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Partial Purification and Properties of L-Asparagine Synthetase from Mouse Pancreas

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L-Asparagine synthetase was partially purified from mouse pancreas to a final mean specific activity of 0.10 unit/mg of protein. The enzyme exhibited an L-glutaminase activity which was not affected by L-aspartate, NH_4Cl, ATP–$MgCl_2$, L-glutamate, AMP (sodium salt) or sodium pyrophosphate. The L-glutamine-dependent L-asparagine synthetase activity of the partially purified enzyme from mouse pancreas was markedly decreased by freezing for 7 days at −87°C in the presence of 1 mM-dithiothreitol, but effectively protected from inactivation by high concentrations (10 mM) of the thiol reagent. The L-glutaminase activity of the enzyme was inhibited by antagonists of L-glutamine (e.g. 6-diazo-5-oxo-L-norleucine, 5-chloro-4-oxo-L-norvaline, 5-diazo-4-oxo-L-norvaline and NSC-163501) and thiol-reactive compounds (e.g. 2-amino-4-arsenophenol hydrochloride, maleimide, mucochloric acid and $ZnCl_2$), but not by aminomalonic acid, the next lower homologue of L-aspartate, nor by L-homoserine β-adenylate, an analogue of the presumed transitory covalent intermediate. The complete forward reaction catalysed by L-asparagine synthetase from mouse pancreas appears to be irreversible and essentially stoicheiometric under the conditions examined. Mouse pancreas contains a proteolytic inhibitor of L-asparagine synthetase separable from the enzyme by ion-exchange column chromatography. The inhibitor is activated by incubation at 4°C for 110 h and inactivated by soya-bean trypsin inhibitor, di-isopropyl phosphorofluoridate and boiling.

Previously, we reported that, of the seven major organs studied, the normal pancreas of mammalian and avian species had the greatest ability to synthesize L-asparagine (Milman & Cooney, 1974); moreover, the activity of L-asparagine synthetase in the normal pancreas of the mouse was comparable with that found in nodules of leukaemia 5178Y, which is resistant to L-asparaginase (L5178Y/AR). Since several laboratories are actively searching for inhibitors of L-asparagine synthetase of tumoral origin in order to overcome the state of resistance to L-asparaginase (Cooney *et al.*, 1976; Chou & Handschumacher, 1972), it was considered important to purify the pancreatic enzyme partially and to characterize its biochemical properties so that its inhibition *in vitro* by selected agents could be compared with that produced against the analogous enzyme from L5178Y/AR cells. This paper describes the partial purification and properties of L-asparagine synthetase from mouse pancreas.

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Experimental

Enzymes

L-Asparaginase (EC 3.5.1.1) from *Escherichia coli* (340 units/mg of protein) was purified at the Merck Institute for Therapeutic Research, West Point, PA, U.S.A., and provided by the Drug Research and Development Branch of the National Cancer Institute (1 unit is equivalent to 1 μmol of substrate hydrolysed/min at 37°C). L-Aspartate β-decarboxylase from *Alcaligenes faecalis* (EC 4.1.1.12; specific activity 77 units/mg of protein) and L-glutamate decarboxylase from *E. coli* (EC 4.1.1.15; specific activity 50 units/mg of protein) were purified by the methods of Tate & Meister (1968) and of Shukuya & Schwert (1960) respectively. Malate dehydrogenase (EC 1.1.1.37; specific activity 720 units/mg of protein), L-glutamate–oxaloacetate transaminase (EC 2.6.1.1; specific activity 180 units/mg of protein), hexokinase (EC 2.7.1.1; specific activity 140 units/mg of protein), lactate dehydrogenase (EC 1.1.1.27; specific activity 200 units/mg of protein) and L-glutamate dehydrogenase (EC 1.4.1.2; specific activity 45 units/mg of protein) were purchased from Boehringer Mannheim

Corp., New York, NY, U.S.A. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49; specific activity 300 units/mg of protein), pyruvate kinase (EC 2.7.1.40; specific activity 350 units/mg of protein) and myokinase (EC 2.7.4.3; specific activity 1000 units/mg of protein) were procured from Sigma Chemical Co., St. Louis, MO, U.S.A.

Radiochemicals

L-[4-^{14}C]Aspartate (specific radioactivity 12.9–17.4 μCi/μmol), L-[U-^{14}C]aspartate (specific radioactivity 214–231 μCi/μmol), L-[U-^{14}C]asparagine (specific radioactivity 100–185 μCi/μmol), L-[U-^{14}C]-glutamine (specific radioactivity 40–57.3 μCi/μmol), L-[U-^{14}C]glutamate (specific radioactivity 275 μCi/μmol) and [γ-^{32}P]ATP (specific radioactivity 2.43 Ci/mmol) were the products of Amersham/Searle Corp., Arlington Heights, IL, U.S.A. 2-Oxo[1-^{14}C]glutarate (specific radioactivity 10.4–26 μCi/μmol) and sodium [^{32}P]pyrophosphate (specific radioactivity 6550 mCi/mmol) were purchased from New England Corp., Boston, MA, U.S.A.

