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Partial Purification and Properties of L-Glutamine D-Fructose 6-Phosphate Amidotransferase from *Phaseolus aureus*[1]

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ABSTRACT

L-Glutamine D-fructose 6-phosphate amidotransferase (EC 2.6.1.16) was extracted and purified 600-fold by acetone fractionation and diethylaminoethyl cellulose column chromatography from mung bean seeds (*Phaseolus aureus*). The partially purified enzyme was highly specific for L-glutamine as an amide nitrogen donor, and L-asparagine could not replace it. The enzyme showed a pH optimum in the range of 6.2 to 6.7 in phosphate buffer. Km values of 3.8 mM and 0.5 mM were obtained for D-fructose 6-phosphate and L-glutamine, respectively. The enzyme was competitively inhibited with respect to D-fructose 6-phosphate by uridine diphosphate-*N*-acetyl-D-glucosamine which had a Ki value of 13 μM. Upon removal of L-glutamine and its replacement by D-fructose 6-phosphate and storage over liquid nitrogen, the enzyme was completely desensitized to inhibition by uridine diphosphate-*N*-acetyl-D-glucosamine. This indicates that the inhibitor site is distinct from the catalytic site and that uridine diphosphate-*N*-acetyl-D-glucosamine acts as a feedback inhibitor of the enzyme.

L-Glutamine D-fructose 6-phosphate amidotransferase (EC 2.6.1.16), the first enzyme on the pathway for the biosynthesis of uridine diphosphate-*N*-acetyl-D-glucosamine (UDPAG)[3], catalyzes the following irreversible reaction:

D-fructose 6-P + L-glutamine

→ D-glucosamine 6-P + L-glutamate.

This enzyme was first partially purified from rat liver by Pogell and Gryder (11) and was further purified from rat liver, *Escherichia coli*, and *Neurospora crassa* by Ghosh *et al.* (3). Using enzyme preparations devoid of phosphohexose isomerase, the latter workers demonstrated that D-fructose 6-P was the sole hexosephosphate substrate. Proof for the feedback inhibition of the mammalian enzymes by UDPAG and for the absence of such inhibition in bacterial enzymes was shown by Kornfeld *et al.* (6) and Kornfeld (5).

The presence of this enzyme in higher plants was demonstrated by Mayer *et al.* (8) who showed that D-glucosamine 6-P was synthesized by D-fructose 6-P with crude extracts of mung bean seeds (*Phaseolus aureus*), using either L-glutamine or L-asparagine as the amide nitrogen donor. They were able to purify partially the enzyme capable of transferring amide nitrogen from L-glutamine and also to demonstrate the inhibition of this enzyme by UDPAG. However, the type of inhibition was not studied, and also they could not demonstrate amide nitrogen transfer from L-asparagine with their partially purified enzyme fractions.

The present paper will deal with the partial purification of L-glutamine D-fructose 6-P amidotransferase from *Phaseolus aureus* by acetone fractionation and DEAE-cellulose column chromatography. A study of some of the kinetic parameters including feedback inhibition of the partially purified enzyme will be made and compared with the data reported for mammalian and bacterial enzymes.

MATERIALS AND METHODS

Materials. All reagents were obtained from commercial sources. The barium salt of D-fructose 6-P (Sigma Chemical Co.) was converted to its potassium salt by mixing equal volumes of equimolar solutions of the sugar phosphate and K_2SO_4, removal of $BaSO_4$ by centrifugation and adjustment of the solution to the desired pH by the addition of one to two drops of concentrated HCl solution. The barium salt of D-glucosamine 6-P was converted to its free acid by the method of Mayer *et al.* (8).

A "buffer mixture" based on that used by Ghosh *et al.* (3) was frequently employed throughout the experiment. It contained the following reagents in μmoles per ml: L-glutamine, 150; potassium phosphate buffer, pH 7.3, 10; and EDTA-disodium salt, 2.0 (final pH adjusted to 7.3 by adding a few drops of 45% KOH).

Methods. D-Glucosamine 6-P was determined on 0.25-ml aliquots of the incubation mixtures, using a modification of the procedure described by Ghosh *et al.* (3). The modification consisted of decreasing the volume of all reagents except Ehrlich's reagent to one-fourth and increasing the incubation period at 37 C from 20 min to 1 hr for complete color development.

