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REGULATION OF PEANUT GLUTAMATE DEHYDROGENASE BY METHIONINE SULPHOXIMINE

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Key Word Index—*Arachis hypogaea*; Leguminosae; peanut; metabolism, glutamate dehydrogenase; subunits, methionine sulphoximine-regulated.

Abstract—Peanut glutamate dehydrogenase (GDH) was electrophoretically purified to homogeneity. Rotofor IEF fractionated the peanut GDH to 7 isoelectric (charge) isomers, which focused in the pH 5–8 range. Western blot analysis of the charge isomers using anti-GDH serum showed that methionine sulphoximine (MSX) treatment suppressed the b-subunit (69 KDa), but enhanced the a-subunit (45 KDa), and α-subunit (46 KDa) of the enzyme. The MSX-mediated suppression of the b-subunit increased the NH_4^+ K_m value of the acidic charge isomers from 7.7 mM in the control to 50 mM in the MSX-treated peanut, and also increased the 2-ketoglutarate K_m value of the basic charge isomers from 0.4 mM in the control to 7.0 mM in the MSX treatment. Therefore, the control peanut could salvage NH_4^+ with its GDH activity. But by increasing the NH_4^+ K_m value, the MSX rendered the enzyme ineffective in NH_4^+ salvage. In the deamination direction, despite the enhancement of the a-, and α-subunits by MSX, the basic charge isomers of GDH had very high K_m value for L-glu (50 mM), similar to that (25 mM) of the control. Thus, the GDHs of the control, and MSX treatment could not function in the deamination reaction *in vivo*. These results show that the treatment of peanut with MSX impaired the amination function of GDH. © 1997 Elsevier Science Ltd

INTRODUCTION

Glutamate dehydrogenase (GDH, EC 1.4.1.2] is abundant in the storage organs of many crop species [1–9]. Many studies on its catalytic properties had concluded that it was performing a deamination function [6, 10–12] despite the thermodynamic evidence in support of an amination function [13–15]. Studies in support of the deamination function routinely applied the herbicide, methionine sulphoximine (MSX) in order to inhibit glutamine synthetase (GS) activity [16–19]. But MSX inhibited the isomerization of maize GDH [20], while it altered the GDH isoenzyme ratios of *Chlamydomonas reinhardtii* [21]. Whereas the mechanism of inhibition of GS by MSX is understood [17–19, 22, 23], that for the inhibition of GDH is not yet known.

The isomerization of GDH has been repeatedly observed [7, 8, 20]. Despite intensive experimentation involving ^{15}N NMR spectroscopy [10, 11], genetic analysis [6, 22], and enzyme kinetics [24–28], there is still the need for an explanation of the physiological function of the enzyme in terms of the observed isomerization of its subunits, and isoenzymes. The subunit composition of the GDH multiple isoenzymes have been deciphered by genetic and immunochemical analyses [29, 30]. Fractionation of the enzyme to its charge isomers [20], followed by assay of their redox activities, showed that the amination reaction, as well as the isomerization of the isoenzymes, were coupled spontaneous reactions [31]. The inhibition of the GDH isomerization by MSX is therefore a potentially useful reaction for probing the physiological function of the enzyme. It has already been demonstrated that GDH is 25% more efficient than glutamate synthase (GOGAT) in the synthesis of L-glu; and that GDH salvaged 51% of the NH_4^+ released during phytochemical defence response, while GS salvaged the remaining NH_4^+ [31].

Hereunder, our results show that MSX treatment of germinating peanut selectivity suppressed the b-polypeptide, but enhanced the α-, and a-polypeptides, thereby rendering the enzyme dormant in NH_4^+ salvage.

RESULTS AND DISCUSSION

NH_4^+ Accumulation in MSX-treated peanut

NH_4^+ accumulates in MSX-treated plant tissues due to the inhibition of GS/GOGAT cycle [19, 22, 23].

