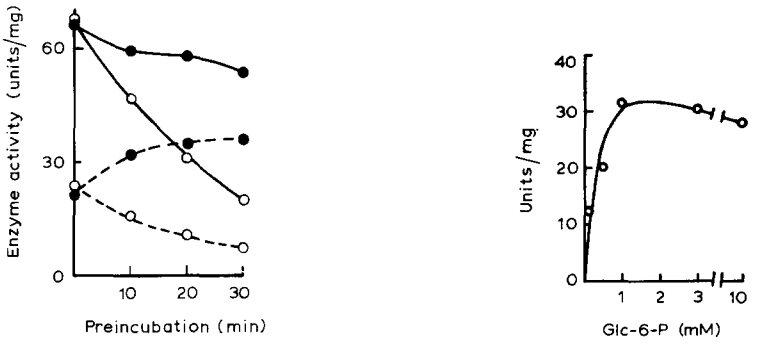

Fig. 1. Incubation of glucosaminephosphate synthase with Glc-6-*P*. The $(NH_4)_2SO_4$-precipitated enzyme fraction was incubated in the absence (○) or presence (●) of Glc-6-*P* under the conditions described in the text. Assays were made in the presence (———) or absence (- - - - -) of dithiothreitol.

Fig. 2. Effect of Glc-6-*P* concentration on the rate of activation of glucosaminephosphate synthase. The $(NH_4)_2SO_4$-precipitated enzyme fraction was incubated as described in the text, except that Glc-6-*P* concentration was varied. The enzyme incubated without Glc-6-*P* served as control. After 10 min, assay was made in the absence of dithiothreitol and the activity above the control value was plotted against Glc-6-*P* concentration.

precipitated enzyme was enhanced by dithiothreitol [3] and inhibited by methylglyoxal [15]. Fig. 1, however, shows that when incubated with 10 mM Glc-6-*P* at 37°C, the enzyme exhibits a time-dependent rise in activity without dithiothreitol, although little change occurs in the total activity (activity with dithiothreitol). The rate of "activation" is dependent on Glc-6-*P* concentration (Fig. 2) and if Glc-6-*P* is absent, the incubation merely leads to a progressive loss of enzyme activity (Fig. 1).

A detailed study of the properties of the enzyme was carried out before and after incubation with Glc-6-*P*. Although affinities for glutamine and UDP-GlcNAc were similar, the preincubated enzyme was inhibited by methylglyoxal much less profoundly than was the untreated enzyme (Fig. 3).

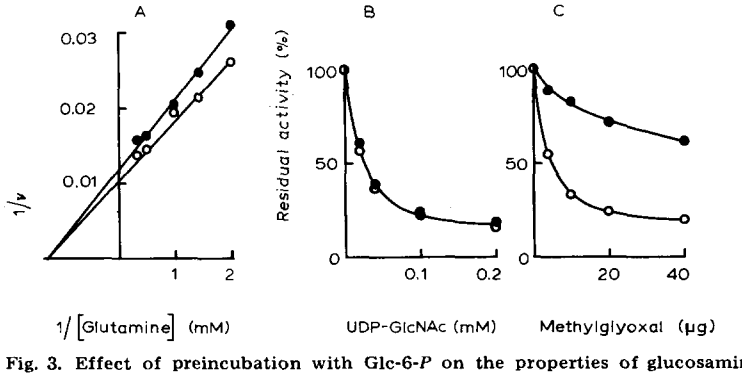

Fig. 3. Effect of preincubation with Glc-6-*P* on the properties of glucosaminephosphate synthase. The $(NH_4)_2SO_4$-precipitated enzyme fraction was examined either directly (○) or after incubation with Glc-6-*P* for 30 min (●) for: A, affinity for glutamine; B, inhibition by UDP-GlcNAc; and C, inhibition by methylglyoxal. Assays were made in the presence (A) or absence (B, C) of dithiothreitol. In A, the velocities were expressed in terms of units/mg of protein.

