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ANION REGULATION OF LUPIN ASPARAGINE SYNTHETASE: CHLORIDE ACTIVATION OF THE GLUTAMINE-UTILIZING REACTIONS

SVEN ERIK ROGNES

Botanical Laboratory, University of Oslo, P.O. Box 1045, Blindern, Oslo 3, Norway

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Key Word Index—*Lupinus luteus*; Leguminosae; yellow lupin cotyledons; asparagine synthetase; glutaminase; anion activation; chloride; kinetics.

Abstract—Small monovalent anions strongly activate glutamine-dependent asparagine synthesis and glutamine hydrolysis catalysed by highly purified asparagine synthetase (EC 6.3.5.4) from cotyledons of *Lupinus luteus* seedlings. Cl^- and Br^- are most effective, but F^-, I^-, NO_3^- and CN^- also stimulate both reactions. The synthetase reactions with NH_3 or NH_2OH are only slightly stimulated by Cl^- and Br^-, indicating that the anions selectively accelerate the reactions involving glutamine cleavage. In asparagine synthesis Cl^- is a competitive activator vs glutamine and a non-competitive activator vs MgATP and aspartate. Addition of Cl^- changes the substrate saturation kinetics of glutamine from negatively cooperative to normal hyperbolic and causes a 50-fold increase in the affinity for glutamine. The inherent glutaminase activity of the enzyme is enhanced up to 30-fold by addition of Cl^-, MgATP and aspartate. Thus, ligands of the synthetase reaction act as allosteric activators of the glutaminase step in the enzyme mechanism.

INTRODUCTION

Enzymes catalysing amide nitrogen transfer from glutamine to aspartate in the presence of ATP and Mg^{2+} have been partially purified and characterized from normal and tumorous mammalian tissues [1–5] and legume seedlings [6–9]. These asparagine synthetases (glutamine-hydrolysing) [L-aspartate: L-glutamine amido-ligase (AMP-forming); EC 6.3.5.4] can also, but less effectively, utilize ammonia or hydroxylamine as nitrogen donors. The important role of asparagine in plant nitrogen transport and storage suggests a wide distribution of the synthetase. Recently, such activity has been detected in crude extracts of several plant tissues [10–13] although assay conditions have varied.

Horowitz and Meister [3] found that the RADA1 mouse cell asparagine synthetase exhibited glutaminase activity and made the important discovery that both activities required Cl^- ions to be present. However, in none of the published studies on the plant enzymes have the anion requirements of the overall process or the partial reactions been examined and clearly defined. For instance, Cl^- has always been added to assay mixtures as Tris–HCl or $MgCl_2$, more or less fortuitously. The possibility that the plant enzymes might also be activated by Cl^- has been suggested [7, 13]. On the other hand, Lea and Fowden [9] stated that the *Lupinus albus* enzyme did not require Cl^-, although no supporting evidence was presented.

It seemed necessary to investigate the influence of anions on a plant asparagine synthetase. This paper reports results of such studies, extending the work on the highly purified yellow lupin cotyledon enzyme [6, 7]. The glutamine-hydrolysing activity of the synthetase has also been examined.

RESULTS

Properties of purified enzyme

The enzyme (*ca* 30% pure) had a specific activity of 3.0–4.5 mkat/kg protein and contained no significant ATPase, 5′-nucleotidase or adenylate kinase activities [7]. The possible presence of two other enzymes was especially checked. Aspartate aminotransferase activity was quite high in crude extracts, but could not be detected in the purified enzyme. Glutamine synthetase is known to catalyse hydrolysis of glutamine in presence of ADP, a divalent metal ion and arsenate (or Pi, by a potential reversal of the forward reaction) [14]. For the subsequent studies of glutamine cleavage it was important to know whether glutamine synthetase was still present. No significant activity (< 1% of asparagine synthetase) was found, indicating effective separation of the two enzymes during purification. NH_4^+, hydroxylamine, glutamine, glutamate, asparagine and aspartate (at 10 mM) were tested as N-donors in the assays of glutamine synthetase and asparagine synthetase, respectively. Results showed that (1) NH_4^+ alone and NH_4^+ plus glutamate gave equal amounts of asparagine synthesized, another indication that glutamine synthetase was absent; (2) no amide-N transfer reaction from asparagine to glutamate did occur, the asparagine synthetase reaction was apparently not reversible; and (3) no exchange reactions between [^{14}C]-aspartate and asparagine, or [^{14}C]-glutamate and glutamine, were catalysed by the enzyme.

