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GLUTAMINE-DEPENDENT ASPARAGINE SYNTHETASE FROM *LUPINUS LUTEUS*

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Key Word Index—*Lupinus luteus*; Leguminosae; yellow lupin; asparagine biosynthesis; glutamine-dependent asparagine synthetase; purification; kinetics; mechanism.

Abstract—Asparagine synthetase (glutamine-hydrolyzing) [L-aspartate: L-glutamine amido-ligase (AMP-forming), E.C. 6.3.5.4] was purified over 500-fold from cotyledon extracts of 1-week-old yellow lupin seedlings. The enzyme was labile and required protection by high levels of thiols; glycerol and the substrates also stabilized it. The reaction products were shown to be asparagine, AMP, PPi and glutamate. The limiting K_m values were for aspartate 1·3 mM, for MgATP 0·14 mM and for glutamine 0·16 mM. Positive homotropic cooperativity was observed for MgATP only, and gel filtration studies indicated that the substrate-free enzyme (MW 160000) associated to a dimer (MW 320000) in the presence of $MgCl_2$ and ATP. The purified enzyme, which had some glutaminase activity, catalyzed an aspartate- and glutamine-independent ATP–PPi exchange reaction at a rate 5–7-fold higher than the rate of asparagine synthesis. Initial velocity studies and exchange data indicated an overall ping-pong mechanism. Compared to similar enzymes isolated from mammalian tumor cells, the lupin enzyme appears to be unique with respect to MW, reaction mechanism and regulatory properties. The allosteric properties observed suggest an important role for this enzyme in the regulation of asparagine biosynthesis.

INTRODUCTION

In recent years two routes of asparagine biosynthesis in higher plants have been studied, the HCN–β-cyanoalanine pathway [1–3], and the direct formation from aspartate with glutamine as the preferred donor of amide group N [4–6]. (For discussions of earlier reports on NH_3-dependent asparagine synthetases from higher plants see Refs [4,6,7].) The two enzymes of the first pathway have been purified [2,3], but several reports have indicated that this pathway does not function as a normal mechanism of asparagine synthesis *in vivo* [6–9]. In 1970, Rognes [4] and Streeter [5] independently detected in extracts of lupin and soybean seedlings a glutamine-linked enzymatic activity which catalyzed asparagine-[^{14}C] synthesis from aspartate-[^{14}C] in the presence of Mg^{2+} and ATP. Later, such activity was also found in several other plant species [6]. The reaction was proposed [4] to be similar to the one catalyzed by mammalian tumor cell asparagine synthetases [10,11]:

$$\text{ATP} + \text{aspartate} + \text{glutamine} \xrightarrow{Mg^{2+}} \text{AMP} + \text{PPi} + \text{asparagine} + \text{glutamate}.$$

Only preliminary studies with slightly (10–20-fold) purified preparations of the plant enzyme have been reported so far [4–6]. The products and the stoichiometry of the reaction have not been determined, and the properties of the enzyme have not been examined in detail. One obstacle has been the instability of the enzyme.

This paper reports a procedure for obtaining highly purified glutamine-dependent asparagine synthetase from yellow lupin and some of its catalytic, kinetic and molecular properties.

Table 1. Purification of asparagine synthetase from *Lupinus luteus*

Step	Fraction	Volume (ml)	Protein (mg)	Total activity (units)	Sp. act. (units/mg)	Purification (-fold)
1	Crude extract	1100	24800	10900	0·44	1
2	0–42% $(NH_4)_2SO_4$ ppt.	200	3840	8830	2·3	5
3	Protamine sulfate supernatant	210	2940	8530	2·9	7
4	Alumina gel supernatant, 0–38% $(NH_4)_2SO_4$ ppt.	30	780	6710	8·6	20
5	Alumina gel eluate	95	142	3850	27·1	62
6	Sephadex G-200 eluate (substrates present)	36	72	2550	35·4	80
7	DEAE-Sephadex eluate	30	24	1870	78·0	177
8	Sephadex G-200 eluate (no substrates)	8	2·4	540	225·0	511

The preparation was obtained from 543 g (fr. wt) of cotyledons from 7-day-old plants.