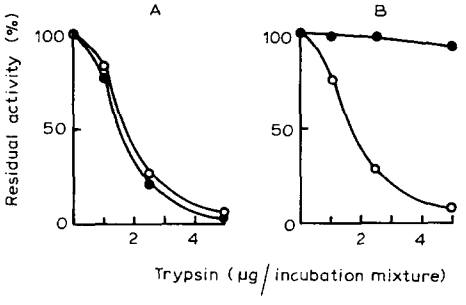

Fig. 4. Effect of preincubation with Glc-6-*P* on the tryptic susceptibility of glucosaminephosphate synthase. The $(NH_4)_2SO_4$-precipitated enzyme fraction was added with water in the ratio of 1 : 9 (v/v) (A) or 100 mM Glc-6-*P* (B) and they were incubated at 37°C. At 0 (○) and 30 min (●), aliquots were removed to incubate in the presence of trypsin. Remaining activities were then determined. The experimental conditions were given in detail in the text.

Perhaps the most interesting finding, however, is that the enzyme preincubated with Glc-6-*P* is no longer inactivated by trypsin (Fig. 4). From the results of control experiments with either Glc-6-*P* or incubation omitted, both Glc-6-*P* and incubation were shown to be essential for the appearance of tryptic insusceptibility. As shown in Table I, Glc-6-*P* could be replaced by Fru-6-*P* or Glc-1-*P* but not by other compounds listed. Fru-6-*P* and Glc-1-*P* were also capable of promoting other alterations caused by Glc-6-*P*.

In order to obtain further insights into the nature of these alterations, the $(NH_4)_2SO_4$-precipitated enzyme fraction was chromatographed on a column of DEAE-Sephadex before and after incubation with Glc-6-*P* (Fig. 5). In both cases, the enzyme activity emerged as a single symmetrical peak, but the peak from untreated enzyme was clearly ahead of the preincubated enzyme peak. The latter was also distinguishable from the former by the lower degree of

TABLE I

EFFECTS OF VARIOUS METABOLIC INTERMEDIATES ON TRYPTIC SUSCEPTIBILITY OF GLUCOSAMINEPHOSPHATE SYNTHASE

The $(NH_4)_2SO_4$-precipitated enzyme fraction was incubated with metabolic intermediate indicated below (10 mM) at 37°C. Before and after incubation, aliquots were removed and treated with 5 μg trypsin for 10 min at 37°C.

Intermediates	Activity after trypsin (%)	
Incubation:	0 (min)	30 (min)
None	7	10
Glc-6-*P*	9	90
Glc-1-*P*	8	93
Fru-6-*P*	10	93
Fru-1,6-P_2		ND *
6-*P*-gluconate	7	13
Glutamine	8	10
Glutamate	7	8

* Tryptic susceptibility could not be determined since the enzyme had totally been inactivated by preincubation with Fru-1,6-P_2.

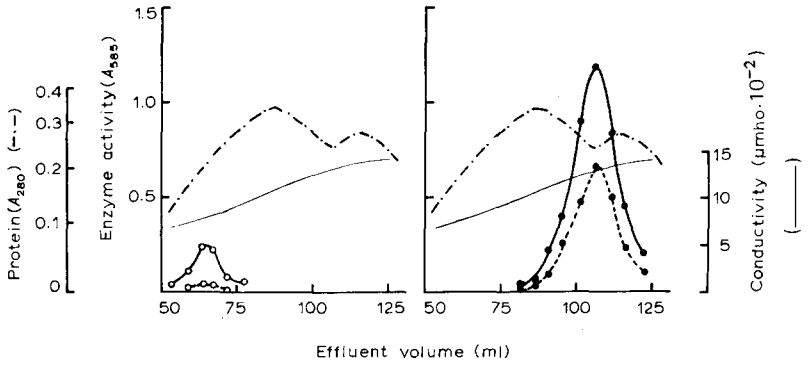

Fig. 5. Effect of preincubation with Glc-6-*P* on the chromatographic behavior of glucosaminephosphate synthase. The $(NH_4)_2SO_4$-precipitated enzyme fraction (approx. 200 mg in protein) was applied to a column of DEAE-Sephadex (1.5 cm × 10 cm) either directly (○) or after incubation with Glc-6-*P* for 30 min (●). The column, which had been equilibrated with 50 mM sodium phosphate (pH 7.5)/50 mM KCl/1 mM EDTA, was washed with 130 ml of the same buffer. A linear gradient, from 50 to 500 mM KCl, was then applied and fractions of 5 ml were collected at a flow rate of 10 ml/h. When the preincubated sample was chromatographed, all the buffers contained 2 mM Fru-6-*P*. The enzyme activity was assayed in the presence (———) and absence (- - - - -) of dithiothreitol.

activation by dithiothreitol (Fig. 5) and relative insensitivity to methylglyoxal and trypsin (data not shown). It is therefore apparent that the incubation with Glc-6-*P* converts the enzyme from one form to another.