Co-purification and chloride stimulation of glutaminase and asparagine synthetase

Glutaminase activity was always associated with the enzyme. In the final Sephadex step, the elution profiles of the synthetase activities (utilizing glutamine, NH_4^+ or hydroxylamine) and glutaminase were identical. The fractions of the single peak observed had a constant ratio between these activities, which suggested glutamine hydrolysis to be an inherent function of the enzyme. This was supported by the responses to Cl^-. When enzyme was isolated and assayed in absence of Cl^-, glutaminase activity was rather weak, but it could be increased 4- to 6-fold by including 20 mM KCl in the assay. Similarly,

glutamine-dependent asparagine synthesis was low in the absence of Cl^- and increased more than 6-fold when 20 mM KCl was added. In the absence of Cl^-, the rates of β-aspartylhydroxamate formation and NH_3-dependent asparagine synthesis were 50% higher than the rate of glutamine-dependent asparagine synthesis. However, these reactions were only 30–60% stimulated by KCl. Omission of Cl^- had no appreciable influence on the pH-dependence of the activities, or on the linearity of the time course. These results suggested that certain anions, like Cl^-, act more or less selectively on one part of the enzyme mechanism, by accelerating the process of glutamine utilization.

Effect of anions

To gain information on the specificity of the Cl^- effect, the influence of several K^+ and Na^+ salts on three reactions catalysed by the enzyme was studied (Table 1). Control assays contained Pi, SO_4^{2-} and acetate; these anions were not activators.

Glutaminase activity was most effectively increased by Cl^- and Br^- and also markedly enhanced by small monovalent anions in the order $CN^- > I^- > NO_3^- > F^-$. Borate had a positive effect, but other di- and trivalent anions tested (including PPi, arsenate, malate, fumarate, tartrate and citrate) were without effect or somewhat inhibitory. The monovalent cation did not seem to be important as K^+ and Na^+ salts gave similar results.

A similar pattern of activation was found for glutamine-dependent asparagine synthetase. Cl^- and Br^- were superior to F^-, I^-, NO_3^- and CN^-. Citrate and PPi were strongly inhibitory.

NH_3-dependent asparagine synthesis was differently affected. A slight increase in activity was observed with Cl^-, Br^- and CN^-, but most other anions were without effect. Citrate, borate and PPi again caused inhibition.

Among anions occurring in sufficiently high concentrations in plant tissues to be of regulatory importance, Cl^- and perhaps NO_3^- appeared to be the best activators.

Effect of chloride on the substrate kinetics of glutamine-dependent asparagine synthetase

The relationship between Cl^- and each of the three substrates glutamine, MgATP and aspartate was analysed. When the glutamine concentration was varied at different fixed Cl^--levels, typical results, as shown in Fig. 1, were obtained. In the absence of Cl^-, or at a low Cl^- concentration, the affinity for glutamine was low. The saturation plot was biphasic with an intermediary plateau at *ca* 1 mM glutamine. In the double-reciprocal plot the curve was strongly concave downward (Fig. 1). The R_s value [15] was $\gg 81$. At 0.3 mM Cl^-, the Hill coefficient (n_H) of glutamine was 0.54 in the range 0.33–2.5 mM.

At 1.5 mM Cl^-, the double-reciprocal plot was less concave downward and n_H higher, 0.85. These diagnostic plots suggested that in the binding of glutamine, the enzyme exhibited negative cooperativity, which was stronger the lower the concentration of Cl^-. At 30 mM Cl^-, the enzyme displayed normal Michaelis–Menten kinetics towards glutamine (rectilinear double-reciprocal and Eadie–Hofstee plots, $R_s = 81$, $n_H = 1.0$), and the $K_{0.5}$ showed a large decrease. Since V_{max} was not affected, Cl^- can be considered a competitive activator with respect to glutamine. Saturation with Cl^- progressively changed the kinetics vs glutamine from negatively cooperative to non-cooperative and increased the affinity for glutamine at least 50-fold.

In similar experiments, the Cl^- concentration was varied at several fixed glutamine levels (Fig. 2). At a glutamine level below 5 mM, the double-reciprocal plot for Cl^- appeared concave downward or biphasic, suggesting symmetry in the responses of glutamine and Cl^-. The $K_{0.5}$ of Cl^- was reduced as the glutamine concentration increased, the double-reciprocal plot also indicated a gradual transition to Michaelis–Menten kinetics (10 mM glutamine). At infinitely high glutamine concentration,

Table 1. Effect of anions on glutamine hydrolysis, glutamine-dependent asparagine synthesis and NH_3-dependent asparagine synthesis catalysed by purified enzyme