Chemicals and supplies

5-Chloro-4-oxo-L-norvaline (2-amino-5-chloro-laevulinic acid), 6-diazo-5-oxo-L-norleucine, 5-diazo-4-oxo-L-norvaline, NSC-163501 [L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydroisoxazol-5-ylacetic acid] and 2-amino-4-arsenophenol hydrochloride were obtained through the Drug Research and Development Branch of the National Cancer Institute. L-Homoserine β-adenylate, synthesized by the method of Chang (1978), was kindly given by Dr. Pauline Chang of Yale University School of Medicine, New Haven, CT, U.S.A. Aminomalonic acid was prepared as previously described (Thanassi, 1970) and was a generous gift of Dr. John Thanassi of the University of Vermont, Burlington, VT, U.S.A. Maleimide and mucochloric acid were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. L-Asparagine was purchased from Schwartz/Mann, Orangeburg, NY, U.S.A. L-Glutamine, 2-oxoglutarate, di-isopropyl phosphorofluoridate and dithiothreitol were obtained from Calbiochem, Gaithersburg, MD, U.S.A. NADH, ATP, AMP, NADP$^+$, L-glutamic acid, L-aspartate and phosphoenolypyruvic acid were the products of Sigma Chemical Co. DEAE-Sephadex A-25 was purchased from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Chromatographic columns were obtained either from Pharmacia Fine Chemicals or Kontes Glass Co. (Vineland, NJ, U.S.A.).

Measurement of L-asparagine synthetase

L-Asparagine synthetase activity was measured essentially as described previously (Milman & Cooney, 1974), with the following variations. After the incubation step, the decarboxylation step was conducted either at 25°C for 16h or as follows. A modified decarboxylation mixture composed of 10mg of 2-oxoglutarate, 70μl of 70% (w/v) $ZnSO_4$ and 72 units of L-glutamate–oxaloacetate transaminase in 10ml of 0.66M-sodium acetate buffer, pH5.0, was prepared. To each vessel 20μl of this reagent was added and the open vessels were then incubated at 50°C for 1h in the ventilation hood. Subsequent recovery of L-[4-^{14}C]asparagine was conducted either for 3h at 37°C or for 1h at 50°C as described by Milman & Cooney (1974).

Preparation of di-isopropyl phosphorofluoridate solution

Di-isopropyl phosphorofluoridate in its undiluted form is a powerful inactivator of acetylcholinesterase (Goodman & Gilman, 1970). Small concentrations can be fatal (LD_{50} by cutaneous application to mice is 72mg/kg). Because of the extreme danger when using this reagent, several precautions were implemented when this chemical was in use. A laboratory coat and expendable gloves were always worn and a solution of 1M-KOH was close by. Atropine was available and a physician was ready if needed. Work with the undiluted chemical was conducted in a well-ventilated hood. A 1.5% (v/v) solution of di-isopropyl phosphorofluoridate was prepared in propan-1-ol and used as indicated.

Partial purification of L-asparagine synthetase from mouse pancreas

Step 1: *homogenization.* L-Asparagine synthetase was extracted from the pancreata of BDF_1 mice by homogenizing the tissue in 3vol. of freshly prepared 0.01M-Tris/formate buffer, pH6.0, containing 1mM-dithiothreitol and 0.5mM-disodium EDTA (starting buffer).

Step 2: *ultracentrifugation.* The crude homogenate was centrifuged at 105000*g* for 20min at 4°C in a Beckman model L2-65B ultracentrifuge.

Step 3: *ion-exchange chromatography at pH*6.0. DEAE-Sephadex A-25 (formate form; pH6.0) was equilibrated in a beaker with four rinses of 500ml each of starting buffer. A column (Kontes Glass Co.; 500mm × 25mm) was poured, then washed with 2 bed volumes of starting buffer. Then 20–25ml of step-2 enzyme was loaded on the column, after which the column was eluted with 2 bed volumes of starting buffer by using a peristaltic pump (LKB Instruments, Rockville, MD, U.S.A.) and a flow rate of 1.2ml/min with downward flow. After the void volume was eluted, a linear gradient of 0.01–1.0M-Tris/formate, pH6.0, containing 1mM-dithiothreitol and 0.5mM-disodium EDTA (250ml in each chamber) was applied; 2.5–3ml fractions were collected.

