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AMMONIUM ION-DEPENDENT ISOMERIZATION OF GLUTAMATE DEHYDROGENASE IN RELATION TO GLUTAMATE SYNTHESIS IN MAIZE

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Key Word Index—*Zea mays*; Gramineae; maize; metabolism; glutamate dehydrogenase; ammonium ion-bioregulated.

Abstract—The glutamate dehydrogenase [GDH, EC 1.4.1.2] from maize seeds that were germinated in the presence of different concentrations of NH_4Cl had charge isomers in the pH 5.8–7.5 range. In a three-phase process, different NH_4Cl concentrations sequentially altered the binomial distribution of the GDH isoenzyme population. The first phase of GDH isomerization was by low (<2 mM); the second was by medium (2–58 mM); and the third was by high (>58 mM) NH_4Cl concentrations. Ammonium ion isomerized the GDH by regulating the hexameric subunit assembly reaction. The isomerization was abolished by methionine sulphoximine. The GDH isoenzyme population distribution that was induced by 25 mM NH_4Cl closely approached the theoretical binomial distribution, and it also gave the highest GDH activity. An analysis of the free amino acids of maize seeds, and comparison of the activities of GDH and glutamate synthase (GOGAT), in each of the three phases of GDH isomerization, showed that GDH was 25% more efficient than GOGAT in the synthesis of L-Glu.

INTRODUCTION

L-Glutamate dehydrogenase (GDH) is present in microbes, and in the tissues of animals and higher plants. The aminating activities of the enzyme are altered when plants are supplied with nutrients, especially carbohydrate, amino acid, chitosan, NH_4^+ salts, or other nitrogen nutrients [1–3]. Photoperiodicity, prevalent environmental stresses, growth regulators, and age of plant tissue, also affect the activities of the enzyme [1, 2]. Enzyme kinetics [1–4], immunochemistry [5, 6] and gel electrophoresis [7, 8] have been employed to elucidate the regulatory mechanisms of the enzyme. But the details of the NH_4^+ ion-mediated regulation of the enzyme are still the subject of intensive research [1–5] because some studies have underestimated the contribution which the enzyme makes in the NH_4^+ ion salvage reaction [9, 10]. In order to further elucidate the mechanism of NH_4^+ ion salvage by the enzyme, new experimental techniques need to be employed.

Glutamate dehydrogenase is multi-isoenzymic in many plant tissues [1, 8, 11–14]. Although NH_4^+ ion and other nitrogen nutrients alter the chromatographic, and electrophoretic patterns of GDH isoenzymes [1, 2, 6, 7, 8, 11–14], no comprehensive studies have been conducted to correlate the isoenzyme population patterns with different concentrations of NH_4^+ ion on the one hand, and with the levels of glutamate on the other hand. Such correlations would seem to be essential because they would be expected to provide the biochemical basis for evaluating the contribution which the enzyme makes to glutamate synthesis under different NH_4^+ ion concentrations in comparison to the glutamate synthase/glutamine synthetase (GOGAT/GS) system. We now report that NH_4^+ ion modulates the binomial population distribution reaction of the GDH isoenzymes, thereby regulating the participation of the enzyme in glutamate synthesis.

RESULTS AND DISCUSSION

Isomerization of GDH charge isoenzymes

Maize seeds were germinated in the presence of different concentrations of NH_4Cl, and methionine sulphoximine (MSX). The only sign of nutrient toxicity was the emergence at 60 hr of the radicles from the seeds treated with 75 and 100 mM NH_4Cl, and with MSX solutions; whereas radicles emerged at 48 hr from the control seeds and those treated with less than 75 mM NH_4Cl solutions. But at 96 hr when the seedlings were harvested, they all had produced coleoptiles. There was 100% germination in all the seed treatments.

The GDH from each seed treatment was first fractionated using Bio-Rad's free solution isoelectric focusing (IEF) technique [15]. The resulting 20 fractions were then fractionated by non-denaturing PAGE. The resolution achieved is shown in Fig. 1. The free solution IEF resolved the GDH of each NH_4Cl-treated maize into eight to nine charge isomers covering the range of pH