Ehrlich's reagent was prepared by adding 2 ml of 12 N HCl to 2 g of *p*-dimethylaminobenzaldehyde and bringing the volume to 100 ml with glacial acetic acid. Routinely, 0.75 ml of the reagent was used per determination. The potassium tetraborate (Sigma Chemical Co.) was substituted for sodium borate as recommended by Ghosh *et al.* (3). Standard curves were established using known solutions of D-glucosamine 6-P treated in the same manner. D-Fructose 6-P was determined by the

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[3] Abbreviations: UDPAG: uridine diphosphate-*N*-acetyl-D-glucosamine; DEAE: diethylaminoethyl.

Roe method (12) and protein by the procedure of Lowry *et al.* (7).

Enzyme Assay. The standard incubation mixtures for the assay of the enzyme included the following reagents in μmoles in a final volume of 0.5 ml: D-fructose 6-P, pH 6.5, 7.0; L-glutamine, 15; potassium phosphate buffer pH 6.5, 30; EDTA disodium salt, 1.2; potassium phosphate buffer (pH 7.3, originating from the enzyme solution), 1.0; and a given amount of enzyme protein. The mixture was incubated for 1.5 hr at 30 C, and the reaction was inactivated by boiling the mixture for 2 min. After centrifugation, a 0.25-ml aliquot of the reaction mixture was analyzed for D-glucosamine 6-P as described. Control tubes, prepared in the same manner, except for their boiling for 2 min prior to incubation at 30 C, were used to set the colorimeter to zero absorbance.

Preparation of the Crude Enzyme. The crude enzyme extract was prepared by the procedure of Mayer *et al.* (8), except that the weight of the decoated beans used was increased 4-fold. Mung bean seeds were soaked overnight in distilled water; they were rinsed with distilled water several times, blotted with paper towels, and decoated. Sixty-four grams of the decoated beans were chilled at 4 C for 2 hr, ground in a mortar with acid washed sand in 20 ml of 50 mM phosphate buffer made 2 mM in EDTA, pH 6.6, and filtered through two layers of cheesecloth. The extract was then centrifuged at 12,000*g* for 12 min, and the supernatant fluid was employed as the source of crude enzyme.

Acetone Fractionation. To 15 ml of the crude extract, 5.0 ml of cold acetone (−20 C) were gradually added, while the enzyme solution was constantly being stirred. The turbid solution formed was centrifuged at 27,000*g* for 10 min at −5 C. To the entire resulting supernatant solution, 3.1 ml of cold acetone (−20 C) were gradually added, and the turbid solution was centrifuged again as before. The precipitate formed was suspended in 5.0 ml of buffer mixture and left overnight at 4 C for the complete solubilization of the precipitate. The suspension was centrifuged at 27,000*g* for 10 min at 0 C; the supernatant solution is referred to as the "acetone fraction."

Ultrafiltration. To 27 ml of the acetone fraction (18–21 mg protein per ml), buffer mixture was added to a final volume of 50 ml. The solution thus obtained was passed through an XM-300 Diaflo membrane (Amicon Corporation, Lexington, Mass.) under a nitrogen pressure of 25 p.s.i. The solution was concentrated to a final volume of 5 to 7 ml. This treatment results in the removal of most of the proteins having a molecular weight below 300,000 and allows a more efficient purification of the enzyme protein by the DEAE-cellulose column. The final volume of the XM-300 fraction was brought up to 14 ml with buffer mixture prior to application to a DEAE-cellulose column.

DEAE-Cellulose Column Chromatography. The DEAE-cellulose (Schleicher and Schuell Co., Keene, N. H.) was washed with 1 N NaOH, 1 N HCl, and 1 N NaOH and equilibrated with 10 mM phosphate buffer, pH 7.3, containing 2 mM EDTA. A column (25 × 2.5 cm) was packed with the DEAE-cellulose slurry and washed with 500 ml of the buffer mixture. Twelve milliliters of XM-300 enzyme fraction (20–27 mg protein per ml) was applied to the column and eluted with the same buffer. The resulting eluate gave a large protein peak shown in Figure 1 (peak I). The elution was continued with the aid of a linear salt gradient by using two 500-ml containers each containing buffer mixture (pH 7.3) to one of which 1 M KCl was added, while the other had no KCl. Five-milliliter fractions were collected every 15 min. The protein elution pattern was established by reading the absorbance at 280 nm against the buffer mixture. Different protein peaks were pooled separately and concentrated to a final volume of 5 to 7 ml by ultrafiltration over a PM-30 Diaflo membrane (Amicon Corporation) at a nitrogen pressure of 50 p.s.i. The solution from each concentrated peak was assayed for enzyme activity under standard assay condition and the fraction containing the enzyme was stored over liquid nitrogen. This fraction is referred to as "DEAE-cellulose fraction".