Step 4: *ion-exchange chromatography at pH*7.8. DEAE-Sephadex A-25 (formate form; pH7.8) was equilibrated in a beaker with four rinses of 500ml each of starting buffer at pH7.8. A column (Pharmacia Fine Chemicals; 1.5cm×30cm) was poured, then washed with 2 bed volumes of starting buffer at pH7.8. Then 10–12ml of the pooled active fractions arising from the DEAE-Sephadex A-25 chromatography at pH6.0 (step 3) was loaded on the column, after which the column was eluted with a linear gradient of 0.01–1.0M-Tris/formate, pH7.8, containing 1mM-dithiothreitol and 0.5mM-disodium EDTA (75ml in each chamber) by using a peristaltic pump and a flow rate of 1.2ml/min with downward flow; 1.5ml fractions were collected.

Step 5: *sucrose-gradient ultracentrifugation.* Solutions of sucrose (5% and 20%, w/v) in 0.1M-Tris/formate buffer, pH7.8, containing 10mM-dithiothreitol and 1mM-disodium EDTA were prepared. From these, 5–20% linear sucrose gradients were prepared (12ml/tube) by using a gradient maker and a pump (Buchler Instruments, Fort Lee, NJ, U.S.A.) with a flow rate of 1ml/min, and dispensed into Beckman nitrocellulose tubes. The three most active fractions from step 4 were layered separately over the gradients, then the tubes were placed in a Beckman SW40 swinging-bucket rotor and centrifuged at 200000*g* and 3°C for 21h in a Beckman model L2-65B ultracentrifuge. At the termination of the centrifugation a needle was inserted through the bottom of the tubes and a pumping solution [60% (w/v) sucrose in 1M-NaCl, adjusted to pH7.0] was pumped (Harvard Instrument Co., Millis, MA, U.S.A.) through the bottom of the tubes at a flow rate of 1ml/min; 1.5ml fractions were collected. Maximal enzyme activity appeared in two fractions of each tube, which were pooled.

Disc-gel electrophoresis

Disc-gel electrophoresis was conducted by the method of Wright *et al.* (1971). For recovery of enzymic activity, all gels were first pre-run for 2h with 0.05M-Tris/glycine buffer containing 10mM-dithiothreitol to remove excess ammonium persulphate, which would inactivate the enzyme. Subsequently, the buffer was replaced with fresh 0.05M-Tris/glycine buffer containing 10mM-dithiothreitol. At the termination of the electrophoresis, one gel was sliced into 1mm segments and an identical gel was stained with Coomassie Blue. The sliced segments were immersed in an Eppendorf 1500μl conical vessel (Brinkman Instrument Co., Silver Spring, MD, U.S.A.) containing 20μl of 0.5M-Tris/HCl buffer, pH8.4, and 5μl of a mixture containing L-glutamine (48mM), L-[4-^{14}C]aspartate (1.9mM), ATP (30mM), $MgCl_2$ (30mM) and Tris/HCl buffer, pH7.6 (0.5M). All vessels were incubated at 37°C for 2h, after which the decarboxylation step was conducted for 16h at 25°C. Subsequent recovery of synthesized L-[4-^{14}C]asparagine was as described by Milman & Cooney (1974).

Measurement of substrate and product concentrations in the partially purified enzyme preparation from mouse pancreas

Ammonia in the partially purified enzyme preparation from mouse pancreas was measured by a spectrophotometric enzymic technique with L-glutamate dehydrogenase (Cooney *et al.*, 1971*a*); L-aspartate, L-glutamate, L-glutamine and L-asparagine were measured by a radiometric method (Cooney & Milman, 1972) and ATP and AMP by spectrophotometric procedures (Lamprecht & Trautschold, 1965; Adam, 1965). The measurement of Mg^{2+} ions by atomic absorption/emission spectroscopy and Cl^- ions by the Technicon stat/ion system for electrolytes was conducted by Litton Bionetics, Kensington, MD, U.S.A.

Measurement of L-glutaminase activity

A 5μl portion (0.25μCi; 0.0444μmol) of L-[U-^{14}C]-glutamine (specific radioactivity 57.3μCi/μmol) was incubated in triplicate at 37°C for 30min in Eppendorf 1500μl conical vessels containing various additives prepared in 0.5M-Tris/HCl buffer, pH7.6, and 5μl of step-5 enzyme. The reaction, which was linear with time for 75min, was terminated by heating the closed vessels at 95°C for 5min. Subsequent recovery of any formed L-[U-^{14}C]glutamate was measured by the addition of 50μl of a decarboxylation mixture (composed of 5 units of L-glutamate decarboxylase in 1ml of 0.66M-sodium acetate buffer, pH4.2) to each reaction vessel and trapping any liberated $^{14}CO_2$ arising during a 3h incubation at 37°C in a 5μl droplet of 40% (w/v) KOH deposited on the underside of the lids of the closed vessels. At the termination of the incubation, the lids were removed, placed in a scintillant (Cooney *et al.*, 1971*b*) and counted for radioactivity at 83% efficiency in a liquid-scintillation spectrometer. Recovery of exogenous L-[U-^{14}C] glutamate was 79%.