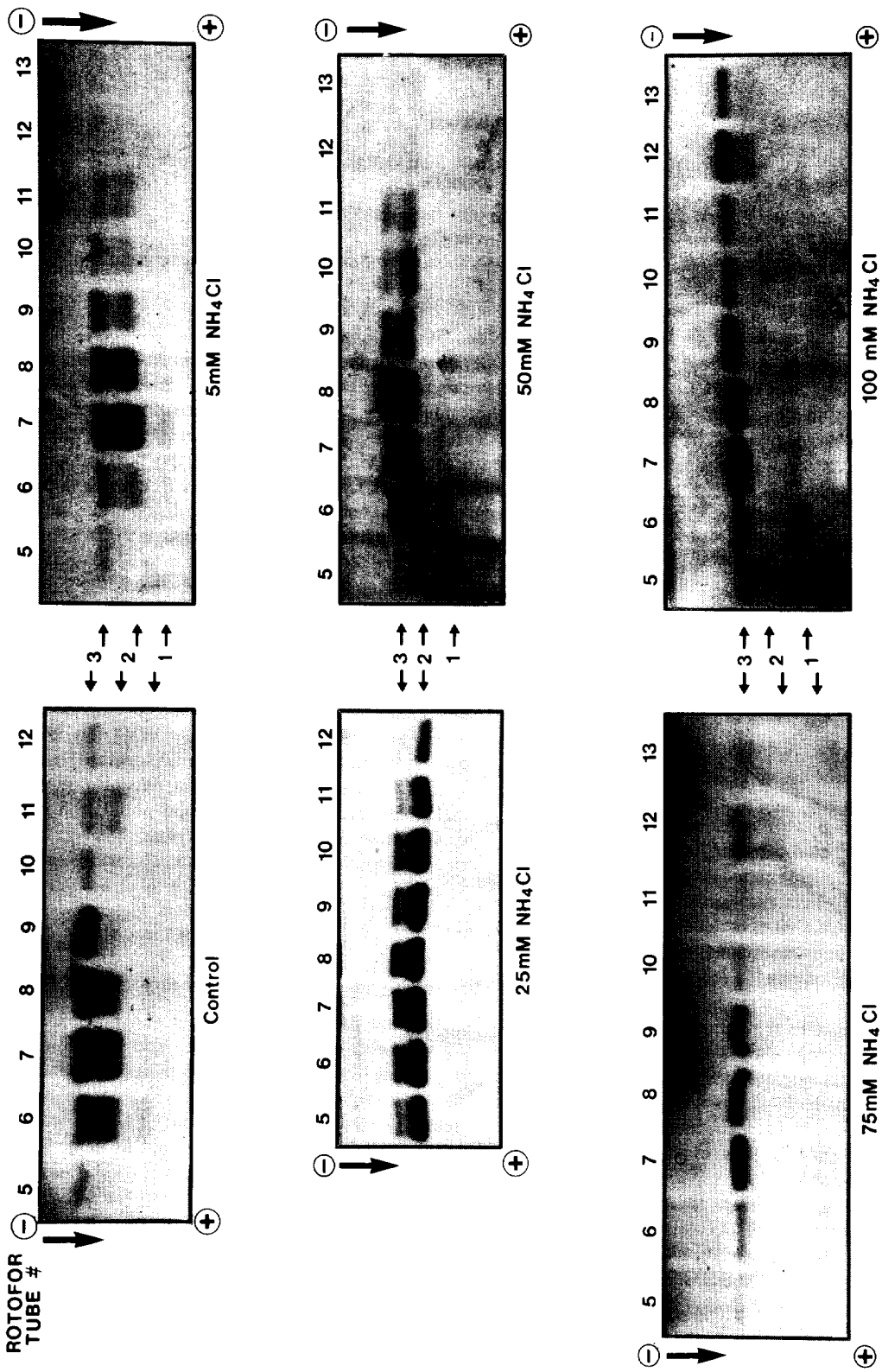

Fig. 1. Ammonium ion-dependent isomerization of maize GDH. The GDH extracted from maize seeds germinated in the presence of distilled water (control), and 5, 25, 50, 75 and 100 mM NH_4Cl solutions were fractionated by free solution IEF, and then by 7.5% native PAGE. GDH activity was detected by staining the electrophoresed PAG with Tetrazolium Blue reagent. The GDH isoenzymes were numbered from the most anodal as number 1, according to IUPAC-IUB recommendation [41].

5.8–7.5. This is the typical multi-isoenzymic pattern reported for many plant GDHs [1, 8, 11, 12]. Bio-Rad's IEF in free solution is a gentle, non-denaturing, and high resolution technique. The subsequent native PAGE of the Rotofor fractions then resolved the charge isomers according to their subunit compositions. The bands in Fig. 1 are, therefore, the GDH isoenzymes. For each of the six NH_4Cl experiments (Fig. 1), the GDH charge isomers in corresponding Rotofor tubes had the same p*I*, i.e. tubes 5, 6, 7, 8, 9, 10, 11, 12, and 13 corresponded to pI values of 5.8, 6.1, 6.3, 6.5, 6.8, 6.9, 7.1, 7.3, and 7.5, respectively. The band intensity of each isoenzyme was quantified by densitometry to permit the calculation of isoenzyme ratios.