Removal of L-Glutamine from Partially Purified Enzyme. Five-milliliters of the DEAE-cellulose fraction was passed through a column of coarse Sephadex G-25 (28 × 2.5 cm) previously equilibrated with a buffer solution containing the following reagents in μmoles per ml: dipotassium salt of D-fructose 6-P, 40; potassium phosphate buffer, pH 7.0, 10; and EDTA, 2. The enzyme was eluted from the column by the same buffer and concentrated over PM-30 Diaflo membrane and stored as mentioned before. The activity of the enzyme

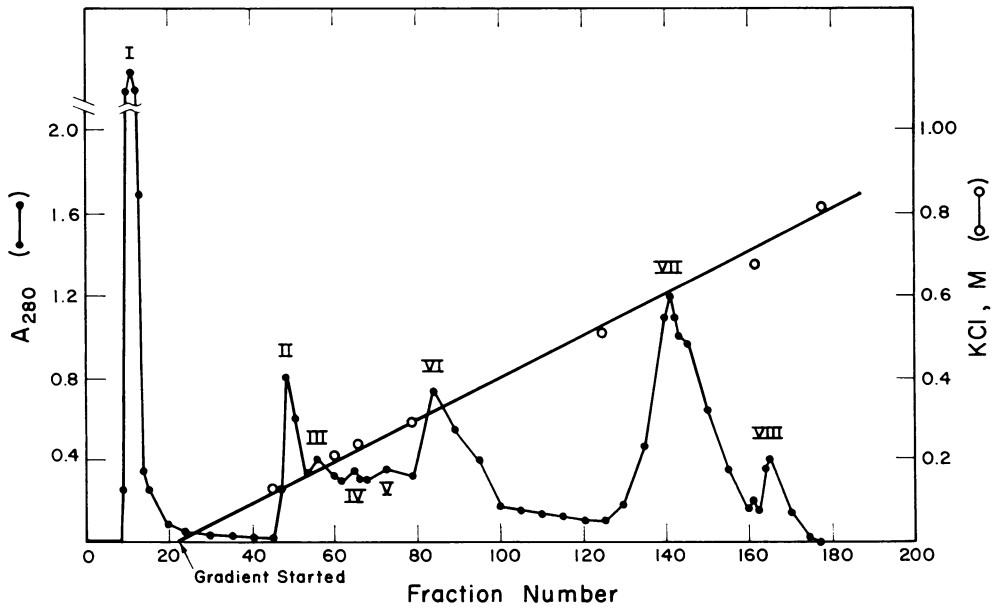

FIG. 1. DEAE-cellulose column chromatogram of mung bean proteins. Twelve milliliters of XM-300 fraction (27 mg protein per ml) were chromatographed on a DEAE-cellulose column as described in the text. The enzyme was eluted with 0.24 to 0.30 M KCl (peak V).

Table I. *Purification of L-Glutamine D-Fructose 6-Phosphate Amidotransferase from Phaseolus aureus Seeds*

Step	Volume	Enzyme Activity	Protein	Specific Activity	Purification Fold	Total Activity	Yield	Total Protein	Protein Recovery
	ml	*units/ml*[1]	*mg/ml*			*units*	%	*mg*	%
1. Crude	69.3	0.133	92.00	0.00144	1	10.22	—[2]	6376	100
2. Acetone fraction	23.1	2.827	20.40	0.1385	96	65.01	100	471.2	7.2
3. Ultrafiltration	12.0	4.693	27.00	0.1738	120	56.32	86	324.0	5.0
4. DEAE-cellulose (peak V)	6.2	2.240	2.59	0.8650	600	13.88	32[3]	14.06	0.22

[1] Enzyme units are expressed as μmoles of D-glucosamine 6-P produced per hr under standard assay conditions at 30 C.