Measurement of L-asparaginase activity

L-Asparaginase activity was assessed by a radiometric procedure as described by Rosenbluth *et al.* (1976).

Determination of protein

This was done on the dialysed fractions from the enzyme-purification procedures by the method of Lowry *et al.* (1951), with bovine serum albumin (Armour Pharmaceutical Co., Chicago, IL, U.S.A.) as the standard.

Stoicheiometry of the L-*asparagine synthetase reaction* (*Table* 3)

Experiment 1. (*a*) A mixture was prepared which contained 0.2M-ATP, 0.05M-$MgCl_2$, 0.04M-L-glutamine and 0.0018M-L-aspartate in 0.5M-Tris/HCl buffer, pH7.6; 200μl of the mixture was incubated at 37°C for 60min with 200μl (60μg) of step-5 enzyme or with 200μl (60μg) of step-5 enzyme which had been previously heated at 95°C for 10min. At the termination of the incubation the vessels were heated at 95°C for 5min. Subsequently, suitable samples of the reaction mixtures were taken for spectrophotometric measurement of any L-aspartate (Cooney *et al.*, 1970), L-asparagine (Cooney *et al.*, 1970) and AMP (Adam, 1965).

(*b*) To 100μl of the above mixture was added 10μl (0.5μCi; 0.0125μmol) of L-[U-^{14}C]glutamine (specific radioactivity 40μCi/μmol); 10μl of this mixture then was dispensed into ten Eppendorf 1500μl conical vessels. To six vessels was added 10μl (3μg) of step-5 enzyme; to the remaining four vessels was added 10μl (3μg) of step-5 enzyme which had been previously heated at 95°C for 10min. The incubation was conducted as described above, after which 5μl of each reaction mixture was analysed for radioactive L-glutamine and L-glutamate by electrophoresis as described by Cooney *et al.* (1976).

(*c*) To 100μl of the mixture described in (*a*) was added 10μl (10μCi; 0.0043μmol) of [γ-^{32}P]ATP (final specific radioactivity 1170μCi/μmol); 10μl of this mixture then was dispensed into ten Eppendorf 1500μl conical vessels. To six vessels was added 10μl (3μg) of step-5 enzyme; to the remaining four vessels was added 10μl (3μg) of step-5 enzyme which had been previously heated at 95°C for 10min. The incubation was conducted as described above, after which 5μl of each reaction mixture was taken for electrophoresis as described by Horowitz & Meister (1972). Also, 5μl of sodium [^{32}P]pyrophosphate was electrophoresed in order to identify the location of the pyrophosphate spots in the experimental channels. ATP and pyrophosphate spots were excised and counted for radioactivity.

Experiment 2. To 200μl of the unlabelled mixture prepared for Experiment 1 was added 200μl (30μg) of step-5 enzyme or 200μl (30μg) of step-5 enzyme which had been heated previously at 95°C for 10min. All vessels were incubated at 37°C for 60min, then at 95°C for 5min. Subsequently, a portion of each incubation mixture was assayed for L-aspartate, L-asparagine and AMP by enzymic spectrophotometric methods (Cooney *et al.*, 1970; Adam, 1965) (Experiment 2*a*) and for L-aspartate and L-glutamate by a radiometric technique (Cooney & Milman, 1972) (Experiment 2b).

Results

Partial purification of L-*asparagine synthetase from mouse pancreas*

In Table 1 the scheme evolved for the partial purification of L-asparagine synthetase from mouse pancreas is presented. A 679-fold purification with a 13% yield of enzymic activity was achieved. L-Asparagine synthetase appeared at a calculated salt concentration of 0.37M when chromatographed on DEAE-Sephadex A-25 (formate form) at pH6.0 (step 3) and at 0.33M when chromatographed on DEAE-Sephadex A-25 (formate form) at pH7.8 (step 4). The enzyme from step 5 (sucrose-gradient-ultracentrifugation step) sedimented to a sucrose concentration of 10%. Peak activity appeared in two fractions (0.5ml each).

Disc-gel electrophoresis

Samples of the various purification steps were subjected to disc-gel electrophoresis. Approx. 12 bands were seen in step-2 enzyme (105000*g* supernatant), whereas only four bands were present in step-5 enzyme (sucrose-gradient-ultracentrifugation step). The enzymic activity was associated with the third major band (Fig. 1).