Salt added	Enzyme activity, pkat (% of control)		
	(a) Glutaminase	(b) Gln-dependent Asn synthetase	(c) NH_3-dependent Asn synthetase
None (control)	2.3 (100)	6.6 (100)	9.5 (100)
NaCl	12.8 (564)	45.6 (689)	14.1 (148)
KCl	13.7 (596)	47.8 (724)	13.5 (142)
KBr	14.6 (642)	42.9 (650)	12.8 (135)
KI	5.7 (251)	15.9 (241)	9.4 (99)
NaF	4.7 (208)	19.3 (292)	10.5 (110)
KCN	7.2 (317)	8.7 (131)	12.3 (130)
KNO_3	5.3 (232)	13.2 (200)	10.3 (109)
$KHCO_3$	2.5 (112)	6.5 (98)	9.7 (102)
K_2SO_4	1.7 (75)	5.4 (82)	9.3 (98)
$Na_2B_4O_7$	4.9 (218)	5.0 (76)	5.8 (61)
K_2HPO_4	2.0 (87)	6.0 (92)	9.1 (96)

20 mM of salt (solns brought to pH 7.8 with HOAc or NaOH) was added to Cl^--free assay mixtures containing 11 μg enzyme protein isolated in buffer B3 ($n = 2$). Control assays contained 50 mM OAc^- and 10 mM Pi (a); 50 mM OAc^-, 10 mM SO_4^{2-} and 10 mM Pi (b); 50 mM OAc^-, 20 mM SO_4^{2-} and 10 mM Pi (c).

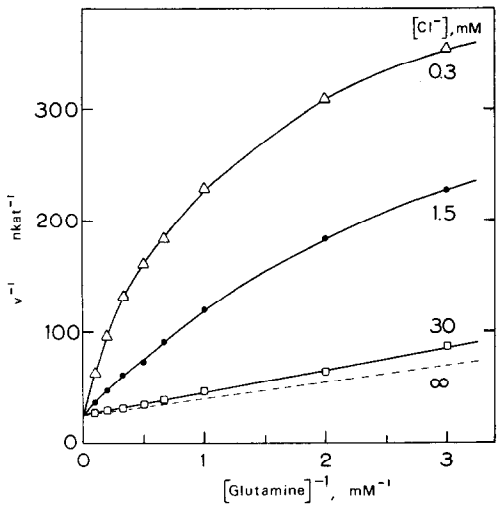

Fig. 1. Effect of Cl^- concentration on the kinetics of glutamine-dependent asparagine synthetase vs glutamine. Double-reciprocal plot. KCl was added to the Cl^--free assay at three fixed levels, glutamine concentration varied as shown, 12 μg protein. Dotted line shows extrapolation to infinitely high Cl^- concentration (from secondary plot, cf. Fig. 2).

Cl^- as a competitive activator is no longer required ($K_{0.5} = 0$). The interdependence of concentrations and $K_{0.5}$ values for Cl^- and glutamine is summarized in Table 2.

The kinetics vs MgATP at different levels of Cl^- is shown in Fig. 3. In agreement with previous findings [7], saturation curves for MgATP were sigmoid, showing positive cooperativity. The pattern was similar at a low or high Cl^- concentration. Secondary plots (Fig. 3, inset) indicated that Cl^- is a non-competitive activator with respect to MgATP. The $K_{0.5}$ of MgATP was not significantly influenced (0.32–0.34 mM) and n_H was 1.65–1.75 at all three Cl^- levels.

The result of an analogous experiment with varying aspartate concentration is shown in Fig. 4. The pattern of

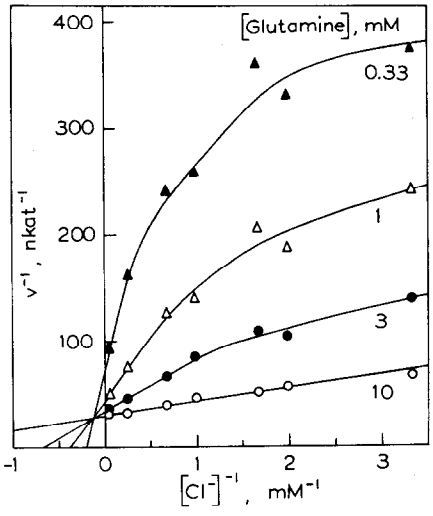

Fig. 2. Effect of glutamine concentration on the kinetics of glutamine-dependent asparagine synthetase vs Cl^-. Double-reciprocal plot, 12 μg protein.