[2] Enzyme activity was found to be very low in the crude extract. Calculations of enzyme yield is based on the activity of the acetone fraction.

[3] Calculated on the basis of 66% of the enzyme activity recovered from the column that was found in fraction V (Fig. 1) and the rest of the activity that was recovered in fraction IV (Fig. 1).

was checked in the standard manner, except that an incubation mixture containing no L-glutamine was also included to check for the complete removal of L-glutamine by this procedure. Only 60% of the enzyme activity put on the column was recovered, and as shown (Fig. 8), the enzyme was completely desensitized to inhibition by UDPAG. This enzyme preparation designated as "Sephadex fraction" was employed in kinetic studies requiring changes in the concentration of L-glutamine.

RESULTS AND DISCUSSION

Enzyme Purification. A typical DEAE-cellulose chromatogram of the enzyme is shown in Figure 1, and a summary of the data on the partial purification of the enzyme is presented in Table I. A 600-fold purification of the enzyme with a protein recovery of 0.22% was obtained.

The acetone fraction suspended in phosphate buffer which was found to be most stable at pH 7.0 completely lost its activity after 24 hr at 4 C. Several reagents such as bovine serum albumin, dithiothreitol, and glycerol were not effective for its stabilization. Addition of high concentrations of D-fructose 6-P (50 mM) rendered the enzyme stable at −20 C, but not at 4 C. However, this acetone fraction could be stabilized at 4 C for several days in 10 mM phosphate buffer, pH 7.0, containing 2 mM EDTA and 0.15 M L-glutamine. The yield of the enzyme from DEAE-cellulose column was always about 20 to 30% of the acetone fraction. The activity was neither increased by adding boiled acetone fraction, nor was there any synergistic effect of the latter fraction on the DEAE-cellulose fraction, indicating the absence of a cofactor for the enzyme, as shown by other workers (3, 5, 8). The rather low yield of the enzyme obtained from DEAE-cellulose column chromatography is probably due to the denaturation of the enzyme upon dilution.

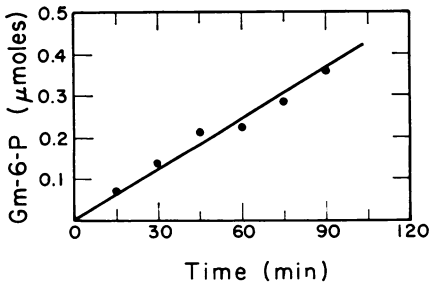

FIG. 2. Enzyme activity as a function of time. The enzyme source was 0.32 mg of the DEAE-cellulose fraction. Assays were done under standard conditions, except that reactions stopped at time intervals indicated. Results expressed as μmoles of D-glucosamine 6-P (Gm-6-P) formed per 0.5 ml of the incubation mixture.

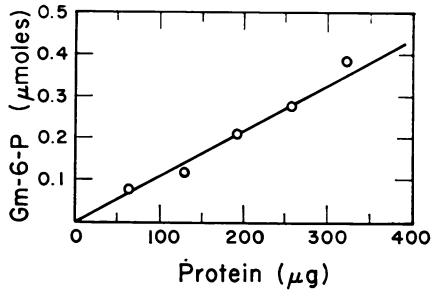

FIG. 3. The effect of protein concentration on the activity of the enzyme. Assays were done under standard conditions. The indicated amounts of protein from DEAE-cellulose fraction were included. The results expressed as μmoles of D-glucosamine 6-P (Gm-6-P) produced per 0.5 ml of the incubation mixture in 90 min.

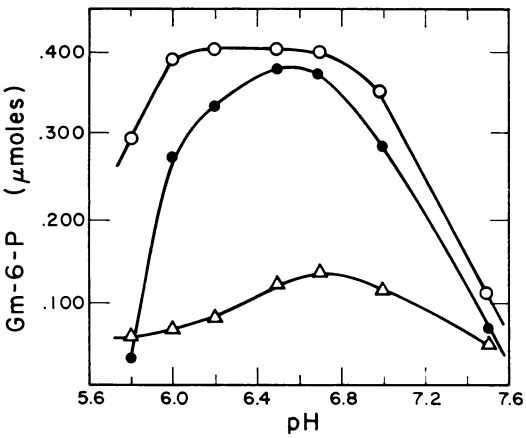

FIG. 4. Mung bean amidotransferase activity as a function of pH. Assays were done according to the standard conditions, except that the pH of D-fructose 6-P solution was adjusted to the indicated pH. The enzyme sources were 0.30 mg of the DEAE-cellulose fraction (○ and △) or 2.1 mg of the acetone fraction (●). The buffers were 30 μmoles of potassium phosphate buffer at the indicated pH (○ and ●) or 30 μmoles of sodium cacodylate buffer at the given pH (△). The results are expressed as in Figure 3.