Table 1. *Partial purification of* L-*asparagine synthetase from mouse pancreas*

L-Asparagine synthetase activity was measured as described in the text. Numbers of experiments are given in parentheses. Mean total enzyme activity is expressed as nmol/h per total volume.

Fractionation step	Volume (ml)	Protein concn. (mg/ml)	Specific activity (nmol/h per mg of protein)	Mean total enzyme activity	Mean yield (%)	Mean purification (fold)
1. Crude homogenate	28.0	45.8±2.8 (12)	8.5± 0.7 (12)	10080	—	—
2. 105000*g* supernatant	15.0	28.3±2.1 (12)	25.3± 2.2 (12)	10881	98	3
3. DEAE-Sephadex A-25, pH6.0	10.0	0.4±0.0 (10)	1160.5± 57.3 (10)	4758	44	137
4. DEAE-Sephadex A-25, pH7.8	4.5	0.2±0.0 (10)	2535.6±267.7 (10)	2510	23	298
5. Sucrose-gradient ultracentrifugation	3.0	0.1±0.0 (5)	5768.6±662.3 (5)	1384	13	679

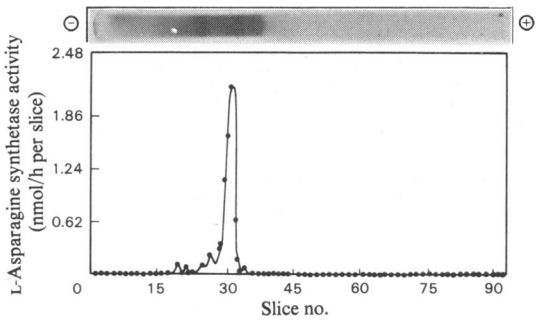

Fig. 1. *Disc-gel electrophoresis of step-5 enzyme from mouse pancreas*

Step-5 enzyme (40 μg; 500 μl) was subjected to disc-gel electrophoresis as described, in the presence of 10 mM-dithiothreitol, after the gels were first pre-run to remove all traces of ammonium persulphate. At the termination of the electrophoresis, the gel was sliced into 1 mm-thick sections. Each section then was incubated for 2½ h at 37°C in the presence of 20 μl of 0.5 M-Tris/HCl buffer, pH 8.4, and 5 μl of the radioactive mixture containing L-glutamine for the measurement of L-asparagine synthetase. Subsequent steps are as described in the text.

Substrate concentration in the partially purified enzyme preparation

To ensure that partially purified L-asparagine synthetase from mouse pancreas was free of contaminating substrates or products, the concentrations of the various substrates and products were determined in the enzyme preparation. The concentration of ammonia in step-4 or step-5 enzyme preparations was 28 μM, but the concentrations of all the other substrates or products were well below this concentration in both preparations (results not shown).

Biochemical characterization of partially purified L-asparagine synthetase from mouse pancreas

Partially purified step-3 enzyme was found to be unstable at 4°C. To explain the instability of the enzyme it was conjectured that an intrinsic protective agent might have been removed in the void volume during the ion-exchange chromatography at pH 6.0. To test this hypothesis, the 105000*g* supernatant from mouse pancreatic homogenate (step 2) was chromatographed on DEAE-Sephadex A-25 (formate form) at pH 6.0 as described. The void-volume fractions were then incubated at 4°C for 110 h, after which 5 μl of the pooled active fractions were assayed for L-asparagine synthetase activity in the presence of 5 μl of each of the void-volume fractions. L-Glutamine (0.013 M) was used as the amide donor for this study. Under these conditions, however, the enzyme activity was decreased to 25% of its initial activity (Fig. 2).

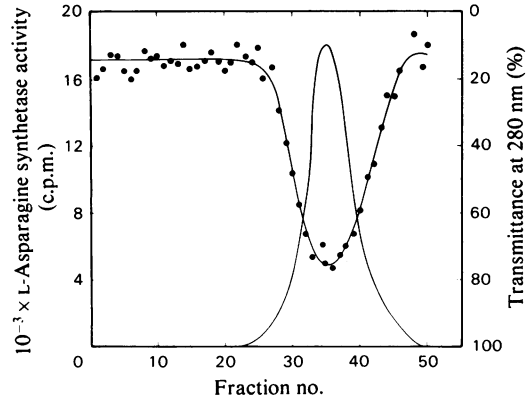

Fig. 2. *Presence of an inhibitor of L-asparagine synthetase in the void-volume fractions from DEAE-Sephadex A-25 chromatography at pH 6.0*

The 105000*g* (20 min) supernatant of mouse pancreatic homogenate was chromatographed on DEAE-Sephadex A-25 (formate form) at pH 6.0 as described in the text. The active fractions then were frozen at −87°C for 110 h; the void volume was incubated at 4°C during that time. Subsequently, 5 μl of the peak active fraction was assayed for L-asparagine synthetase activity in the presence or absence of 5 μl of each of the void-volume fractions, with L-glutamine (0.013 M) as amide donor. ●, Enzyme activity; ——, transmittance at 280 nm.