RESULTS

Purification of crude extract

Cotyledons of 1-week-old seedlings gave crude extracts with the highest sp. act. of the enzyme (0·3–0·6 units/mg). This age corresponds to the period of the most rapid asparagine accumulation *in vivo* [12]. Crude extracts had high levels of ATPase (E.C. 3.6.1.3) and phosphatase activities. A linear relationship between protein concentration and activity was obtained with 0–80 μg protein/100 μl assay mixture. The results of a typical purification are given in Table 1, the final preparation was purified over 500-fold in about 5% yield. Disc gel electrophoresis of this fraction revealed four protein bands, and the enzyme was estimated to be 20–30% pure. The purified enzyme was free of ATPase, 5′-nucleotidase (E.C. 3.1.3.5) and adenylate kinase (E.C. 2.7.4.3) activities, but still had some inorganic pyrophosphatase (E.C. 3.6.1.1) and glutaminase (E.C. 3.5.1.2) activity. Glutaminase activity remained associated with the enzyme during the last two purification steps and showed a similar elution profile.

Table 2. Thiol requirement of asparagine synthetase

Protein dissolved in	Days	Asparagine formed (nmol/30 min) Dithiothreitol	No thiol
Buffer A (no thiol)	0	19·4	3·6
	9	0·9	0·1
Buffer A plus 14 mM mercaptoethanol	0	37·4	37·1
	9	35·5	22·3
Buffer A plus 14 mM mercaptoethanol plus 0·5 mM dithiothreitol	0	38·1	36·9
	9	36·2	35·0

Step 4 protein (sp. act. 8 units/mg) was reprecipitated with $(NH_4)_2SO_4$ (0–40%) at pH 7·5 and washed once with 40% saturated $(NH_4)_2SO_4$ soln. The pellets were dissolved in three different chilled buffers, with thiols added as indicated, to give protein concentrations of 6·4 mg/ml. Protein (160 μg) from each solution was assayed after 1 hr (0 days) and after 9 days of storage at −25°, with or without 2 mM dithiothreitol included in the standard assay.

Thiol requirement and stability

The general instability of the enzyme prompted a search for conditions giving maximum protection. The enzyme had an absolute requirement for an environmental thiol. When preparations were dissolved in a thiol-free buffer, activity was rapidly lost (Table 2). A combination of high levels of mercaptoethanol and dithiothreitol was satisfactory. In conjunction with thiols improved stability was observed after addition of glycerol and KCN to the buffers.

In the absence of substrates the enzyme was extremely thermolabile, and half-lives in buffer B at 37 and 56° were found to be 90 and 30 sec, respectively. However, under assay conditions (37°) rates of asparagine formation were linear with time for up to 60 min, from which it can be inferred that substrate binding must result in a considerably more stable conformation. Concentrated enzyme solutions in buffer D were stored at −25° for extended periods with little loss of activity. The protecting effect appeared mainly to be due to $MgCl_2$ + ATP. The sp. act. of the purified enzyme in buffer B decreased to 40% of its initial value after 3 months at −25°.

Table 3. Product formation and stoichiometry of the reaction catalyzed by asparagine synthetase

Reaction mixture	Time (min)	Product formed (μmol) Asparagine	AMP	PPi	Pi	Glutamate
Complete	60	0·69	0·70	0·66	0·02	0·96
Complete	30	0·36	0·34	0·38	0·01	0·53
Minus aspartate	30	0*	0·02	0·01	0·01	0·15
Minus ATP	30	0	0	0	0	0·12
Minus $MgCl_2$	30	0	0·01	0·01	0	0·13
Minus glutamine	30	0	0·01	0·02	0·01	0
Minus NaF	30	0·37	0·34	0·08	0·58	0·55
Minus enzyme	30	0	0	0	0	0

* A zero means that less than 0·01 μmol of product was found.

Complete reaction mixtures comprised in 0·50 ml: 12 enzyme units (60 μg protein), 5 mM aspartate-[^{14}C], 4 mM ATP, 8 mM $MgCl_2$, 5 mM glutamine, 50 mM Tris–HCl (pH 7·6), 2·5 mM NaF and 2 mM dithiothreitol. Components were omitted as indicated. After incubation at 37° for 30 or 60 min, 0·10 ml icecold 10% TCA was added, and the tubes were chilled in ice. Suitable aliquots were removed from the supernatants after centrifugation for determination of asparagine-[^{14}C], AMP, PPi, Pi and glutamate (see Experimental). Values are corrected for a zero time control.