In the control maize (distilled water treatment), the cathodal isoenzymes (isoenzymes in row 3) were the most abundant, followed in a decreasing order by isoenzymes in rows 2 and 1. For the 5 mM NH_4Cl-treated seeds, the ratio between GDH isoenzymes in rows 2 and 3 changed so that isoenzymes in row 2 became the more abundant. Isoenzymes in row 2 gained maximum prominence over the isoenzymes in row 3; in addition there were increases in the levels of the isoenzymes in Rotofor tubes 9–12 (pH 6.8–7.3) for the 25 mM NH_4Cl-treated seeds. Treatment of maize seeds with 50 mM NH_4Cl solution initiated a new redistribution of the charge isoenzymes because the isoenzymes in row 3 started to gain in relative abundance as compared to the situation under the 25 mM NH_4Cl treatment; in addition, there were decreases in the levels of the isoenzymes in Rotofor tubes 9–13 (pH 6.8–7.5). In the 75 mM NH_4Cl-treated seeds, isoenzymes in Rotofor tubes 10–13 and in row 2 were suppressed, thereby completing the trend initiated by the 50 mM NH_4Cl treatment. The 100 mM NH_4Cl treatment stabilized the trend observed in the 75 mM NH_4Cl-treated seeds.

The densitometric ratios of the isoenzymes permitted the graphical interpretation of the charge isomerizations in Fig. 1. The process (Fig. 2) by which NH_4^+ ion concentrations isomerized the charge isoenzymes of maize GDH occurred in three phases. The first phase was by low (<2 mM) NH_4Cl concentrations; the second was by medium (2–58 mM) NH_4Cl concentrations; and the third was by high (>58 mM) NH_4Cl concentrations. The isomerization of GDH isoenzymes by different concentrations of NH_4^+ ion (Fig. 2) is evidence in support of the participation of the enzyme in NH_4^+ ion salvage *in vivo* [1–8, 11–14]. Many studies [9, 10, 16, 17] have questioned the participation of the enzyme in NH_4^+ ion salvage reaction.

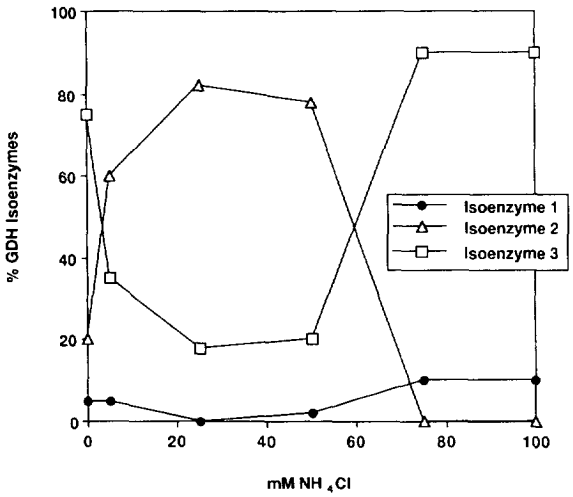

Fig. 2. The three phases of the ammonium ion-dependent isomerization of maize GDH. Maize GDH was first isomerized by germinating the maize seeds in the presence of different concentrations of NH_4Cl. The isomerized GDH was fractionated by free solution IEF to the charge isomers, and finally by native PAGE to the isoenzymes. Percentage ratios of the isoenzymes 1–3 were determined by densitometry after staining the polyacrilamide gel for GDH activity.

Isomerization of GDH isoenzyme subunits

The GDH_1 and GDH_2 nonallelic gene structure [8, 11], with the gene (GDH_1) encoding the more acidic subunits (α and a), being heterozygous, and codominant; and the gene (GDH_2), encoding the less acidic subunit (b), being homozygous [18, 19], explain the isoenzyme population patterns in Fig. 1. The characteristic eight to nine charge isoenzyme distribution in the figure is also suggestive of an hexameric subunit structure for the enzyme as has been reported for many plant GDHs [1, 8, 11, 18]. The binomial statistical distribution of the population of maize GDH isoenzymes based on the existence of three different types of subunits: a, α and b is shown in Table 1. Altogether, 28 isoenzymes are theoretically expected based on the two codominant allelic forms of GDH_1 which control, respectively, the synthesis of subunits α, and a [18, 19]. The experimentally derived distribution of the population of GDH isoenzymes in Fig. 1 resembles the binomial statistical distribution in Table 1; but there are important deviations caused by the NH_4^+ ion-dependent isomerization of GDH. The figure shows that treatment of maize with up to 50 mM NH_4Cl concentrations gave from 16 to 22 GDH isoenzymes. This is in good agreement with the theoretically expected number because of possible incompleteness in the resolution by native PAGE of some of the isoenzymes, especially those of the same charge but made up of all the three different kinds of subunit polypeptides. Based on the densitometric quantitation of the isoenzyme bands, the isoenzymes in Rotofor tubes 6–9 in the control experiment accounted for 85% of the isoenzyme population, whereas according to the binomial distribution (Table 1), they should have accounted for only 54% of the population. Therefore the control maize displayed a strong preference for the usage of subunits α and a (products of the allelic forms of GDH_1) in the construction of the hexameric structure of GDH, thereby giving rise to a skewed distribution of the isoenzyme population in which isoenzymes in the 3rd row in Rotofor tubes 7–9 (pH 6.3–6.8) were the most abundant. But 5 mM NH_4Cl treatment of maize gave an isoenzyme population distribution that approached the theoretical