Exhaustive dialysis of the void-volume fractions against 0.1 M-Tris/HCl buffer, pH 7.6, containing 0.01 mM-dithiothreitol and 0.5 mM-disodium EDTA did not prevent the inhibition of L-asparagine synthetase, whereas heating the void-volume fractions at 95°C for 10 min totally abolished the inhibitory activity. Assay of step-3 enzyme in the presence of the endogenous inhibitor and soya-bean trypsin inhibitor (0.005 mg) restored full enzymic activity. Similar findings were observed when step-3 enzyme was assayed in the presence of the void-volume fractions which had been treated previously with di-isopropyl phosphorofluoridate (1 mM) for 30 min at 4°C.

Stability. Step-3 enzyme was highly unstable at 4°C, but could be stored at −87°C for 16 h without appreciable loss of enzymic activity. Addition of di-isopropyl phosphorofluoridate (1 mM) to the crude homogenate (step-1 enzyme) did not alter the instability of the enzyme preparation.

The utilization of L-glutamine, but not NH_4Cl, by step-4 enzyme was completely abolished by freezing the enzyme preparation at −87°C for 24 h. That the elimination of the so-called 'L-glutamine site' was not due to peroxidation, which may occur as a result of the degradation of dithiothreitol (Trotta *et al.*, 1973), was shown by the lack of inhibitory potency

of H_2O_2 (0.01M–95μM) towards the utilization of L-glutamine by step-4 enzyme.

To prevent the loss of the 'L-glutamine site' which occurred on freezing of step-4 enzyme at −87°C, various additives were tested as prospective protective agents. Only 11% of L-asparagine synthetase activity remained (as measured with L-glutamine as amide donor) when step-4 enzyme was stored at −87°C for 7 days, whereas 60% of the enzymic activity was recovered when NH_4Cl was used as the amide donor in the assay. L-Glutamine (10mM), KCl (20mM), NaCl (20mM), Na_2HPO_4 (20mM), K_2HPO_4 (20mM), sucrose (10%, w/v) and bovine serum albumin (3%) were ineffective in preventing the destruction of the 'L-glutamine site' of the enzyme when it was frozen at −87°C; $(NH_4)_2SO_4$ (100mM) and glycerol (20%, v/v) were partially effective (approx. 55% activity surviving) in protecting the 'L-glutamine site'. Of the agents tested, only dithiothreitol (5mM and 10mM) and dimethyl sulphoxide (20%, v/v) were almost fully capable of protecting the enzyme's ability to utilize L-glutamine after freezing at −87°C for 7 days.

Freezing step-3, step-4 or step-5 enzyme in liquid N_2 for indefinite periods of time provided full protection of both sites; however, freeze-drying of step-3, -4 or -5 enzymes yielded 95, 66 and 30% of the enzymic activity respectively with L-glutamine as amide donor.

Step 5 (sucrose-gradient ultracentrifugation) was conducted in the presence of 10mM-dithiothreitol, since at 1mM-dithiothreitol, in the presence or absence of 20% (v/v) dimethyl sulphoxide, there was a total loss of the ability to utilize L-glutamine.

Assay of the L-glutamine-site-inactivated enzyme for L-asparagine synthetase activity in the presence of 10mM-dithiothreitol and with L-glutamine (0.013M) as amide donor produced partial restoration of the utilization of L-glutamine (45% when compared with the activity obtained with NH_4Cl as amide donor).

Studies of the partial reactions. Step-5 enzyme catalysed the breakdown of L-glutamine (approx. 3000nmol of L-glutamate formed/h per mg of protein) and this activity was not influenced by the presence of other substrates. Addition of ATP (4mM) plus $MgCl_2$ (8mM), L-aspartate (1.5mM) or $MgCl_2$ (8mM) did not appreciably augment the L-glutaminase activity of the enzyme; NH_4Cl (0.02M) neither stimulated nor inhibited. Of the products, L-glutamate (4mM), AMP (4mM) and pyrophosphate (4mM) did not significantly alter the L-glutaminase activity. L-Asparagine (4mM), however, completely abolished this activity when present alone or in combination with L-glutamate (4mM), AMP (4mM) or pyrophosphate (4mM).