Reaction products and stoichiometry of the glutamine reaction

The enzyme catalyzed the aspartate-, glutamine-, ATP- and Mg^{2+}-dependent formation of asparagine, AMP, PPi and glutamate (Table 3). The formation of nearly equimolar amounts of AMP, PPi and asparagine required the simultaneous presence of all substrates. Glutamate formation was partially independent of aspartate, Mg^{2+} and ATP. However, the results indicated an approximate stoichiometry between aspartate-dependent glutamate production and formation of the three other products. The independent glutaminase activity amounted to 30–50% of the glutamate formation coupled to asparagine synthesis. No formation of ADP was detected in standard assay mixtures. Addition of NaF to the reaction mixtures had no appreciable effect upon the formation of asparagine, AMP or glutamate, but was necessary to obtain stoichiometric quantities of PPi. F^- completely inhibited the inorganic pyrophosphatase remaining in the preparation, thereby preventing the formation of 0·57 μmol Pi from 0·30 μmol PPi.

Net synthesis of asparagine catalyzed by the enzyme was established by use of PC and *E. coli* L-asparaginase [4].

Specificity and catalytic properties

ATP could not be replaced by ADP or AMP and Mg^{2+} could not be replaced by Zn^{2+}, Fe^{2+}, Co^{2+}, Ba^{2+} or Ca^{2+}. Ca^{2+} was also strongly inhibitory when added to the standard assay, 0·3 mM Ca^{2+} inhibited the reaction by 50%. Simultaneous inclusion of 5 mM EDTA eliminated this inhibition. Mn^{2+}, at lower concentrations than were optimal for Mg^{2+}, gave *ca* 15% of the activity obtained in the standard assay. Addition of 50 mM KCl to the standard assay had no effect.

NH_4Cl, at 10–20-fold higher concentrations than used for glutamine, could replace glutamine for asparagine formation. V_{max} with NH_4Cl as N-donor was 30% of the V_{max} with glutamine at pH 7·8. The ratio of the NH_3-linked and glutamine-linked activities did not change appreciably during purification, and the two activities were not separated. The products of the NH_3 reaction were asparagine, AMP and PPi. When NH_2OH replaced glutamine, β-aspartylhydroxamate was identified as a product. Hydroxamate formation required aspartate, ATP and Mg^{2+}. Ten units of enzyme (standard assay) catalyzed the formation of 8·8 nmol/min of β-aspartylhydroxamate in the hydroxamate assay. After step 5, the activity causing aspartate-dependent hydroxamate formation was copurified with asparagine synthetase and followed exactly the same elution pattern.

The purified enzyme exhibited high activity over a broader pH range than the cruder preparation [4]. An optimum at pH 7·6 was found for the glutamine reaction in Tris–HCl, triethanol-amine–HCl and imidazole–HCl buffers, with 80% of maximal activity at pH 6·9 and 8·3.

Initial velocity kinetics with glutamine as N-donor

The effect of varying the Mg^{2+} concentration at three different levels of ATP was examined.

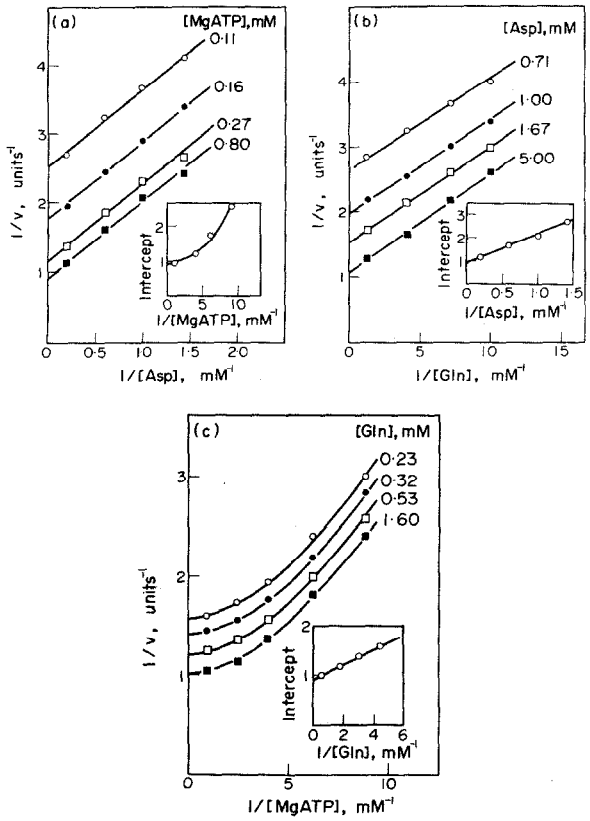

Fig. 1. Initial velocity pattern of asparagine synthetase. In all radioactive assays a 4 mM excess of Mg^{2+} over ATP was maintained. (a) Reciprocal velocity vs reciprocal concentration of aspartate at four fixed levels of MgATP and constant glutamine concentration (10 mM). Inset: replot of intercepts on the ordinate vs reciprocal concentration of MgATP. (b) Reciprocal velocity vs reciprocal concentration of glutamine at four fixed levels of aspartate and constant MgATP concentration (1·25 mM). Inset: replot of intercepts vs reciprocal aspartate concentration. (c) Reciprocal velocity vs reciprocal MgATP concentration at four fixed levels of glutamine and constant aspartate concentration (5 mM). Insct: replot of intercepts vs reciprocal glutamine concentration.