This conclusion was substantiated by gel filtration experiments on Sephadex G-200 (Fig. 6). The activity of preincubated enzyme appeared in a volume corresponding to a molecular weight of 340 000, whereas the volume for untreated enzyme corresponded to 410 000. This difference, however, probably reflects a difference in conformation rather than molecular weight (see Discussion).

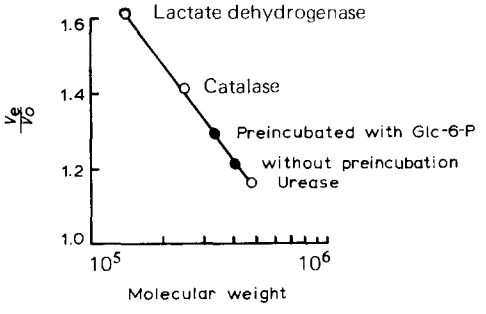

Fig. 6. Effect of preincubation with Glc-6-*P* on the gel filtration of glucosaminephosphate synthase. The $(NH_4)_2SO_4$-precipitated enzyme fraction was applied, either directly or after preincubation with Glc-6-*P* for 20 min, to a column of Sephadex G-200 (2.5 cm × 90 cm) previously equilibrated with 50 mM sodium phosphate (pH 7.5)/50 mM KCl/1 mM EDTA. The same buffer was used for elution. Fractions of 5 ml were collected at a flow rate of 10—15 ml/h. When the preincubated sample was chromatographed, the buffer contained 2 mM Glc-6-*P*. Rabbit muscle lactate dehydrogenase (mol. wt. 140 000), beef liver catalase (mol. wt. 250 000) and soybean urease (mol. wt. 480 000) were used as markers. ● shows glucosaminephosphate synthase.

TABLE II

PURIFICATION OF TWO FORMS OF GLUCOSAMINEPHOSPHATE SYNTHASE

Details of the procedure were given in text.

Steps	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (-fold)	Recovery (%)	—Dithiothreitol / +Dithiothreitol
Form A:						
1. 105 000 × *g* supernatant	14 427	589	24.5	1	100	1.0
2. $(NH_4)_2SO_4$-precipitate	23 258	191	122	5	161 *	0.39
3. DEAE-Sephadex	7 891	21.0	360	15	54	0.38
4. Hydroxyapatite	4 141	1.7	2 264	94	29	0.17
Form B:						
3. Incubation with Glc-6-*P*	22 362	191	117	5	155	0.61
4. DEAE-Sephadex	13 536	13.1	1 033	42	94	0.64
5. Hydroxyapatite	11 240	0.8	14 051	574	78	0.55

* This increase was due to the removal of inhibitors by $(NH_4)_2SO_4$ precipitation (3,15).

The nature and interrelationship of the two forms of glucosaminephosphate synthase were investigated through purification. In the experiment in Table II, half of the $(NH_4)_2SO_4$-precipitated enzyme was incubated with Glc-6-*P* and further purified in the presence of 2 mM Fru-6-*P* (the reason will be shown below). An almost 600-fold purification could be achieved with 78% recovertry of activity (Table II, form B). If the incubation and use of Fru-6-*P* were omitted, the purification achieved was only 90-fold with 30% recovery of activity (Table II, form A).

The effects of trypsin and methylglyoxal on enzyme activity were examined to see whether the two forms could have been purified without any gross change in their properties. The results given in Fig. 7 (solid lines) show that after purification, the enzyme preincubated with Glc-6-*P* (form B) still retained its insensitive nature to these agents while the untreated enzyme (form A) remained extremely susceptible. The higher degree and yield of purification observed for the preincubated enzyme (Table II) are therefore indicative of form B being much more stable than form A under the conditions of purification. In Fig. 7, form A was much less sensitive than form B to UDP-GlcNAc. But this is probably due to "desensitization" during the purification [2,7], since the conversion reaction per se does not affect UDP-GlcNAc inhibition (Fig. 3).

Fig. 7 also demonstrates that Fru-6-*P* is vitally important to the purification of glucosaminephosphate synthase. If form B is purified in the absence of Fru-6-*P*, one obtains a product that is almost identical with form A. Form A on the other hand gives rise to a product having properties intermediate of A and B if Fru-6-*P* is present during purification. These results leave little doubt that forms A and B are interconvertible, the equilibrium being shifted in favor of B if Fru-6-*P* is present.