In Table 2 are summarized the results obtained when several agents were examined as inhibitors of the L-glutaminase activity of the step-5 enzyme. Except for aminomalonic acid and L-homoserine β-adenylate, all the agents tested (6-diazo-5-oxo-L-norleucine, 5-chloro-4-oxo-L-norvaline, NSC-163501, mucochloric acid, maleimide, 2-amino-4-arsenophenol hydrochloride and $ZnCl_2$) inhibited the enzyme's ability to hydrolyse L-glutamine. These

Table 2. *Effects of several agents on the* L-*glutaminase activity of the partially purified enzyme preparation from mouse pancreas*
The inhibition of the L-glutaminase and L-asparagine synthetase activities of the partially purified enzyme preparation from mouse pancreas (step-5 enzyme) was investigated as described in the text.

Drug	Concn. (mM)	Inhibition (%) L-Glutaminase activity	Inhibition (%) L-Asparagine synthetase activity
Aminomalonic acid	5	5	70
	3	0	60
6-Diazo-5-oxo-L-norleucine	3	100	84
	0.5	99	65
5-Chloro-4-oxo-L-norvaline	10	98	88
	3	98	82
5-Diazo-4-oxo-L-norvaline	7.5	98	71
	3	97	50
NSC-163501	3	92	95
	0.3	90	77
L-Homoserine β-adenylate (in the presence of 30mM-$MgCl_2$)	1	0	98
	0.2	0	85
Mucochloric acid	5	76	65
	3	64	52
Maleimide	3	57	70
	1	27	45
2-Amino-4-arsenophenol hydrochloride	3	83	66
$ZnCl_2$	10	75	100
	3	74	100

results suggest the presence of critical thiol functions at the 'L-glutamine site' of the enzyme. 5-Diazo-4-oxo-L-norvaline appears to inhibit the L-glutaminase activity of the enzyme to a greater degree than the L-asparagine synthetase activity. But 0.02M-L-glutamine was used to detect L-asparagine synthetase activity, and 0.28mM-L-glutamine for the measurement of L-glutaminase activity, and this might have contributed to the difference in the observed inhibition by this compound.

The synthesis of [^{32}P]ATP from [^{32}P]pyrophosphate (0.017mM) and AMP (5mM) in the presence of $MgCl_2$ (10mM) or other substrates by step-5 enzyme could not be detected (results not shown).

Studies of the exchange reactions. Step-5 enzyme was unable to catalyse an L-aspartate⇌L-asparagine exchange or an L-glutamate⇌L-glutamine exchange under the conditions examined (results not shown). Addition of the other substrates or products of the reaction, individually or in various combinations, could not elicit either exchange reaction.

Studies of the reverse reaction. By using a 30–240min incubation period, no reversal of the forward reaction catalysed by L-asparagine synthetase from mouse pancreas (step-5 enzyme) could be demonstrated at pH6.0, 7.6, 8.4 or 9.5 (results not shown). Formation of L-[U-^{14}C]glutamine from L-[U-^{14}C]-glutamate (0.012mM), NH_4Cl (20mM), L-asparagine (5mM), AMP (5mM), pyrophosphate (5mM) and $MgCl_2$ (15mM) with 50μl of step-5 enzyme (final reaction volume 60μl) could not be demonstrated even when the reaction was conducted for 180min at 37°C (paper electrophoresis was used to identify L-glutamine).

Stoicheiometry of the reaction. By using partially purified step-5 enzyme, approximately stoicheiometric amounts of products formed and substrates consumed were observed (Table 3, Expt. 1), the exceptions being the concentrations of L-glutamine used and L-glutamate formed. The apparently greater amounts of L-glutamine consumed (524nmol; Expt. 1) than L-glutamate formed (348nmol; Expt. 1) might be due to the method of detection. Thus, although L-glutamate is readily measurable, the amount of L-glutamine consumed was equivalent to only 10% of the total L-glutamine present. This decrement in the concentration of the amino acid is not within the sensitivity of the analysis used (Table 3).

The complete L-asparagine synthetase reaction was therefore repeated (Table 3; Expt. 2) and the amounts of L-aspartate consumed and L-glutamate formed were assayed by an enzymic radiometric method (Expt. 2b). By using this procedure, stoicheiometry was observed.