Results showed that free ATP was strongly inhibitory, and that maximal rates were obtained with a 0·8–10 mM excess of Mg^{2+} over ATP. Under such conditions all ATP would be complexed [13]. Aspartate, glutamine and MgATP were considered to be the three actual substrates.

The initial velocity pattern of the reaction was studied by making reciprocal plots for one substrate (variable substrate) at different fixed concentrations of a second substrate (changing fixed substrate), while the concentration of the third substrate was kept constant at a high, saturating level [13,14].

When aspartate was the variable substrate, sets of parallel lines were obtained when MgATP was the changing fixed substrate at constant glutamine concentration (Fig. 1a, or when glutamine was the changing fixed substrate ([MgATP] constant). Similarly, when glutamine was the variable substrate, a parallel pattern was found at different fixed concentrations of aspartate [MgATP] constant) (Fig. 1b, or with MgATP as the changing fixed substrate ([aspartate] constant). When MgATP was the variable substrate, all reciprocal plots were parabolic, concave up (Fig. 1c). Plots of $1/v$ vs $1/[MgATP]^2$ were essentially linear and parallel. Hill plots (log $[v/(V_{max} - v)]$ vs log $[S]$) of data collected from several experiments with different batches of enzyme gave interaction coefficients $n_H = 1·0$ for aspartate, $n_H = 1·0$ for glutamine and $n_H = 1·8$–$2·0$ for MgATP.

Secondary plots of the intercepts (Fig. 1, insets) gave the following limiting Michaelis constants, for aspartate $K_m = 1·3$ mM, for glutamine $K_m = 0·16$ mM and for MgATP $K_m = 0·14$ mM.

ATP–PPi exchange

The purified enzyme catalyzed a vigorous incorporation of PPi-[^{32}P] into ATP. The rate of the ATP–PPi exchange was constant for the first 25 min of reaction and proportional to the amount of enzyme added. Incorporation of labeled PPi required Mg^{2+}, ATP and enzyme, however, it was independent of added aspartate

Table 4. Characteristics of the ATP–PPi exchange reaction catalyzed by asparagine synthetase

Incubation system	PPi-[^{32}P] incorporated in ATP (cpm × 10^{-3})	% Of complete system
Complete	23·0	100
0·5 enzyme	12·9	56
Minus enzyme	0·38	2
Minus ATP	1·9	8
Minus $MgCl_2$	2·6	11
Plus aspartate (25 mM)	23·6	103
Plus glutamine (20 mM)	23·2	101
Plus aspartate (25 mM), plus glutamine (20 mM)	19·8	86
Plus Pi (20 mM)	23·7	103
Plus PPi (12 mM)	3·4	15

Standard reaction mixtures (see Experimental) were used with omissions and additions as designated. 0·25 units of enzyme/tube, except where noted. The sp. act. of the labeled PPi was $1·2 \times 10^6$ cpm/μmol.

or glutamine (Table 4). Addition of both aspartate and glutamine to the reaction mixture, making it a complete system for asparagine synthesis, depressed the exchange by 14%. Individual additions of Pi (20 mM), acetate (10 mM), NH_4Cl (10 mM) or asparagine (20 mM) had no significant effect, NH_2OH (200 mM) inhibited the exchange by 25%. In the range of 1–10 mM F^- identical results were obtained, omission of F^- gave 15% lower incorporation. Addition of non-labeled PPi, as expected, led to diminished incorporation of radioactivity into ATP, showing that the reaction really was an exchange of PPi.

A preparation of sp. act. 165 asparagine synthetase units/mg protein catalyzed an exchange of 880 nmol PPi/min . mg protein. It was calculated that the rate of the ATP-PPi exchange reaction was 5–7-fold higher than the rate of overall asparagine synthesis catalyzed by the enzyme.