Table 1. Binomial statistical distribution of the a, α, b subunits in the hexameric structure of the isoenzymes of maize GDH

ααααα	αααααb	αααααbb	ααabbb	ααbbbb	αbbbbb	bbbbbb
aaaaaa	aaaaab	aaaabb	aaabbb	aabbbb	abbbbb	
ααααα	αααab	αααabb	ααabbb	αabbbb		
αααaaa	αααaab	αααaab	ααabbb			
αααaaa	αaaaab	ααaabb				
αaaaaa						

The a and α subunits are more acidic than the b subunit.

binomial distribution. Therefore, 5 mM NH_4Cl treatment of maize enhanced the participation of all the three different kinds of subunits (α, a and b) in the construction of the hexameric structure of GDH. This trend developed further in the GDH of maize treated with 25 mM NH_4Cl solution because all the isoenzymes consisting of two different kinds of subunits (3rd row isoenzymes) were present, while the isoenzymes consisting of three different kinds of subunits (2nd row isoenzymes) increased in abundance dramatically. In the 50 mM NH_4Cl-treated maize, the isoenzymes consisting of two different kinds of subunits started to increase in abundance again, with concurrent decrease in those isoenzymes with three different kinds of subunits. The 75 mM NH_4Cl completely inhibited those isoenzymes made up of three different kinds of subunits, and at the same time, the total number of isoenzymes dropped by 50%. The 100 mM NH_4Cl stabilized the trend initiated by 75 mM NH_4Cl.

From the above, concentrations of NH_4^+ ion in the first phase of isomerization enhanced the usage of the more acidic subunits (α and a) of GDH; concentrations in the second phase of isomerization enhanced the usage of all the three different subunits (α, a and b); while concentrations in the third phase of isomerization permitted the usage of only two (b and either a or α) of the subunits, in the construction of the hexameric structure. The isoenzyme patterns in the third phase did not permit a conclusion as to which of the allelic subunits (α and a) was preferentially employed in the hexameric structure of the enzyme.

The isoenzyme population distribution induced by 25 mM NH_4Cl approximated closely to the theoretical binomial distribution; then the deviations from the binomial increased as NH_4Cl concentrations departed from the optimal 25 mM concentration. Ammonium ion, therefore, isomerized the GDH charge isoenzymes by regulating the subunit compositions of the isoenzymes themselves. This means that NH_4^+ ion is responsible for the assembly of subunits to form the hexameric structure of GDH. This function of NH_4^+ ion has not been reported before, and so it was not taken into account in the previous evaluations [9, 10, 16, 17] of the contributions of GDH in glutamate synthesis. The regulation of the subunit composition of GDH by NH_4^+ ion is strong evidence in support of the participation of the enzyme in NH_4^+ ion salvage *in vivo*. The results in Figs. 1 and 2 suggest that the bioregulation is mediated by the binding of NH_4^+ ion to domains which become sequentially saturated by the ion as the concentration of the ion increases *in vivo*.

The operation of NH_4^+ ion-binding domains was tested by germination of maize seeds treated with 5 mM MSX solution. Thereafter, the GDH was fractionated and activity-stained as was done for the treatments presented in Fig. 1. No bands were obtained after activity staining of the electrophoresed native PAG. Therefore, the MSX blocked the NH_4^+ ion binding domains, thereby abolishing the NH_4^+ ion-dependent isomerization of GDH isoenzymes. The assembly of the subunits to give the hexameric structure of GDH is, therefore, a critical reaction step in the synthesis of glutamate by GDH. The assembly of the GDH subunits by NH_4^+ ion was abolished by MSX. The elegant resolution of GDH by free solution IEF, followed by native PAGE, revealed the unique regulatory function of NH_4^+ ion in the assembly of GDH subunits.

The NH_4^+ ion concentrations that induced maize GDH isomerization, especially the first phase of isomerization, were well below the true Michaelis constant (K_m) of the enzyme [1–3, 21, 22]. This is, therefore, further evidence in support of the hypothesis that the NH_4^+ ion-dependent isomerization (bioregulation) of GDH is different from the binding of the substrate to the enzyme [1]; the former being most likely a transcriptional and/or translational event [8].