Discussion

In the course of the partial purification of L-asparagine synthetase from mouse pancreas, it was found that an inhibitor of the enzyme was being eluted in the void volume produced during the DEAE-Sephadex A-25 ion-exchange chromatography at pH6.0 (step 3). This inhibitor was tentatively identified as trypsin or an analogous proteinase on the following basis: its inhibition by di-isopropyl phosphorofluoridate, an agent which binds L-serine residues irreversibly, and by soya-bean trypsin inhibitor; its resistance to dialysis; its elimination by

Table 3. *Stoicheiometry of the reaction catalysed by partially purified L-asparagine synthetase from mouse pancreas*
The techniques are as described in the text. Values are the means of four to six determinations (Expt. 1) or duplicate determinations (Expt. 2) corrected for blank values. Individual values did not vary from the means by more than 5%. Blank values obtained for boiled enzyme (expressed in nmol equivalents/400μl) were: Expt. 1, L-asparagine, 0; AMP, 0; L-glutamate, 38; PP_i, 212; Expt. 2, L-asparagine, 0; AMP, 0; L-glutamate, 4.

Amount (nmol/400μl reaction volume)

	Substrate consumed			Product formed			
Expt. no.	L-Asp	L-Gln	ATP	L-Asn	L-Glu	AMP	PP_i
1	207	524	268	200	348	232	262
2a	84			71		80	
2b	112				116		

Ratio

Expt. 1		Expt. 2a		Expt. 2b	
L-Asn:L-Asp	0.96	L-Asn:L-Asp	0.84	L-Glu:L-Asp	1.03
L-Glu:L-Gln	0.66	L-Asn:AMP	0.88		
AMP:ATP	0.86				
PP_i:ATP	0.97				
L-Asn:L-Glu	0.57				
L-Asn:AMP	0.86				
L-Asn:PP_i	0.76				

boiling. Furthermore, the inhibitor was observed only after the void-volume fractions were aged for 110h at 4°C; under these conditions trypsinogen is known to be converted into trypsin (Allan *et al.*, 1964).

A second observation of significance was the apparent strong susceptibility of the partially purified enzyme to oxidation. This is not surprising, since L-asparagine synthetase from a mutant form of *Escherichia coli* (Cedar & Schwartz, 1969), 6C3HED Gardiner lymphosarcoma (Chou, 1970), L178Y/AR cells (Jayaram *et al.*, 1976), RADA1 leukaemia (Horowitz & Meister, 1972) and Novikoff hepatoma (Patterson & Orr, 1967) also are susceptible to oxidation. With the enzyme from mouse pancreas, however, this was particularly evident when L-glutamine, but not NH_4Cl, was the amide donor. When a partially purified (step 4) L-asparagine synthetase preparation from mouse pancreas was stored at −87°C in the presence of 1 mM-dithiothreitol, nearly total loss of the ability of the enzyme to utilize L-glutamine as the amide donor was produced, whereas utilization of NH_4Cl continued unaltered. However, 10mM-dithiothreitol or 20% (v/v) dimethyl sulphoxide completely prevented the loss of the 'L-glutamine site'. [Dithiothreitol (10mM) also was partially effective in reversing the oxidation of the 'L-glutamine site' (45% reversal).] Neither L-glutamine (10mM), phosphate ions [previously shown by Trotta *et al.* (1973) to be effective stabilizers of carbamoyl phosphate synthetase from *E. coli*], Cl^- ions [found to stimulate the utilization of L-glutamine by L-asparagine synthetase from RADA1 leukaemia (Horowitz & Meister, 1972)], bovine serum albumin [shown by Chou (1970) to increase the half-life of partially purified L-asparagine synthetase from 6C3HED Gardiner lymphosarcoma] nor sucrose (10%) could effectively prevent the loss of the enzyme's ability to utilize L-glutamine that occurs on freezing the enzyme preparation at −87°C. Apparently, agents that are known protectors of thiol functions on proteins (e.g. dithiothreitol) or cryopreservatives (e.g. dimethyl sulphoxide), if present in sufficient quantity, are able to prevent the loss of the synthetase's ability to utilize L-glutamine as amide donor, whereas L-glutamine or agents that in some cases are capable of stimulating the utilization of L-glutamine (e.g. Cl^- and phosphate ions) are ineffective. On the basis of these observations, it can be concluded that there are critical thiol functions at the 'L-glutamine site' which are absent from or inaccessible at the 'ammonia site'.

The partially purified enzyme preparation from mouse pancreas was observed to catalyse the hydrolysis of L-glutamine in the absence of any other substrate or product of the complete forward reaction. Moreover, this activity was markedly inhibited by antagonists of L-glutamine (e.g. 6-diazo-5-oxo-L-norleucine, 5-chloro-4-oxo-L-norvaline, 5-diazo-4-oxo-L-norvaline and NSC-163501) and thiol-reactive compounds (e.g. mucochloric acid, maleimide, 2-amino-4-arsenophenol hydrochloride and $ZnCl_2$), thereby supporting the suggestion of the presence of critical thiol functions at the 'L-glutamine site'.

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