Gel filtration behaviour: substrate effects on apparent MW

When subjected to Sephadex G-200 gel filtration in the absence of substrates (buffer B), the 60-fold purified enzyme migrated essentially as one band of MW *ca* 160000. On the other hand, when samples of the same preparation were filtered under identical conditions, except for the presence of all substrates (buffer D), activity was always eluted in a single symmetrical peak of MW *ca* 320000. This behaviour was reproduced with three different batches of enzyme, a similar effect of substrates was also observed during ion-exchange chromatography. The results were tentatively interpreted as a change in MW of the enzyme, possibly a dimerization, specifically induced by one or more of the substrates [15]. Benefit was drawn from this behaviour to achieve purification of the enzyme (see steps 6 and 8 of purification procedure). In the final step enzyme of MW 160000 was obtained.

Slightly purified (20-fold or less) enzyme frequently gave one broad, composite peak or two peaks of activity, corresponding to both the MW 160000 and the MW 320000 species, when filtered in the absence of substrates. Incomplete removal of residual bound ligands might explain this, since a more thorough preliminary removal of substrates by precipitation and desalting techniques always tended to displace the equilibrium in favour of the MW 160000 form.

Table 5. Effect of substrates on Sephadex G-200 gel filtration behaviour of asparagine synthetase

Additions to filtration buffer (buffer B)	Ratio V_e/V_0*	Apparent MW (daltons)
(a) None	1·52	163000
(b) Plus aspartate (5 mM), plus glutamine (5 mM)	1·53	159000
(c) Plus ATP (1 mM), plus $MgCl_2$ (5 mM)	1·26	323000
(d) As (c), plus aspartate (5 mM)	1·27	318000
(e) As (d), plus glutamine (5 mM)	1·25	328000

* V_e = elution volume of asparagine synthetase activity peak, V_0 = void volume.

Filtration experiments (a)–(e) were carried out with portions (3 mg) of concentrated step 7 enzyme protein. The columns were equilibrated and run with buffer B containing additions as designated.

To study the effect of specific substrate combinations on the apparent MW 170-fold purified enzyme was used. As shown in Table 5, $MgCl_2$ and ATP were required for aggregation to the MW 320000 form. In the presence of aspartate and glutamine only the enzyme was eluted with MW 160000.

DISCUSSION

By the procedure developed glutamine-dependent asparagine synthetase was purified to a sp. act. of 225 units/mg protein. This represents the first highly purified preparation from a plant source, and its sp. act. is higher than reported for the enzymes from the Novikoff hepatoma [10] (90 units/mg) and mouse leukemia cells [11] (180 units/mg) (units as defined in this paper).

The results indicated that asparagine synthesis followed the proposed reaction scheme and was coupled to stoichiometric cleavage of ATP to AMP and PPi, which is in agreement with data for the glutamine-dependent tumour enzymes [10,11] and the NH_3-dependent *E. coli* enzyme [16]. Glutamine-hydrolyzing activity was tightly associated with the enzyme. However, substantially more glutamate was formed in the complete assay system, and aspartate-dependent glutamate formation approached the amount of asparagine formed. High glutaminase activity was also reported for the soybean [6] and mouse cell [11]

preparations. In the latter case glutamate production was found to be Cl^- -dependent, but independent of added aspartate. Possible Cl^- effects on the lupin enzyme have not as yet been explored. By analogy with several other glutamine-linked N-transferases the glutaminase activity most likely represents a partial reaction of the overall process, possibly located to a special subunit in the enzyme structure [17–19].

The complex reaction involves three substrates and four products. The parallel pattern obtained throughout in the initial velocity studies gave evidence for an overall ping-pong [13] or enzyme substitution [14] mechanism, in which the substrates aspartate, glutamine and MgATP combine with different enzyme forms not interconnected through reversible steps. This could be fulfilled by release of a product, and hence, formation of a substituted enzyme species, before the next substrate enters the reaction sequence. The first substrate which combines with the enzyme must be MgATP or glutamine. It is indicated that MgATP adds, and PPi is released, giving an adenylylated enzyme intermediate, before aspartate adds to the enzyme. This hypothesis requires an aspartate-*in*dependent ATP–PPi exchange, in agreement with the data obtained. The high rate of the exchange reaction supports the idea that it represents a not rate-limiting partial reaction of the complete catalytic process. For the mouse cell enzyme, in contrast, some evidence was presented in favour of sequential addition of aspartate and MgATP and involvement of enzyme-bound β-aspartyladenylate as an intermediate [11]. However, the reported rates of aspartate-dependent ATP-PPi exchange were very low (only 2% of the rate of asparagine synthesis), which is rather puzzling. The NH_3-dependent *E. coli* enzyme was shown by kinetic analysis to have sequential addition of aspartate and MgATP, and in this case the rate of the aspartate-dependent ATP–PPi exchange closely approached the rate of asparagine synthesis [20]. To elucidate the complete mechanism of the lupin enzyme, further work, including product and competitive inhibitor studies, is necessary.