Contrary to the results with crude enzyme (Table I), Glc-6-*P* could not replace Fru-6-*P* in the experiments shown in Fig. 7. When the $(NH_4)_2SO_4$-precipitated fraction was tested for glucosephosphate isomerase, sufficient en-

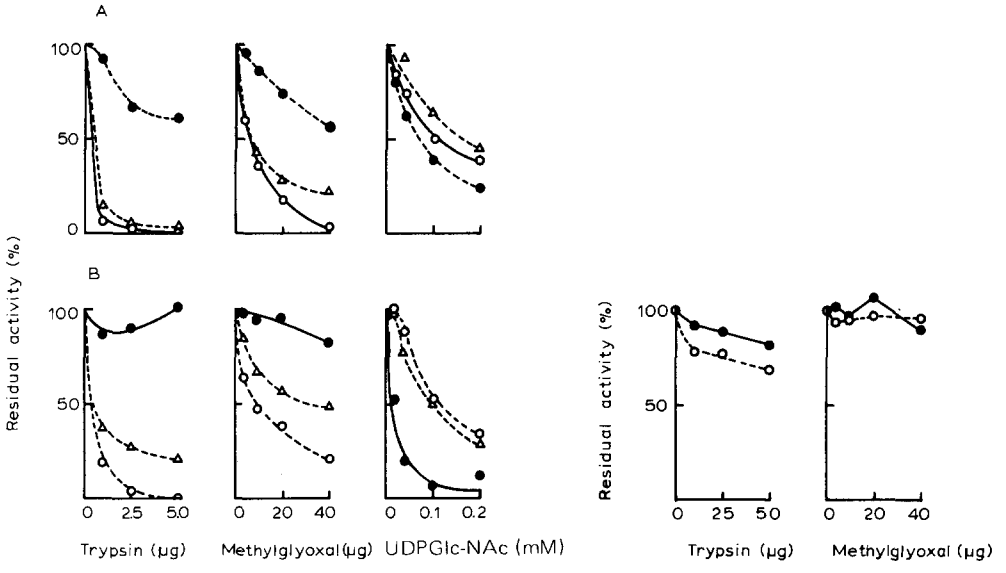

Fig. 7. The properties of glucosaminephosphate synthase purified by DEAE-Sephadex followed by hydroxyapatite. The $(NH_4)_2SO_4$-precipitated enzyme fraction was purified, either directly (A) or after incubation with Glc-6-*P* (B), by the procedure described in the text. The chromatography was executed with buffers only (○), with buffers containing 2 mM Fru-6-*P* (●) or 2 mM Glc-6-*P* (△). The products were examined for tryptic susceptibility, inhibition by methylglyoxal and inhibition by UDP-GlcNAc. UDP-GlcNAc inhibition was measured in the presence of 10 mM Glc-6-*P* [7]. The solid lines in A and B represent form A and form B of Table II, respectively.

Fig. 8. Sensitivity to trypsin and methylglyoxal of glucosaminephosphate synthase in rat liver extracts. Rat liver was homogenized in 0.154 M KCl/1 mM EDTA (○) or in 0.154 M KCl/1 mM EDTA/12 mM Glc-6-*P* (●) and the 105 000 × *g* supernatant was used for examinations. Assays were made in the absence of dithiothreitol.

zyme was found to cause complete equilibration of Fru-6-*P* and Glc-6-*P* almost instantly [7]. The enzyme, however, is completely removed at the DEAE-Sephadex step [7]. A conclusion can be drawn from these observations that Fru-6-*P*, but not Glc-6-*P*, is essential for the conversion of form A to B.

As previously reported [3,5,8], the presence of Glc-6-*P* during homogenization of rat liver yields extracts with a much higher activity of glucosaminephosphate synthase. The Glc-6-*P*-mediated conversion of form A to B, however, may not be used for interpreting this effect of Glc-6-*P*, since, judging from the behavior towards trypsin and methylglyoxal (Fig. 8), even the extracts prepared in the absence of Glc-6-*P* contain the enzyme mostly in form B. The observation then indicated that the $(NH_4)_2SO_4$ precipitation may facilitate the B to A conversion.

In a separate experiment, form B of glucosaminephosphate synthase, prepared by incubating the $(NH_4)_2SO_4$-precipitated fraction with Glc-6-*P* was recovered from the incubation mixture by precipitation with $(NH_4)_2SO_4$. The enzyme obtained in this manner was readily inactivated by trypsin. Therefore, there is no doubt that $(NH_4)_2SO_4$ precipitation converts form B to form A.