Mechanism of inhibition of GDH by MSX

Maize seeds were germinated in the presence of 1.0 mM MSX, and in that way, we captured a stage in the inhibition of GDH by the herbicide. The GDH isoenzyme distribution pattern induced by MSX is shown in Fig. 3. Comparison with the patterns in Fig. 1 shows that MSX permitted only six instead of the usual eight to nine GDH charge isomers produced by Rotofor IEF. Furthermore, the MSX-treated maize gave only six GDH isoenzymes instead of 12–22 observed in Fig. 1. The six isoenzyme system suggests a tetrameric subunit arrangement. Therefore, in abolishing the isomerization of GDH, MSX first of all disengaged the hexameric structure, and replaced it with the tetrameric arrangement. GDH, therefore, has different domains for the interaction of its hexameric and tetrameric subunits. Photometric assays showed that the six isoenzymes exhibited insignificant ($< 3\ \mu mol\ min^{-1} ml^{-1}$) aminating

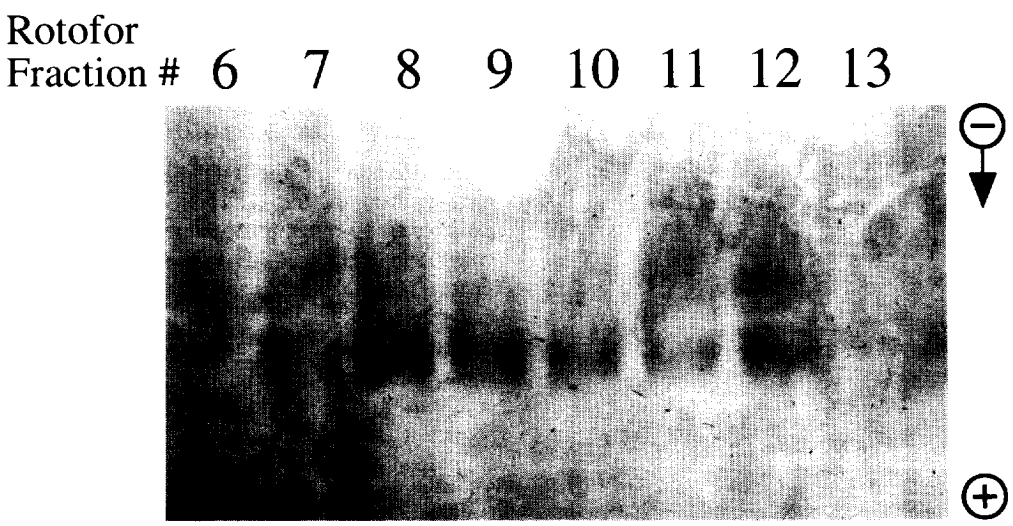

Fig. 3. Inhibition of maize GDH byMSX. The GDH extracted from maize seeds that were germinated in the presence of 1 mM MSX solution was fractionated by free solution IEF, and then by 7.5% native PAGE. GDH activity was detected by staining the electrophoresed PAG with Tetrazolium Blue reagent.

and deaminating activities, thereby indicating that the tetrameric subunit arrangement is not the physiological form of the enzyme.

The herbicide, MSX is classified as exerting its primary effect on glutamine synthetase (GS) [23] because NH_3 accumulates in MSX-treated plants; and GS is accepted as the primary means by which plants recycle NH_3 [24]. GDH was regarded as being resistant to MSX, so that the non-assimilation of NH_3 by MSX-treated plants was interpreted to mean the non-involvement of GDH in NH_4^+ ion assimilation [9, 25, 26]. In $^{15}NH_4^+$ ion labelling experiments in the presence of MSX, it was concluded that less than 1% of the total NH_4^+ ion assimilated was attributable to GDH [25]. Evidence showing the MSX inhibition of GDH (Fig. 3) now means that the role of the enzyme in NH_4^+ ion salvage reaction has been grossly underestimated; and there is the need to review and revise the physiological functions of the enzyme.

Activities of GDH charge isomers

Rotofor free solution IEF technique [15] permitted the assay of the activity of GDH charge isomers after IEF. The aminating activities are presented in Table 2. The peak activity was in the 25 mM NH_4Cl-treated seeds. This is in agreement with the isoenzyme population distribution which closely approached the theoretical binomial distribution in the 25 mM NH_4Cl-treated seeds also. GDH activities were expressed as units ml^{-1} but not as units mg^{-1} protein so as to retain the comparative isoenzyme population distribution patterns since there were other proteins in addition to GDH in the Rotofor fractions. The NH_4^+ ion-induced shift from a skewed (control maize) towards a normal binomial distribution of GDH isoenzyme population (5 mM NH_4Cl treatment), now explains the previously observed change from an α-ketoglutarate-inhibited amination mechanism ($K_i = 7.0$) in control maize, to a normal mechanism in which the inhibition by α-ketoglutarate was relieved ($K_m = 0.24$) in the 5 mM NH_4Cl-treated maize [21]. Therefore, the well documented increase in GDH activities following the treatment of plant tissue with NH_4^+ salts [1, 2, 4, 6–14, 18–22] is due to the NH_4^+ ion-mediated rearrangement of the isoenzyme population from a skewed, towards the normal binomial distribution, and vice versa.