Any time this fraction was rechromatographed on a column, only about 20 to 30% of the enzyme activity applied to the column was recovered. Because of this low yield, it was difficult to purify the enzyme further. In order to prevent the loss of activity during the removal of L-glutamine from the enzyme, D-fructose 6-P was added to the eluting buffer in Sephadex

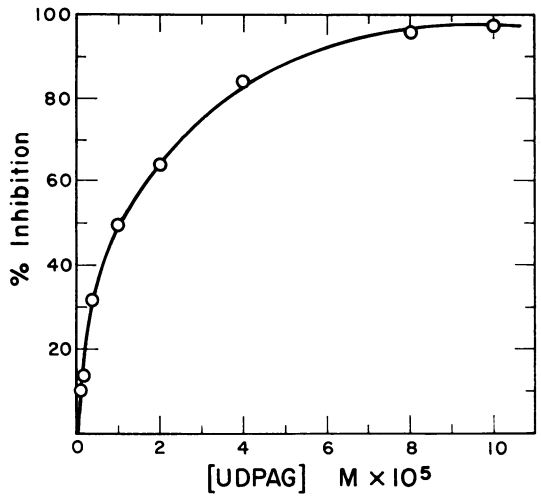

FIG. 5. Inhibition pattern of the mung bean enzyme by UDPAG. Enzyme source 0.13 mg of the freshly prepared DEAE-cellulose fraction. Assays done under standard conditions with the inclusion of the designated concentrations of UDPAG.

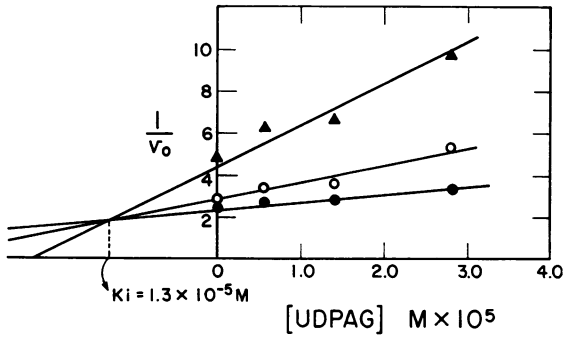

FIG. 6. Competitive inhibition of mung bean amidotransferase by UDPAG. The incubation mixtures contained the same amount of reagents per 0.5 ml as the standard conditions except that D-fructose 6-P concentrations were 14 mM (●), 7.0 mM (○) and 2.8 mM (▲). Enzyme source was 0.26 mg protein from the DEAE-fraction. UDPAG was added as indicated. Incubations done at 30 C for 20, 40, 60, and 80 min and the initial velocities (v_0) were calculated from the slope of activity against time plots and expressed as μmoles of D-glucosamine 6-P (GM-6-P) produced per hr per ml of the incubation mixture.

column chromatography which stabilized the enzyme to some extent. The DEAE-cellulose fraction in the buffer mixture, when concentrated, maintained its stability up to 2 weeks by keeping over liquid nitrogen.

Effects of Time, Enzyme Concentration and pH on Enzyme Activity. Figure 2 demonstrates the time course of the partially purified enzyme. The effects of enzyme concentration and pH on enzyme activity are shown in Figures 3 and 4, respectively. The reaction follows a straight line up to 90 min (Fig. 2), and the activity is linearly proportional to protein concentration up to 0.32 mg of protein used per 0.5 ml of the incubation mixture (Fig. 3). The partially purified enzyme shows a pH optimum in the range of pH 6.2 to 6.7 in phosphate buffer and an optimum of about pH 6.7 in cacodylate buffer; however, the activity in phosphate buffer is approximately 3-fold that of cacodylate buffer (Fig. 4). The pH optimum is slightly lower than those reported for bacterial and mammalian enzymes by other workers (3–5).