Table 2. Relationship between concentrations and kinetic constants of glutamine and chloride

Kinetic parameter	Glutamine concn (mM)					
	0.33	1.0	5.0	10.0	26.3	∞
$K_{0.5}$ for Cl^-	4.3	2.5	0.9	0.6	0.18	0*
	Cl^- concn (mM)					
	0	0.3	1.5	10.0	30.0	∞
$K_{0.5}$ for glutamine	33.0	14.8	4.4	0.9	0.67	0.55*

Glutamine and KCl concentrations varied, otherwise conditions of the Cl^--free asparagine synthetase assay. $K_{0.5}$ = ligand concentration (in mM) giving 0.5 V_{max} under the given conditions.

* By extrapolation.

straight lines crossing on the abscissa led to the conclusion that Cl^- activation is also essentially non-competitive with respect to aspartate ($K_{0.5}$ = 1.6 mM). At 100 mM Cl^-, inhibition of activity occurred at aspartate levels below 5 mM (not shown), but V_{max} was higher than at 20 mM Cl^-. Thus, there was an inhibitory effect on the slope, but not on the intercept on the ordinate in a double-reciprocal plot. Such an effect, termed competitive substrate inhibition, is characteristic of ping-pong mechanisms [16]. It indicated that Cl^-, at high levels, may interact with and inhibit aspartate binding.

Effect of chloride and ligands on glutamine hydrolysis

Cl^- promoted the partial reaction of glutamate formation from glutamine in the same way as the complete synthetase reaction; by increasing the affinity for glutamine manifold and by changing the kinetics vs glutamine from negatively cooperative into non-cooperative (Fig. 5). A biphasic saturation curve (zero or

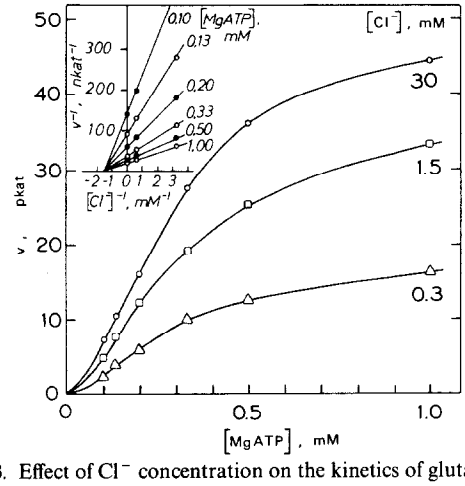

Fig. 3. Effect of Cl^- concentration on the kinetics of glutamine-dependent asparagine synthetase vs MgATP. Saturation plot. KCl added at three fixed levels, ATP concentration varied as shown; 6 mM $MgSO_4$, 12 μg protein. Inset shows reciprocal velocity vs reciprocal Cl^- concentration.

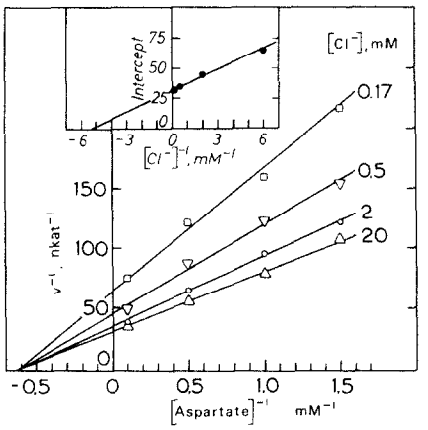

Fig. 4. Effect of Cl^- concentration on the kinetics of glutamine-dependent asparagine synthetase vs aspartate. Double-reciprocal plot. KCl and aspartate concentrations varied as shown; 26.3 mM glutamine, 10 μg protein. Inset shows intercepts on the ordinate vs reciprocal Cl^- concentration.

low Cl^-) was gradually changed into a normal hyperbola (20 mM Cl^-). The $K_{0.5}$ of glutamine decreased from *ca* 30 mM (no Cl^- present) to 1.2 mM (20 mM Cl^-). The Hill coefficient increased from 0.6 to 1.0. There was no apparent effect of Cl^- on V_{max}.

Previous work [7] indicated that the enzyme protein associates to a dimer upon addition of MgATP. Addition of $MgSO_4$ and ATP approximately doubled the glutaminase activity whether Cl^- was present or not. However, the kinetics of glutamine saturation, as related to the Cl^- level, was largely similar to the pattern found in absence of MgATP (Fig. 5).

The ratio of glutamate to asparagine formed in the asparagine synthetase reaction was 1.35–1.45. By comparison, the ratio of glutaminase to asparagine synthetase activity (Cl^- and glutamine levels similar in both assays) was only *ca* 0.3 (Table 1; cf. also Table 3 in ref. [7]). This means that the rate of glutamine hydrolysis must increase 4- to 5-fold when MgATP and aspartate are also present. Apparently, the glutaminase activity is modulated by ligands of the synthetase reaction, as well as by Cl^-. These effects were further explored (Table 3).

