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Glucosamine synthesis in mast cells

Heparin is found in mast cells and it is generally accepted that its synthesis takes place in these cells[1]. Several nucleotide sugars which are apparently involved in heparin biosynthesis and some of the enzymes which catalyze their formation, have been shown to be present in transplantable mast cell tumors[2–5]. Mastocytoma preparations are also known to be active in sulfation reactions required for the formation of heparin[6,7].

One of the necessary steps in the early stages of the sequence toward heparin synthesis is the formation of glucosamine (2-amino-2-deoxy-D-glucopyranose). The present study was made to characterize this reaction in mast cell tumors. It has been shown previously that rat liver and various micro-organisms contain an enzyme, L-glutamine:D-fructose-6-phosphate amidotransferase (EC 2.6.1.16) which catalyzes the reaction:

D-Fructose-6-*P* + L-glutamine ⇌ D-glucosamine-6-*P* + L-glutamate

This report describes the purification of such an enzyme from mast cell tumors and defines some of its characteristics.

The source of mast cells was the transplantable FURTH mastocytoma[9] growing subcutaneously in LAF mice. Tumors were homogenized at 0–3° for 3 min in a Potter–Elvehjem homogenizer with a solution (4 ml/g) containing 0.15 M KCl, 0.004 M EDTA and 0.001 M mercaptoethanol adjusted to pH 7.5. The mixture was centrifuged at 1800 × *g* for 1 h and the supernatant solution (Fraction I) was used for subsequent purification.

The pH of Fraction I was adjusted to 7 and 0.1 vol. of a 2% solution of protamine sulfate was added dropwise. The mixture was stirred for 5 min, centrifuged and the precipitate was discarded. 10 ml of the clear supernatant was stirred for 5 min with 500 mg DEAE-cellulose which was previously equilibrated with a mixture of 0.2 M KCl and 0.01 M phosphate, pH 7. The DEAE-cellulose was collected by filtration and washed with 10 ml of buffer to which was added 1.0 ml 0.01 M mercaptoethanol. The absorbed enzyme was then eluted with two 5-ml portions of a solution composed of 0.2 M KCl, 0.01 M phosphate, pH 7.2, and 0.001 M mercaptoethanol. The combined eluates were designated as Fraction II. Addition of 0.25 vol. of ethanol to this fraction at −10° yielded a precipitate which was collected by centrifugation. This was redissolved in 0.15 M KCl containing 0.004 M EDTA and 0.2% mercaptoethanol, pH 7.2 (Fraction III).

A certain degree of purification could also be achieved by ethanol fractionation of the original extract. To 10 ml of Fraction I at −10° were added 1.75 ml of ethanol and the precipitate was removed by centrifugation. Further addition of ethanol, 1.8 ml per 10 ml solution, yielded a precipitate which was collected and redissolved in the original extracting medium (Fraction I-A).

The assay for enzyme activity was based on determination of the amount of hexosamine formed when aliquots from desired fractions were incubated with excess substrate. The standard mixture contained 20 μmoles D-fructose 6-phosphate, 20 μmoles of L-glutamine, 30 μmoles sodium phosphate buffer, pH 7.3, 10 μmoles

glutathione and the desired amount of protein in volume of 1 ml. After incubation of the mixtures for 30 min at 30°, they were placed in boiling water for 1 min, centrifuged, and an aliquot was assayed for hexosamine as described by GHOSH *et al.*[8]. Alternatively, 1.0 ml of 0.4 M trichloroacetic acid was added to the incubation mixture and hexosamine was determined by the acetylacetone method[10]. Controls contained the same components except that L-glutamine was added just before the denaturation step.

A unit of enzyme activity is defined as the amount of enzyme which causes the formation of 1 μmole of glucosamine 6-phosphate per h under conditions of the assay. Specific activity is equal to the number of units/mg of protein. Protein was determined by the procedure of LOWRY *et al.*[11]. The results of the purification are shown in Table I.

TABLE I

PURIFICATION OF THE FRUCOSE PHOSPHATE AMINOTRANSFERASE
Total units are given per 10 g of tissue.

Fraction	*Specific activity*	*Total units*	*Percent yield*
I	0.048	5.12	100
I-A	0.130	1.74	34
II	0.141	0.92	18
III	0.454	0.72	14

The enzyme was comparatively unstable even when kept at 0°. The original extract (Fraction I) lost over 60% of its activity in 24 h. With Fraction I-A, only 15% of the activity was retained when kept overnight. Addition of glucose 6-phosphate had only a slight stabilizing effect. Extended purification could thus not be carried out and all assays had to made on the same day as the extraction and fractionation.

The glucosamine 6-phosphate was identified from a scaled up incubation mixture by paper chromatography and paper electrophoresis in a manner similar to that described by GHOSH *et al.*[8].

The pH optimum for the reaction was found to be 7.2–7.6. The apparent K_m for fructose-6-*P* calculated by the method of LINEWEAVER AND BURK[12] is about $1.9 \cdot 10^{-3}$. The value for glutamine is $1.1 \cdot 10^{-3}$ M.

When fructose-6-*P* was replaced by glucose-6-*P* or fructose in the reaction mixture, glucosamine-6-*P* was not formed. Similarly glutamic acid or ammonium chloride could not be substituted for glutamine. ATP and magnesium are not required for the reaction; in fact, when added in equimolar concentration with the substrates, there was an over 50% decrease in activity.

Both *O*-diazoacetyl-L-serine (azaserine) and 6-diazo-5-oxo-L-norleucine inhibit the reaction as they do in other transaminations involving glutamine. 6-Diazo-5-oxo-L-norleucine is a more potent inhibitor in that 0.1 μmole causes 42% inhibition. With azaserine 1.0 μmole effects only 22% inhibition.

The results show that mast cells contain an aminotransferase which catalyzes the formation of glucosamine-6-*P* from fructose-6-*P* and glutamine. This reaction may thus be involved in the biosynthesis of the hexosamine required as a component of heparin.

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