The gel filtration studies indicated that the substrate-free enzyme is an oligomer of 160000 daltons which associates to a di-oligomer only upon binding of MgATP. The enzyme was only 20–30% pure and some binding to inert protein during gel filtration might be possible. However, the specificity and reproducibility of the substrate effect tend to give such an argument less weight. Ligand-induced association–dissociation behaviour as a means of control has been shown for a number of regulatory enzymes, including glutamine-dependent ligases [21–23]. Dimerization of CTP synthetase from *E. coli* was largely independent of enzyme purity [15,21]. It is necessary to measure the state of polymerization of an enzyme under assay conditions when the state of polymerization of the active species is desired [21]. In the case of the lupin enzyme the catalytically active species must be the aggregated form of 320000 daltons. This MW is much larger than the values estimated for the mouse cell (105000 daltons) and *E. coli* (82000 daltons) enzymes [11,16].

Consistent with the requirements for dimerization and the proposed ping-pong mechanism is the positive homotropic cooperativity observed for MgATP and the absence of such effects for aspartate and glutamine. The Hill coefficient close to 2 would suggest two strongly interacting binding sites for MgATP. According to Sumi and Ui [24], allosteric enzymes with ping-pong mechanism will exhibit cooperative kinetics only with respect to the substrate which directly binds to the enzyme form undergoing allosteric transition. The pieces of evidence derived from different types of experiments with the lupin enzyme are thus in agreement.

The lupin enzyme and the slightly purified soybean enzyme studied by Streeter [6] have several general properties in common (e.g. stability properties, thiol requirement, much lower effectivity and higher K_m of NH_3 compared to glutamine, roughly similar K_m values). In contrast, the lupin enzyme is widely different from the NH_3-dependent activity described by Nair [25]. In comparison with mammalian asparagine synthetases the lupin enzyme is unique in certain respects, which might well reflect different functions of the amide in plant and animal metabolism. Product inhibition by asparagine is much weaker, and the MW and mechanism of reaction appear to be different. Asparagine synthesis can be viewed as a one-enzyme pathway, and one might expect the plant enzyme to be fitted with properties allowing some type of metabolic control. Par-

ticularly intriguing is the suggested role of ATP level and adenine nucleotide pool sizes in the regulation of asparagine synthetase as compared to another key enzyme of aspartate metabolism, aspartate kinase [26]. Many *in vivo* studies have shown that conditions which affect the energy level of tissues (e.g. light/darkness [27], sugar feeding [28]) also profoundly influence the channeling of carbon and nitrogen into asparagine compared to other protein amino acids of the aspartate family.

EXPERIMENTAL

Plant material. About 0·3 kg of seeds (*Lupinus luteus* L.), from the same batch as used previously [4], were treated with a hypochlorite soln, rinsed and soaked in H_2O for 15 hr at 20°. Seedlings were grown in vermiculite in darkness at 25° with abundant H_2O supply. Cotyledons from 6- to 7-day-old plants were excised, rinsed briefly with dist H_2O and immediately used for enzyme preparation.

Buffers. The following solns, with thiols freshly added, were used during purification. Buffer A: 20 mM Tris–HCl (pH 7·5), 15% (by vol.) glycerol. Buffer B: buffer A plus 56 mM mercaptoethanol, 0·5 mM dithiothreitol and 4 mM KCN. Buffer C: 100 mM Tris–HCl (pH 8·5), 15% (by vol.) glycerol, 56 mM mercaptoethanol and 4 mM KCN. Buffer D: buffer B, containing in addition 1 mM ATP, 5 mM $MgCl_2$, 4 mM aspartate and 2 mM glutamine.

Enzyme purification. All solns were kept at 0–4°. The first five steps should be carried out with as little delay as possible. Usually the procedure was interrupted by freezing the enzyme at −25° after completion of step 5.

Step 1. Chilled cotyledons (0·5 kg fr. wt) were homogenized with buffer C (800 ml) in a Waring Blendor for 3 × 30 sec. The homogenate was passed through 4 layers of cheesecloth and the filtrate was centrifuged (30 min, 13000 *g*). The supernatant soln (crude extract) contained 95–98% of the total activity present in the filtered homogenate.

Step 2. The crude extract was made to 42% saturation with solid $(NH_4)_2SO_4$, pH being kept at 7·5 by addition of NH_3. Additional 0·5 ml/l. of mercaptoethanol was added. After stirring for 30 min, the ppt was collected by centrifugation and dissolved in sufficient buffer B to give a protein concentration of *ca* 20 mg/ml.