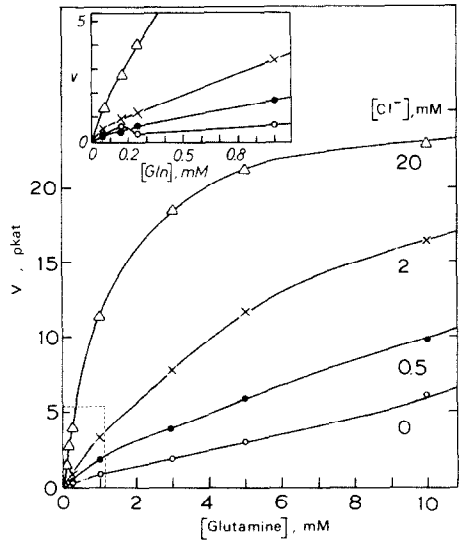

Fig. 5. Glutaminase activity of the enzyme as function of glutamine concentration at four levels of Cl^-. KCl and glutamine were added as indicated to Cl^--free glutaminase assay mixtures; 26 μg protein. Inset: magnification of the area in the lower left corner.

Remarkable and clearly synergistic activation was caused by each of the combinations $MgSO_4$ + AMP + PPi, $MgSO_4$ + ATP, $MgSO_4$ + AMPCPP (an ATP-analog [17, 18]) and $MgSO_4$ + ADP. As Pi was present, all of the combinations might fit into a binding site designed for MgATP. The first two combinations led to an approximate doubling of the rate. In the absence of Cl^-, only $MgSO_4$ + ATP had this effect. Since neither $MgSO_4$ nor ATP activated alone, Cl^- contamination of the reagents was ruled out as an explanation in this case. Aspartate addition to $MgSO_4$ alone, $MgSO_4$ + AMP + PPi, $MgSO_4$ + ADP or $MgSO_4$ + AMPCPP did not increase the rates of glutamine hydrolysis above those found in absence of aspartate. No asparagine formation occurred in these mixtures (e.g. the α,β-methylene-phosphonate bond of AMPCPP was not cleaved). Aspartate addition to $MgSO_4$ + ATP produced a complete synthetase system, leading to amide-N transfer and asparagine formation. A great increase in glutamate production was observed.

Table 3. Effect of substrates, analogs and products on glutaminase activity in presence and absence of chloride

Additions	Glutaminase activity, pkat (% of control)	
	+ Cl^-	− Cl^-
None (control)	9.9 (100)	1.9 (100)
$MgSO_4$	7.6 (77)	1.7 (90)
Asp	9.6 (97)	1.8 (97)
ATP	10.0 (101)	1.8 (97)
AMPCPP	9.9 (100)	n.d.
$MgSO_4$ + PPi	10.9 (111)	n.d.
$MgSO_4$ + AMP	7.4 (75)	n.d.
$MgSO_4$ + PPi + AMP	20.2 (204)	1.5 (79)
$MgSO_4$ + ADP	16.6 (168)	1.9 (100)
$MgSO_4$ + ATP	19.5 (197)	3.8 (203)
$MgSO_4$ + AMPCPP	17.3 (175)	n.d.
$MgSO_4$ + Asp	8.0 (81)	n.d.
$MgSO_4$ + Asp + PPi + AMP	18.8 (190)	n.d.
$MgSO_4$ + Asp + ADP	16.7 (169)	n.d.
$MgSO_4$ + Asp + ATP	54.1 (545)	6.2 (326)
$MgSO_4$ + Asp + AMPCPP	17.1 (173)	n.d.

Enzyme protein (10 μg) isolated in buffer B3 was added to the Cl^--free glutaminase assay; additions as listed at the following concentrations (in mM): KCl (+ Cl^- series) 32; aspartate (Asp): 10; ATP, ADP, AMP and AMPCPP 2.5; PPi 2; $MgSO_4$ 10. All incubations contained 10 mM Pi (n = 2). n.d. = not determined.

At zero or low Cl^-, aspartate produced little stimulation of glutamine hydrolysis and the curves had plateau regions (Fig. 6). At higher Cl^- levels, aspartate activation was substantial and the kinetics more or less hyperbolic. The aspartate-dependence of glutamate formation is evident, but the kinetics vs aspartate may be more complex than in asparagine formation (Fig. 4).