Kinetics of L-Glutamine D-Fructose 6-P Amidotransferase. The inhibition pattern of the freshly prepared enzyme in the

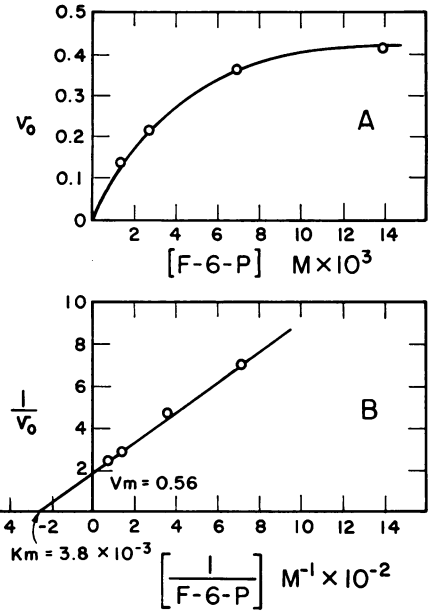

FIG. 7. A: Rate of amidotransferase activity as a function of D-fructose 6-P concentration; B: the double reciprocal plot of A. The assays were done according to the standard conditions, except that D-fructose 6-P concentrations were varied as indicated. The enzyme source was 0.26 mg of the DEAE-cellulose fraction per 0.5 ml of the incubation mixture. Initial velocities (v_0) were determined and expressed as in Figure 6.

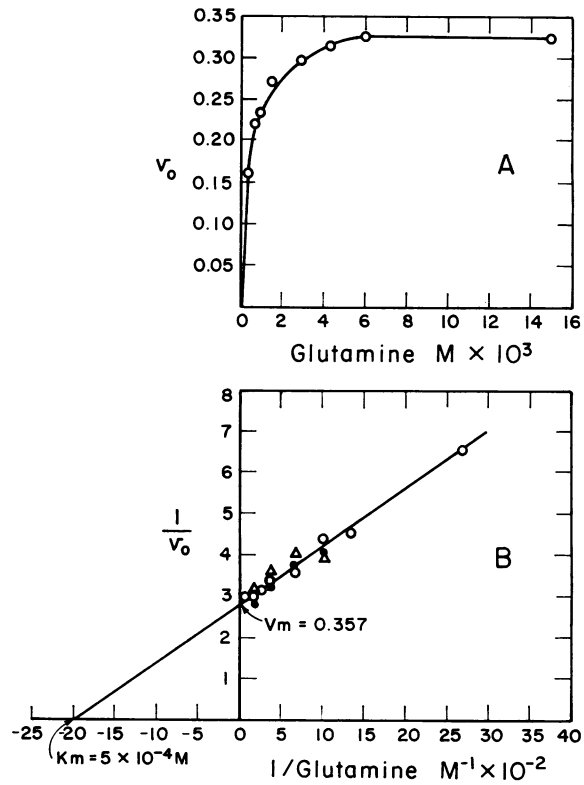

FIG. 8. A: Rate of mung bean amidotransferase activity as a function of L-glutamine concentration; B: Lineweaver-Burk double reciprocal plot. The enzyme source was 0.35 mg of the desensitized "Sephadex fraction" per 0.5 ml of the incubation mixture. Assays done according to the standard conditions except that L-glutamine was included as indicated and UDPAG concentrations were 0 (○), 14 μM (△) and 28 μM (●). Initial velocities (v_0) were determined and expressed as in Figure 6.

buffer mixture is shown in Figure 5, and a Dixon plot (2) showing the type of inhibition is demonstrated in Figure 6. As shown, the freshly prepared enzyme in the buffer mixture is inhibited up to 98% by UDPAG (Fig. 5); a *Ki* value of 13 μM was obtained for the inhibitor acting competitively with respect to D-fructose 6-P (Fig. 6).

The initial velocity of the amidotransferase as a function of D-fructose 6-P concentration at a fixed concentration of L-glutamine together with the Lineweaver-Burk double reciprocal plot is shown in Figure 7, A and B. A *Km* value of 3.8 mM and a V_{max} value of 0.56 μmole D-glucosamine 6-P per hr per ml of the incubation mixture was obtained for D-fructose 6-P.