Step 3. A freshly prepared 2% soln of protamine sulfate in buffer B was added dropwise to the enzyme soln, with rapid stirring over 45 min, to yield a final concentration of 0·16 mg protamine sulfate/mg protein. The ppt which formed was removed by centrifugation (20 min, 30000 *g*) and discarded.

Step 4. To the protamine-treated enzyme soln a suspension of alumina C_γ gel (11 mg dry wt/ml) in dist H_2O was added to give a final gel/protein ratio of 0·5. The concn of mercaptoethanol was kept at 56 mM. After stirring for 30 min, the gel with adsorbed inactive material was centrifuged down and discarded. The supernatant soln was fractionated with solid $(NH_4)_2SO_4$. The 0–38% protein ppt. was dissolved in buffer B (30 ml) and desalted by passage over a 4·2 × 22 cm Sephadex G-25 column equilibrated and eluted with buffer B. The active fractions of the eluate were combined.

Step 5. To the enzyme soln a suspension of alumina C_γ gel (29 mg dry wt/ml) in dist H_2O was added to give a gel/protein ratio of 3–4. The gel, with almost all protein adsorbed, was centrifuged down after 30 min. The enzyme was eluted by washing the gel × 4 with portions (25 ml) of buffer B containing 0·35 M $(NH_4)_2SO_4$. The supernatant solns after centrifugation were combined (95 ml), and the enzyme was reprecipitated by adding more $(NH_4)_2SO_4$ to 38% saturation.

Step 6. The enzyme protein was dissolved in buffer D (6 ml) and placed on a Sephadex G-200 column (2 × 40 cm) equilibrated with buffer D. Filtration was performed with the same buffer at 10 ml/hr, 2·7 ml fractions were collected. Under these conditions (substrates present) enzyme activity emerged as one homogeneous peak ($V_e/V_0 = 1·27$). The most active fractions were pooled.

Step 7. The active filtrate was applied to a DEAE-Sephadex A-50 column (2 × 5 cm) equilibrated with buffer B, and the column was washed with 15 ml buffer B. Elution was accomplished with a linear gradient of NaCl (0–0·6 M) in buffer B, each chamber containing 50 ml of soln. Enzyme activity appeared as a peak near the end of the gradient.

Step 8. The enzyme was carefully precipitated from the pooled active fractions of the DEAE-Sephadex eluate by addition of $(NH_4)_2SO_4$ (0–40%), and the protein pellet was dissolved in buffer B (2 ml). The enzyme soln was filtered on a Sephadex G-200 column similar to the one used in step 6, equilibrated with buffer B (no substrates present). The enzyme was eluted with buffer B, essentially as a single peak ($V_e/V_0 = 1·52$), with a small protein peak coinciding. The fractions of highest sp. act. were combined, distributed in 0·5 ml portions and stored at −25°. This fraction (purified enzyme) was used in the experiments described unless otherwise stated.

Asparagine synthetase assays. Standard assay: reaction mixtures, incubated at 37° for 30 min, contained in 50 or 100 μl: 6 mM L-aspartate-[^{14}C] (sp. act. 10^6 cpm/μmol), neutralized with Tris, 5 mM ATP, 10 mM $MgCl_2$, 10 mM L-glutamine, 2 mM dithiothreitol, 100 mM Tris–HCl (pH 7·6 at 37°) and a rate-limiting amount of enzyme protein. The reaction was initiated by addition of enzyme and stopped by adding 25 μl of a mixture of 10% TCA and 0·2 M L-asparagine (7:1). After centrifugation, 25 μl of the supernatant soln was subjected to paper electrophoresis and asparagine-[^{14}C] was determined as described [4]. Aspartate-[4-^{14}C] was used during the first four purification steps to eliminate interference from other activities, during later steps aspartate-[U-^{14}C] was used.

Hydroxamate assay: In 0·6 ml were contained: 60 mM L-aspartate, 10 mM ATP, 15 mM $MgCl_2$, 400 mM NH_2OH–HCl (brought to pH 7·6 with Tris), 2 mM dithiothreitol and enzyme protein. Incubation was for 60 min at 37°. After addition of 0·9 ml $FeCl_3$-reagent [29] and centrifugation, $A_{540\,nm}$ was determined. A value of $\epsilon_{540\,nm} = 600\ M^{-1}\ cm^{-1}$ for β-aspartylhydroxamate was employed [29].