Effect of keto acids

Pyruvate, oxaloacetate and 2-oxoglutarate (as K^+ salts) were tested at various concentrations for effect on glutamine-dependent asparagine synthesis. With purified enzyme, lacking aspartate aminotransferase activity, the keto acids were rather weak inhibitors. At 20 mM, oxaloacetate gave 40% inhibition, whereas pyruvate and 2-oxoglutarate only inhibited 12–15%. At 5 mM, none of the keto acids inhibited more than 10%.

DISCUSSION

The present findings establish the important role of small anions, particularly halides, in the glutamine-linked reactions catalysed by the *L. luteus* enzyme. The similar responses of glutamine-dependent asparagine synthetase and glutaminase to anions, and other observations, provide evidence that these activities are functions of the same enzyme molecule. In these respects, the lupin enzyme resembles the enzyme from a very different source, the RADA1 mouse tumor [3, 4]. The enzyme from other plants might also prove to be Cl^--activated [13], as recently found for a preparation from 4-day-old wheat seedlings (Ramnefjell and Rognes, unpublished results). Experimental evidence to the contrary [9] has not been reported.

Small halide ions like Cl^- and Br^- (ionic diameters 0.35 and 0.39 nm [19]) seem to have an optimal size, but activation occurred with anions of diameter between 0.27 (F^-) and 0.45 nm (I^-). Among the non-halide anions tested, NO_3^- and CN^- were the best. Larger ions can not possibly be accommodated in the cleft designed for the anion on the enzyme.

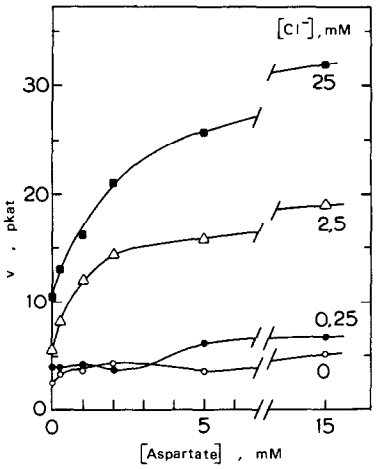

Fig. 6. Effect of aspartate and Cl^- concentration on glutamine hydrolysis in the presence of MgATP. Glutamate formation was determined in reaction mixtures containing (in mM): [^{14}C]-glutamine 6.3; ATP 2.5; $MgSO_4$ 5; Tris–HOAc (pH 7.7) 50; dithiothreitol 2.5; EDTA 0.5; plus various concentrations of KCl (0–25) and aspartate (0–15). 8 μg protein.

In Cl^- activation, there is clearly direct interaction between the anion and the glutamine-binding sites, resulting in strongly altered kinetic responses to changes in glutamine concentration. Although the major function of the anion is associated with glutamine utilization, a minor influence on binding of, for example, NH_3 cannot be excluded as the reactions with ammonium and hydroxylamine were also somewhat stimulated by Cl^- and Br^-. Horowitz and Meister [3] reported a 10% increase in NH_3-dependent asparagine synthesis upon adding Cl^-.

These authors could not detect any glutaminase activity when Cl^- was replaced by Pi, HCO_3^-, SO_4^{2-}, citrate, OAc^- or F^- [3]. Nevertheless, glutamine-dependent asparagine synthesis was observed in the presence of five of these ions; again no activity was found with F^-. In the present study a small basal glutaminase activity was found with these ions and F^- was definitely activating. Glutamine-dependent asparagine synthesis was stimulated 3-fold by F^- compared with the other ions above.

Anion activation has been reported for several glutaminases [20]. Borate, Pi, citrate and dicarboxylic acids frequently have effects, but halide activation does not seem to be common. Interestingly, a γ-glutamyltransferase from the mushroom *Lentinus edodes* was strongly activated by Cl^- and Br^- [21]; SCN^- and NO_3^- were also effective, but organic anions, HCO_3^-, Pi and SO_4^{2-} failed to substitute for the halides, as in the present work. Some enzymes display a shift in the pH optimum towards more basic values upon anion addition [22]. However, no major change in the pH dependence was found for the RADA1 synthetase [4] or the lupin enzyme.