Discussion

The present work leaves little doubt that glucosaminephosphate synthase of rat liver can exist in two different forms: one, called form B, is highly resistant to tryptic digestion and the other, form A, is susceptible to this proteolytic attack. These forms are interconvertible, the equilibrium being shifted in favor of A or B depending on the presence or absence of Fru-6-*P*, respectively. We have also shown that form B is readily converted to form A upon precipitation with $(NH_4)_2SO_4$.

Winterburn and Phelps [11,12] have reported that Fru-6-*P* stabilizes glucosaminephosphate synthase. They showed that the enzyme purified from rat liver with Fru-6-*P* as stabilizer (preparation II) is more stable than that without Fru-6-*P* (preparation I). These preparations differed from each other kinetically and were convertible to each other relatively slowly. Although preparations I and II of Winterburn and Phelps have not been tested for proteolytic digestion, they may be identical or similar to form A and B, respectively, of the present work.

The present finding that Fru-6-*P* converts the enzyme to a tryptic-insusceptible form (form B) has therefore enabled us to provide a rational explanation for the stabilizing effect of Fru-6-*P* and also for the usefulness of Fru-6-*P* for purification of the enzyme, which was first reported by Winterburn and Phelps [11,12] and has been confirmed by the present work. The stabilizing effect of Glc-6-*P* [2,3,5,10] appears to be exerted after its conversion to Fru-6-*P*. However, as already noted (Fig. 8) all of the stability confered by Glc-6-*P* on the enzyme cannot be explained by this mechanism.

In the experiment shown in Fig. 9, the $(NH_4)_2SO_4$-precipitated fraction used for incubation with Glc-6-*P* was 21 or 4 mg per ml. It is evident that the rate of conversion from A to B is lower if the enzyme concentration is lower. While the reason is as yet unclear, the finding may be useful for explaining why the conversion does not take place under the assay conditions.

The data in Fig. 6 exclude the possibility that either form is a dimer or aggregate of the other. The possibility of the A to B conversion being associated with the loss of peptide would not be in keeping with the ease of

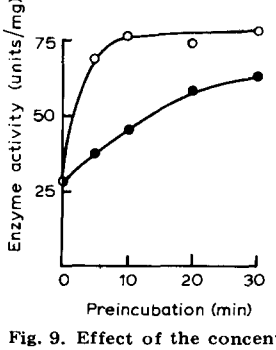

Fig. 9. Effect of the concentration of the $(NH_4)_2SO_4$-precipitated enzyme fraction on the activation of glucosaminephosphate synthase by Glc-6-*P*. The fraction was incubated with Glc-6-*P* at two different protein concentrations (21.6 mg/ml, ○; and 4.3 mg/ml, ●). Aliquots were removed with time and assayed for glucosaminephosphate synthase activity in the absence of dithiothreitol.

reconversion of form B to form A. Although the lower sensitivity of form B to dithiothreitol and methylglyoxal, compared with form A, may suggest that the SH-groups of the enzyme are involved in the interconversion, dithiothreitol alone could not confer tryptic-insusceptibility on the enzyme. Rather, it enhanced inactivation of the enzyme when incubated together at 37°C. ATP plus cyclic AMP neither enhanced nor reversed the A to B conversion, thereby excluding the possibility that the two forms are products of phosphorylation or dephosphorylation (unpublished observations).

The most likely explanation, after all, appears to be that the two forms of glucosaminephosphate synthase differ only conformationally. According to Frieden [16], hysteretic enzymes are defined as those enzymes which respond slowly to a rapid change in ligand concentration. Glucosaminephosphate synthase of rat liver appears to fulfill this criterion together with a number of enzymes occurring at a strategic point in a metabolic pathway. For glucosaminephosphate synthase, however, a hysteretic response appears primarily as a decrease in proteolytic susceptibility rather than an alteration in velocity.

At present, it is not known if the interconversion has any physiological significance. Although the two forms have been distinguished primarily on the basis of tryptic susceptibility, they may also differ in their stability in vivo. An indication of difference is the behavior of the two forms towards purification. If so, the interconversion could contribute to the control of degradative rate of glucosaminephosphate synthase.

Acknowledgements

The present work was supported by scientific research funds from the Ministry of Education of Japan.

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