Examination of Rotofor tubes 7 and 8 under all the NH_4Cl treatments shows that the majority of the most aminating GDH charge isoenzymes possess higher ratios of the more acidic than the less acidic subunits. This is because, the more acidic isoenzymes in the three kinds of subunit system (Table 1), statistically outnumber the less acidic ones. By enhancing the levels of the less acidic GDH isoenzymes, medium (2–58 mM) NH_4^+ ion concentrations redistributed the catalytic reaction of NH_4^+ ion salvage among more molecules of the enzyme. Therefore, the isomerization of GDH by NH_4^+ ion appears to be a specific metabolic device that ensures the expression of the optimal population of GDH isoenzymes, for the rapid salvage of the prevalent NH_4^+ ion concentration. We have used Northern blot analysis with a GDH cDNA as probe, to demonstrate that the NH_4Cl treatments also dramatically increased the GDH mRNA levels (unpublished results). These are the expected bioregulatory function of NH_4^+ ion, if GDH is the enzyme that salvages the ion.

The amination reaction of most GDHs is optimal in the region of pH 7.5–8, whereas the deamination reaction is optimal at pH 8.5–9 [27, 28]. Therefore, according to the reaction mechanism for GDH [28], the mild acid p*I* 5.8–7.5 of the isoenzymes of maize seed GDH is more favourable for an amination than a deamination function. The deaminating activities of the isoenzymes were also very low, being generally below

Table 2. Aminating activities of the GDH charge isomers in control and NH_4Cl-treated germinating maize seeds

Rotofor fractions*	5 (5.8)	6 (6.1)	7 (6.3)	8 (6.5)	9 (6.8)	10 (6.9)	11 (7.1)	12 (7.3)	13 (7.5)
GHD activities†									
Control	107.1 ± 8.0	95.2 ± 7.0	131.0 ± 11.0	166.7 ± 14.0	186.6 ± 15.0	83.2 ± 6.0	131.0 ± 12.0	71.3 ± 5.0	—
5 mM NH_4Cl	107.1 ± 9.0	131.0 ± 12.5	154.3 ± 12.0	186.2 ± 16.0	186.6 ± 15.0	119.3 ± 10	131.0 ± 12.0	71.3 ± 5.0	48.0 ± 3
25 mM NH_4Cl	143.0 ± 11.0	179.2 ± 15.3	298.3 ± 21.0	190.4 ± 17.2	179.3 ± 16.1	179.3 ± 15	131.0 ± 12.0	107.1 ± 8.0	—
50 mM NH_4Cl	107.1 ± 9.6	119.5 ± 13.4	131.0 ± 11.0	298.3 ± 21.0	107.8 ± 9.1	107.8 ± 9.2	71.3 ± 5.2	83.3 ± 5.1	71.5 ± 5.4
75 mM NH_4Cl	60.1 ± 3.4	71.1 ± 5.7	131.0 ± 11.0	95.0 ± 6.8	83.3 ± 5.4	83.4 ± 5.2	60.1 ± 3.5	71.0 ± 5.0	60.1 ± 3.0
100 mM NH_4Cl	71.0 ± 3.3	71.0 ± 5.8	95.7 ± 6.6	83.8 ± 7.0	83.3 ± 7.6	83.3 ± 7.6	107.0 ± 9.1	95.0 ± 8.1	107.7 ± 8.1

*The isoelectric point (pH) of each charge isomer is in parentheses.
†Unit of activity is μmol NADH converted to NAD^+ min^{-1} ml^{-1} of each Rotofor fraction.

5 μmol min^{-1} ml^{-1}. Maize seed GDH was, therefore, aminating in activity.

Percentage participation of GDH in L-glutamate synthesis

Information on the NH_4^+ ion-mediated sequential isomerization of the GDH isoenzymes now makes it possible to investigate the degree of involvement of the enzyme in NH_4^+ ion salvage because the isomerization process (Figs. 1 and 2) is a complete functional test for the enzyme.

The free amino acid compositions of control maize seeds, and seeds treated with 5 and 100 mM NH_4Cl solutions are shown in Table 3; the three treatments represent seeds at the first, second, and third isomerization phases of GDH, respectively. The results in Table 3 show that the level of free L-glu was low in the control seeds. Previous studies [21], had shown that GDH, and GS were inhibited by α-ketoglutarate ($K_i = 7.0$), and L-glu ($K_i = 6.0$) respectively, in the control maize seed; while GOGAT was not inhibited. This means that in the control maize seeds, the GOGAT activities accounted for the low free L-glu level. Other amino acids that derive their amino groups from L-glu by transamination were also low in concentration in the control seeds.