In *E. coli* CTP synthetase, the anionic effector GTP is selectively involved in glutamine utilization [23]. GTP strongly enhances the glutaminase activity of the enzyme and stimulates CTP synthesis when glutamine is N-donor by enhancing the formation of an intermediary γ-glutamyl-enzyme and bound NH_3 from the enzyme–glutamine Michaelis complex. As discussed previously [7], the CTP synthetase and the lupin asparagine synthetase show several common features (e.g. ATP-induced association; positive and negative cooperativity towards different ligands). The analogy could conceivably extend to the mechanism of glutamine utilization, since the Cl^- activation results reported here bear a striking resemblance to the GTP effects on CTP synthetase. Addition of Cl^- considerably reduces the $K_{0.5}$ of glutamine and eliminates the strongly negative cooperativity towards glutamine. GTP has the same effects on CTP synthetase. The mechanism of Cl^- activation might be similar to the one proposed for GTP [23, 24].

Another analogy to CTP synthetase is found in the properties of the glutaminase activity, which increases in response to addition of ligands of the synthetase reaction. The similar effects of Mg^{2+} + ATP, Mg^{2+} + AMP + PPi and Mg^{2+} + AMPCPP suggest that a similar conformational change is brought about by the three combinations. However, it is not known if the enzyme dimerization induced by Mg^{2+} + ATP is also produced by the others. The effect of Mg^{2+} plus nucleotide is clearly different from the Cl^- effect on glutaminase activity. The lupin enzyme is quite different from the RADA1 tumor enzyme, in which glutaminase activity was found to be aspartate-independent, and thus uncoupled from asparagine synthesis [3]. Unlike the lupin enzyme, the RADA1 synthetase did not show cooperative kinetics or allosteric behaviour.

Lea and Fowden [9] found that 62.5 mM of several salts, including chlorides, severely inhibited the activity of the *L. albus* enzyme. However, they used a low assay concentration of aspartate, close to the apparent K_m value (0.8 mM). Inhibition caused by high Cl^- levels is apparently related to the Cl^-/aspartate ratio (cf. comments to Fig. 4 above). The keto acid inhibitions reported by the same authors have not been confirmed in this study. Due to a calculation error, the reported specific activities of their white lupin preparations (cf. Table 1 in ref. [9]) were too high by a factor of 10. The enzyme used in this study was probably 6- to 7-fold more pure and had no aspartate aminotransferase activity. Although keto acids undoubtedly have a key role in amino acid synthesis, they may not have much effect on the synthetase enzyme *per se*.

Cl^- is an important ion in the total ionic flux of many plant cells. The present findings suggest that Cl^- transport within cells and tissues may substantially contribute to the regulation of glutamine utilization for asparagine synthesis, thereby forming a link between ion transport and nitrogen metabolism.

EXPERIMENTAL

Materials. Cotyledons from 6-day-old, dark-grown *Lupinus luteus* L. seedlings were harvested and extracted [7]. L-[4-^{14}C]-Aspartic acid, L-[U-^{14}C]-glutamine and L-[U-^{14}C]-glutamic acid were from The Radiochemical Centre, Amersham. L-Amino acids, ATP, ADP and AMP (Na salts) were Sigma products. Adenylyl(α,β-methylene)diphosphonate (AMPCPP) was obtained from Serva Feinbiochemica, Heidelberg. Other chemicals were of analytical purity grade, or prepared as described [7].

Buffers. Concns in mM, except for glycerol. Buffer B2 contained: Tris–HCl (pH 7.5) 20; KCl 20; 2-mercaptoethanol 42; dithiothreitol 0.5; KCN 1; Na_2EDTA 0.1 and 20% (v/v) glycerol. The Cl -free buffer B3 comprised: K-Pi (pH 7.5) 25; 2-mercaptoethanol 42; dithiothreitol 0.5; Na_2EDTA 0.1 and 20% (v/v) glycerol. Buffer C2: Tris–HCl (pH 8.5) 100; 2-mercaptoethanol 42, KCN 1, Na_2EDTA 0.1 and 25% (v/v) glycerol. Buffer D2: buffer B2 with addition of ATP 1; $MgSO_4$ 5 and glutamine 2.

Enzyme assays. Asparagine synthetase (EC 6.3.5.4). The radioactive standard assay [7] was slightly modified. In the glutamine-dependent assay, reaction mixtures contained in a vol. of 100 μl (in mM): L-[4-^{14}C]-aspartate (neutralized with Tris; 10^6 cpm/μmol) 5; ATP 5; $MgSO_4$ 10; L-glutamine 10; Tris–HCl (pH 7.8 at 20°) 50; dithiothreitol 2; EDTA 0.5 and enzyme protein. Incubations were for 30 min at 37°. In Cl^--free assay mixtures, Tris–HOAc replaced Tris–HCl. In the ammonia-dependent assay, reaction mixtures contained the same components, except that $(NH_4)_2SO_4$ (10 mM) replaced glutamine. When β-aspartylhydroxamate formation was studied, 10 mM hydroxylammonium sulfate (freshly neutralized with Tris) was added in place of glutamine. Reactions were terminated by adding 25 μl 10% (w/v) TCA containing 20 mM asparagine (or 20 mM β-aspartylhydroxamic acid in the hydroxamate assay). Labelled asparagine or β-aspartylhydroxamate formed was isolated by high voltage paper electrophoresis and determined as described earlier [6].