One unit of asparagine synthetase activity will catalyze the formation of 1 nmol/min of asparagine under standard assay conditions.

ATP–PPi exchange. PPi-[^{32}P] incorporation into ATP was measured after separation of labelled ATP from uncharged PPi-[^{32}P] by adsorption of ATP to acid-washed charcoal [30]. Standard assay mixtures contained in 0·2 ml: 2 mM ATP, 1 mM PPi-[^{32}P] (sp. act. 0·5 to 1·5 × 10^6 cpm/μmol), 7 mM $MgCl_2$, 2·5 mM NaF, 2 mM dithiothreitol, 50 mM Tris–HCl (pH 7·5) and *ca* 0·2 units of purified enzyme. After 15 min at 37°, the reaction was stopped by addition of 0·2 ml of non-labeled 0·4 M PPi in 7% (w/v) $HClO_4$, followed by 0·15 ml of a 9% (w/v) suspension of acid-washed charcoal. The mixtures were filtered through Whatman GF/C glass fiber discs. The filter discs were washed with 20 ml icecold H_2O, dried and assayed for radioactivity. Exchange rates were calculated according to Ref. [31].

Other enzyme assays. Inorganic pyrophosphatase, ATPase and 5'-nucleotidase were assayed by measuring the release of Pi during incubations with 1 mM substrate ($Na_4P_2O_7$, ATP or AMP), 5 mM $MgCl_2$, 100 mM Tris–HCl (pH 7·6) and protein in a vol. of 0·5 ml. After 15 min at 37°, the tubes were placed in ice and 0·3 ml of 10% (w/v) TCA was added. Samples of the supernatant soln after centrifugation were analyzed for Pi. Glutaminase was assayed by measuring glutamate formation as described in ref. [32].

MW estimation. Gel filtration on calibrated Sephadex G-200 columns was used [33]. For standardization the columns (2 × 40 cm) were run at 4° with buffer A containing 7 mM mercaptoethanol. Reference materials were applied singly or in sequence in a 1 ml vol. Blue Dextran was measured at 620 nm, catalase was assayed by measuring the disappearance of H_2O_2 at 240 nm [34], alcohol dehydrogenase by reduction of NAD^+ at 340 nm [35], and urease by a microdiffusion method followed by Nesslerization. Other proteins were followed by their $A_{280\,nm}$.

Analytical methods. Paper electrophoresis at pH 5·0 (50 mM Na-acetate, 48 V/cm, 40 min) separated glutamate from aspartate and asparagine–glutamine. Following electrophoresis, glutamate was determined with ninhydrin [32]. Electrophoresis at pH 4·4 (50 mM Na-citrate, 35 V/cm, 90 min) separated ATP, ADP, AMP and PPi [36]. AMP was eluted with 0·1 N HCl and determined from $A_{257\,nm}$ ($\epsilon^{AMP}_{257\,nm} = 1{\cdot}5 \times 10^4\,M^{-1}\,cm^{-1}$). PPi and Pi were determined in deproteinized reaction mixtures by a semimicro modification of the method of ref. [37]. 2,4-DIAMINOPHENOL diHCl replaced 1,2,4-aminonaphtholsulfonic acid as eikonogen. Protein in soln was determined with the Folin-Ciocalteu reagent and crystalline bovine serum albumin as a standard. Since the buffered solns contained high levels of mercaptoethanol, preliminary precipitation with TCA was carried out as described in ref. [38]. Analytical disc gel electrophoresis was done in 5 and 7·5% polyacrylamide gels [39]. Only the separating gel was used and the buffer included 14 mM mercaptoethanol. Electrophoresis was run at 4° with 1–2 mA/tube for 90–120 min. Gels were stained for protein with Coomassie Brilliant Blue [40].

Chemicals. Alumina C_γ gel was prepared and aged as described by Bauer [41]. β-aspartylhydroxamic acid was synthesized according to ref. [42]. All other reagents were obtained commercially. DL-aspartic acid-[4-^{14}C] was from New England Nuclear. L-aspartic acid-[U-^{14}C] and $Na_4P_2O_7$-[^{32}P] were from The Radiochemical Centre. The uniformly labeled L-aspartic acid was further purified over a Dowex 1-X8 column. Protamine sulfate (ex salmon roe) and the following proteins (MW in parentheses) were from Koch-Light: chymotrypsinogen A, ex bovine pancreas (25000), chicken egg albumin (45000), bovine serum albumin (68000), yeast alcohol dehydrogenase (150000), beef liver catalase (250000) and jack bean urease (480000).

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