Figure 8 A and B, demonstrates the initial velocity of the amidotransferase as a function of L-glutamine concentration at a fixed concentration of D-fructose 6-P together with the Lineweaver-Burk double reciprocal plot. As shown in Figure 8 B, a *Km* value of 0.5 mM with a V_{max} value of 0.357 μmole of D-glucosamine 6-P per hr per ml of the incubation mixture was obtained for L-glutamine. This enzyme preparation was desensitized to UDPAG and no inhibition of the enzyme activity was noted at the two levels of the inhibitor indicated (Fig. 8B).

The *Km* value of 3.8 mM obtained for D-fructose 6-P and 0.5 mM reported for L-glutamine are comparable to values of 2.0 mM and 0.65 mM for *E. coli* enzyme reported for D-fructose 6-P and L-glutamine (3, 4).

Kornfeld (5) demonstrated the feedback inhibition of the rat liver and HeLa cells enzymes by UDPAG and obtained *Ki* values of 5 μM and 11 μM, respectively for the competition of UDPAG for D-fructose 6-P. Our *Ki* value of 13 μM reported for the mung bean enzyme (Fig. 6) is in good agreement with that reported by Kornfeld (5) for HeLa cell enzyme.

Upon removal of L-glutamine from our partially purified enzyme preparations and storage in D-fructose 6-P buffer over liquid nitrogen for 24 hr, the enzyme lost its sensitivity to inhibition by UDPAG completely at the two inhibitor levels tested (Fig. 8). Our enzyme preparations in buffer mixture that had been stored over liquid nitrogen for more than a week also showed a partial desensitization to the inhibitor without any appreciable loss of catalytic activity. Instead of 95% inhibition reported for the freshly prepared enzyme in the buffer mixture at 70 μM UDPAG (Fig. 5), only a 22% inhibition was noticed. Kornfeld (5) has been able to desensitize partially the rat liver enzyme to UDPAG by heating the enzyme at 37 C for 30 min in the presence of D-glucose 6-P prior to its incubation with the substrates and the inhibitor. Changeux (1) reported the desensitization of biosynthetic L-threonine deaminase to its feedback inhibitor L-isoleucine by heat treatment in the presence of 7.5 μM *p*-chloromercuribenzoate with the retention of the catalytic activity of the enzyme. He was able to preserve the sensitivity of the enzyme to L-isoleucine by heating in the presence of its substrate L-threonine. Similar papers and reviews (9, 10) describe the desensitization of several regulatory enzymes to their feedback inhibitors by different treatments and explain the presence of different enzyme sites for the substrate and the inhibitor.

Inclusion of high concentrations of L-glutamine not only stabilized our enzyme preparations, but also kept the inhibitor site sensitive to the inhibitor for a longer period of time (compare Fig. 5 and 8B). The reason that Mayer *et al.* (8) could not obtain more than 60% inhibition of the enzyme by UDPAG is probably due to the desensitization of the enzyme to the inhibitor.

Molecular Weight of the Enzyme. A molecular weight of 340,000 has been obtained by Kornfeld (5) for rat liver and HeLa cells enzymes. Our ultrafiltration data by XM-300 Diaflo membrane (Table I), which demonstrates the retention of 86% of the enzyme activity by the membrane, indicates a molecular weight for the mung bean enzyme greater than 300,000. These membranes are supposed to retain compounds with molecular weights greater than 300,000, excluding those with lower molecular weights.

Inability of L-Asparagine to Act as an Amide Nitrogen Donor. Using incubation mixtures containing L-asparagine and including control tubes with boiled enzyme, Mayer *et al.* (8) were able to demonstrate the formation of D-glucosamine 6-P by the crude extracts of mung bean seeds. However, they were not able to demonstrate such synthesis in their partially purified enzymes. Using incubation mixtures with and without L-asparagine, it was observed that even a higher amount of D-glucosamine 6-P is produced in the absence of L-asparagine than in its presence, when the crude enzyme is used. This indicates the possibility of the presence of L-glutamine, in the crude extracts, which presumably acts as an amide N donor. When L-asparagine is added to the medium, it acts as a competitive inhibitor of L-glutamine and reduces the amount of D-glucosamine 6-P formed. The failure of the partially purified enzyme preparations to show any D-glucosamine 6-P formation in the presence of L-asparagine is most probably due to the removal of the small amount of L-glutamine from enzyme solutions upon precipitation of the enzyme by ammonium sulfate (8) or in this case by acetone.

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