Glutaminase (EC 3.5.1.2). A radioactive assay, based on the formation of [^{14}C]-glutamate from [^{14}C]-glutamine, was chosen. Standard reaction mixtures contained in 100 μl (in mM): L-[U-^{14}C]-glutamine (2×10^6 cpm/μmol) 6; Tris–HCl (pH 7.8) 50; dithiothreitol 2; EDTA 0.5 and enzyme protein; incubation for 30 min at 37°. In Cl -free assays Tris–HCl was replaced by Tris–HOAc. Reactions were stopped by adding 25 μl 10% (w/v) TCA containing 20 mM glutamic acid. Labelled glutamate formed was separated from [^{14}C]-glutamine by paper electrophoresis at pH 5.0 [7] and determined by scintillation counting, using zero-time and non-enzymic controls.

Glutamine synthetase (EC 6.3.1.2). The assay principle was similar to the one used for measuring asparagine synthetase. In a vol. of 100 μl were contained (in mM): L-[U-^{14}C]-glutamic acid (neutralized with KOH; 7.8×10^5 cpm/μmol) 5; ATP 5; $MgSO_4$ 10; NH_4Cl 10; KCl 40; Tricine–KOH (pH 7.6) 50; dithiothreitol 2 and enzyme protein. When formation of [^{14}C]-γ-glutamylhydroxamate was measured, 5 mM hydroxylammonium sulfate replaced NH_4Cl. After 30 min at 37°, 25 μl 10% (w/v) TCA was added and the tubes were placed in an ice bath. Radioactive glutamine (or γ-glutamylhydroxamate) formed was separated from [^{14}C]-glutamate as described above and determined by scintillation counting.

Aspartate aminotransferase (EC 2.6.1.1). The reduction of oxaloacetate formed in the reaction was followed at 340 nm using a coupled assay with the reagents GOT-A and GOT-B (AB Kabi, Stockholm). Incubations contained in a final vol. of 3.3 ml (in mM): L-aspartate 28; 2-oxoglutarate 7.2; Tris–HCl (pH 8.0) 20; EDTA 4; NADH 0.15; malate dehydrogenase (11 nkat) and enzyme protein. The temp. was 25° and the reaction was started after a 3 min pre-incubation by adding 2-oxoglutarate.

Protein determination. The method of ref. [25] was used, with crystalline bovine serum albumin as a standard.

Enzyme purification. Asparagine synthetase was purified 450- to 600-fold from cotyledon extracts according to the procedure previously described [7] with some minor modifications. The first three steps were unchanged, except that buffers B2 and C2 replaced buffers B and C. The higher Cl^- and glycerol content of buffer B2 gave improved stability and higher yields. In step 4, alumina C_γ gel suspension (26 mg dry wt/ml) was added to give a gel/protein ratio of 2.5. The elevated ratio did not result in adsorption of enzyme and more inactive protein could be removed by centrifugation. To the supernatant soln $(NH_4)_2SO_4$ was added to 38% satn. The ppt. was dissolved in buffer D2 and passed through a Sephadex G-75 column equilibrated with buffer D2. Step 5 was omitted, since it sometimes did not produce much purification. The Sephadex G-75 treated enzyme soln was applied to a DEAE-Sephadex A-50 column equilibrated with buffer D2. Elution was performed with a linear gradient of KCl in buffer D2 (0–0.6 M). Enzyme activity emerged between 0.20 and 0.35 M KCl, with a peak at 0.27 M. Active fractions were pooled and the enzyme was precipitated by adding $(NH_4)_2SO_4$ to 40% satn. The protein was dissolved in buffer D2 and filtered through a Sephadex G-200 column, using buffer D2 (cf. step 6). After another $(NH_4)_2SO_4$ precipitation, the enzyme protein was dissolved in either buffer B2 (giving a Cl^--containing preparation) or buffer B3 (when a Cl^--free enzyme was desired). This fraction was finally subjected to gel filtration on Sephadex G-200, using buffer B2 or B3 (cf. step 8). All experiments reported were done with enzyme carried through this last step. The enzyme was normally stable for several months at −30° at this stage. Loss of activity during storage could be reversed by fresh addition of dithiothreitol and slow warming of the enzyme soln to 20° before assay.

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