When the GDH isoenzyme population was transformed from the skewed towards the binomial distribution by 5 mM NH_4Cl treatment of maize seeds, the L-glu level rose by 25% (Table 3). Other amino acids that are metabolically related to L-glu also increased their concentrations. The 5 mM NH_4Cl treatment caused the inhibition of GS, and GOGAT by L-glu ($K_i = 6.0$) and α-ketoglutarate ($K_i = 0.4$), respectively; but relieved the α-ketoglutarate-mediated inhibition of the GDH [21]. Therefore, GDH activities accounted for the increased level of free L-glu in the 5 mM NH_4Cl-treated seeds.

In the third phase of GDH isomerization (100 mM NH_4Cl-treated seeds), the level of L-glu increased by 59% over the level in the 5mM NH_4Cl-treated seeds (Table 3). The level of L-gln was doubled; and other amino acids that are metabolically related to L-glu also experienced spectacular increases. The dramatic increases in the levels of free L-glu and L-gln were due to the activation of all of the three enzymes, i.e. GDH, GS and GOGAT, by the 100mM NH_4Cl treatment of the seeds [21]. Similar activation of the GOGAT and GS of wheat cells by high concentrations of exogenous NH_4^+ ion has been reported [17].

Therefore, GDH took part in L-glu synthesis because when it was inhibited, while GOGAT was functional, the level of the amino acid was very low; but when GOGAT and GS were inhibited, while the GDH isoenzymes had been isomerized, the level of the amino acid rose by as much as 25%. This means that GDH was 25% more efficient than GOGAT in L-glu synthesis. These results agree with those obtained earlier using $^{15}NH_4Cl$ tracer technique [29]. Similar high involvement of GDH in L-glu synthesis has been reported for *Chlamydomonas*

Table 3. Free amino acid composition of control maize seeds (GOGAT synthesis of L-glu), 5 mM NH_4Cl-treated maize seeds (GDH synthesis of L-glu) and 100 mM NH_4Cl-treated maize seeds (GDH and GOGAT synthesis of L-glu)

Amino acids	Control nmol (g fr. wt)$^{-1}$	5 mM NH_4Cl nmol (g fr. wt)$^{-1}$	100 mM NH_4Cl nmol (g fr. wt)$^{-1}$
Glu	262	328	520
Gln	242	370	754
Asp	272	400	370
Asn	552	644	662
Ser	366	480	490
Gly	526	696	644
His	192	200	222
Thr	300	360	360
Ala	1340	1420	2266
Arg	294	336	402
Pro	548	606	662
Tyr	256	252	218
Val	450	470	450
Met	120	154	122
Ile	194	216	198
Leu	454	488	440
Phe	114	106	94
Trp	40	34	26
Lys	294	372	402

[30], *Canavalia ensiformis* [31], and *Brassica napus* [32]. This clearer understanding of the NH_4^+ ion-mediated isomerization of the subunits of GDH isoenzymes; and of the inhibition of not only GS but also GDH by MSX [9, 23–26] has permitted a more accurate estimation of the role of the enzyme in NH_4^+ ion assimilation. MSX herbicidal action is targeted not only at GS [23], but also at GDH activities; and so explains the observed non-incorporation of $^{15}NH_4^+$ ion by MSX-treated plant tissues [9, 10, 16]. MSX abolishes the NH_4^+ ion-mediated isomerization of GDH in much the same way as the combined NH_4Cl/chitosan treatment of germinating maize seeds [29]. The mechanism of NH_4^+ ion-mediated isomerization of GDH, taken together with the normal kinetic mechanisms of GS, GOGAT and GDH, therefore permitted the identification of those NH_4^+ ion concentrations that are suitable for evaluating the specific roles of GDH in L-glu synthesis.

The complicated response of GDH isoenzymes and subunits to NH_4^+ ion and MSX suggests that the GDH genes possess multiple domains that specify enzyme activity. We have observed many of these in our studies on the GDH RFLPs of crop species (unpublished results). These domains need to be completely mapped [33] before conclusions can be made from experiments involving mutants of the enzyme [9], because knocking-out of a few of the domains might not completely inactivate the mutant enzyme.

The charge isomerization of GDH by NH_4^+ ion is a bioregulatory process that could be useful for probing other physiological functions of the complex enzyme, especially its role in NH_4^+ ion toxicity syndrome [34, 35] that retards plant growth by up to 60%. The suppression by 75 and 100 mM NH_4Cl (third isomerization phase), of those GDH isoenzymes consisting of the three kinds of subunits (Fig. 1), suggests that it is the biochemical explanation for NH_4^+ ion toxicity syndrome, because the suppression correlates with the observed 12 hr delay in the germination of those seeds treated with the higher levels of the ion. A genetic analysis of NH_4^+ ion-induced isomerization of GDH, similar to the GDH-dependent cold temperature sensitivity [19], will be necessary for further elucidation of the mechanism of NH_4^+ ion toxicity syndrome.

Previous studies in this laboratory suggested the involvement of GDH in the storage protein metabolism of chitosan-treated yam tuber, sweetpotato and maize [3, 21, 29, 36, 37]. Chitosan, like NH_4^+ ion, is a bioregulator of plant GDH [3, 29, 37]. Therefore, the sequential isomerization of GDH isoenzymes by NH_4^+ ion and the concomitant increase in the level of L-glu are in support of the enzyme's involvement in storage protein enhancement in crops.

EXPERIMENTAL

Germination of maize seeds. Maize seeds (*Zea mays* L. Pioneer 3369A variety) were soaked overnight in 1 and 5 mM MSX solns, H_2O, or in 5, 25, 50, 75 and 100 mM NH_4Cl solns. They were then germinated between filter papers in 9-cm diameter petri dishes, in a growth chamber (29°, 12 hr night/day cycle). The filter papers were

moistened with MSX soln, H_2O or the appropriate NH_4Cl soln, daily. Germination was stopped at 96 hr by freezing the seedlings on dry ice. There was 100% germination in each treatment. The endosperms were dissected out by hand, and immediately stored at $-80°$.

Extraction of GDH. GDH was extracted from the endosperms (25 g) and partially purified by fractional precipitation with solid $(NH_4)_2SO_4$ [35]. The protein pellet was dissolved in the min volume of 10 mM Tris–HCl (pH 8.2), and dialysed against 3 changes of 3.5 l of the same buffer at 3°. Proteir precipitates at the end of dialysis were removed by centrifugation (10 000 *g*, 15 min, 4°).

Free solution IEF of GDH. Partially purified GDH extract containing approximately 1 g protein was made 4 M with deionized urea and 2% with Bio-Lyte ampholyte (pH 3–10, 40% w/v). This soln (50 ml) was applied to the Rotofor cell [15]. Focusing of each extract in the Rotofor cell required 3.5 hr at 15 W constant power. The Rotofor cell was cooled by recirculation of 4° water. Electrolytes were 0.1 M H_3PO_4 and 0.1 M NaOH in the anode and cathode chambers, respectively. The initial conditions were approximately 40 mA and 300 V. At equilibrium, the conditions were 400 V, 10 W and 12 mA. The 20 Rotofor fractions were collected, and their pH values measured. Removal of ampholytes was accomplished by making each fraction 1.0 M with NaCl, and then dialysing at 4° against 3 changes of 3.5 l of 10 mM Tris–HCl (pH 8.2).

Assay of GDH activity. GDH activity of the Rotofor fractions was determined by photometry at 340 nm as described before [36]. For the aminating reaction, all substrates were prepared in 0.1 M Tris–HCl (pH 8.2). Concentrations of 16 mM α-ketoglutarate, 50 mM NH_4Cl, 1.0 mM $CaCl_2$ and 5 mM NADH were used. Reaction was started by addition of 0.1 ml of dialysed Rotofor fraction. Total reaction vol was 3.0 ml.

In the deaminating direction [8], all substrates were prepared in 0.1 M Tris–HCl (pH 9.4). Concentrations of 50 mM L-glu, 0.5 mM $CaCl_2$, 1.0 mM NAD^+ and 0.1 ml of dialysed Rotofor fraction were used. Total reaction vol was 3.0 ml.

Protein concentrations were determined by the Lowry method [38], using bovine serum albumin as standard. GDH activities presented are the average of two independent experiments, each with 3 replicate assays of GDH activity.

Native PAGE. Aliquots of the Rotofor fractions containing the GDH activities were concd 10-fold by vacuum centrifugation, and electrophoresed at 4° through a 7.5% slab gel as described before [36]. GDH activity was detected by staining the electrophoresed gel in L-glutamate–NAD^+–phenazine methosulphate–Tetrazolium Blue soln [11, 36]. L-Glutamate was omitted in the blank test. Gels stained without L-glu showed no bands.

Free amino acid analysis by HPLC. Free amino acids were extracted from maize endosperms by homogenization with twice their vol. (w/v) of 1% picric acid [39], and centrifugation at 10 000 *g* for 15 min. The supernatant was passed through a 2 cm column of Dowex-1 in order to remove the picric acid. Effluents were used for free amino acid analysis by HPLC. The free amino acid extract (10 μl), as well as internal standard amino acids, were derivatized with phenylisothiocyanate. A Millipore, Waters Pico-Tag column, and eluents for physiological amino acid analysis, were used according to the manufacturer's manual [40].

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