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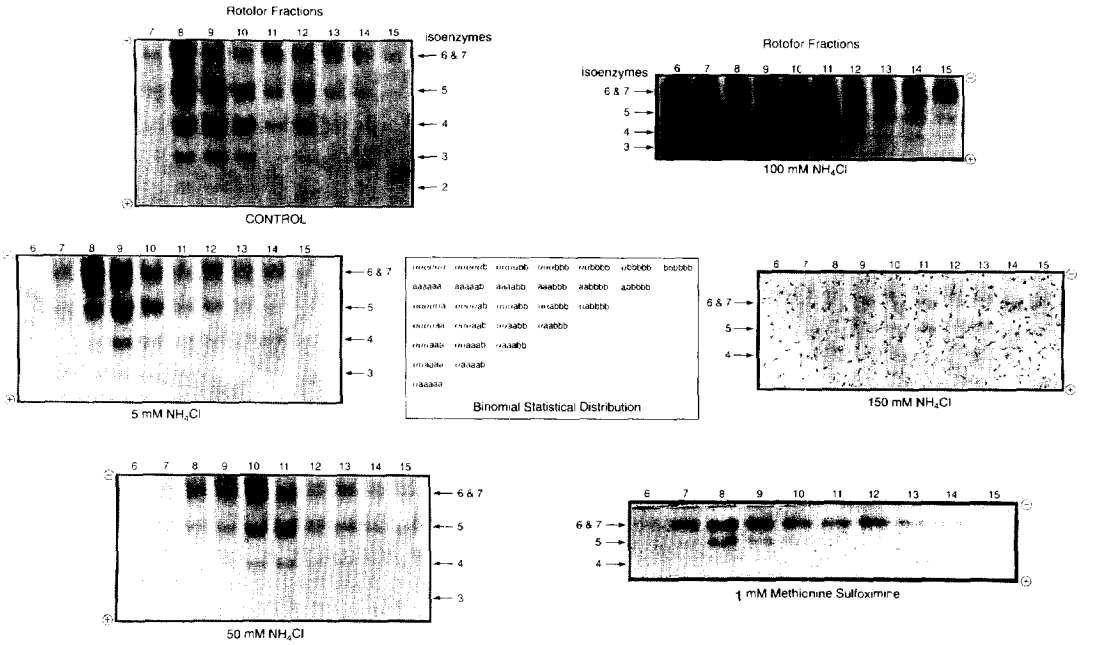

Fig. 1. Isomerization of Peanut GDH by NH_4Cl and MSX Solutions. The GDH extracted from peanut seeds germinated in the presence of distilled water, MSX, and 5, 50, 100, and 150 mM NH_4Cl solutions were fractionated by Rotofor IEF, and then by 7.5% native PAGE. GDH activity was detected by staining the electrophoresed gel with tetrazolium bromide reagent. The inserted binomial distribution is for comparison with the GDH isoenzyme population patterns.

This accumulation was evaluated on the basis of the NH_4^+-induced isomerization of peanut GDH. Peanut seeds were germinated in the presence of 1 mM MSX, and different concentrations of NH_4Cl. The $(NH_4)_2SO_4$-purified GDH from each seed treatment was fractionated using Rotofor IEF technique. The resulting 20 fractions were dialysed and then aliquots were fractionated by native PAGE, followed by GDH activity staining of the electrophoresed gel. The isomerization is shown in Fig. 1. The GDH focused in Rotofor chambers 7–15. This is the unique 7–9 charge isomer pattern of plant GDHs [2, 29].

The GDH charge isomers in Rotofor chambers 7–14 had pI values of 5.2, 5.5, 5.9, 6.3, 6.6, 7.1, 7.4, and 7.8, respectively in the control, NH_4Cl, and MSX treatments. Figure 1 shows that whereas Rotofor IEF fractionated the GDH to 7–9 charge isomers, the native PAGE fractionated each charge isomer to its constituent isoenzymes. Native PAGE fractionates isomeric proteins to their component isomers according to their charge and size [32]. Figure 1 shows that only the isoenzyme distribution of the control GDH resembled closely the binomial statistical distribution expected. Also, except the control GDH which displayed five rows of isoenzymes, the GDHs from the NH_4Cl treatments displayed progressively fewer rows of isoenzymes until at 150 mM NH_4Cl treatment only three rows were detectable. The gels for the 100, and 150 mM NH_4Cl treatments were incubated in the tetrazolium bromide reagent for 15, and 24 hr, respectively, before the GDH bands became visible, thus indicating the decreased activity of the enzyme with increasing NH_4Cl treatment of the peanut. The acidic charge isomers (Rotofor fractions 7–9) also suffered progressive depression with increasing concentration of the NH_4Cl treatment. A similar effect was reported for maize GDH [20]. Also in the control, and NH_4Cl treatments, the most cathodal GDH isomers consisted of two isoenzymes that were partially separated by the native PAGE.

In the MSX treatment, the GDH had only three rows of isoenzymes. On that basis, the 1 mM MSX was as toxic as the 150 mM NH_4Cl treatment. A similar suppression of the GDH isoenzymes of maize by MSX has been reported [20]. Therefore, from the severe depression of GDH by MSX (Fig. 1), the amount of NH_4^+ accumulation in the peanut seedlings was similar to that due to the 150 mM NH_4Cl treatment. The population distribution of the detectable GDH isoenzymes in the MSX treatment is the pattern theoretically expected if the b-subunit of the enzyme were partially suppressed (Fig. 1). It therefore became necessary to study the MSX-dependent regulation of GDH at the subunit level of the isoenzymes. This was done by immunochemical procedures.

The GDH isoenzyme population distributions presented in Fig. 1 were obtained when the IEF was performed with the pH 3–10 Bio-Lyte ampholyte. When the focused isoenzymes were pooled, dialysed and then re-focused in a narrow pH gradient (pH 5–7) ampholyte, the GDH isoenzymes focused in over 14 chambers instead of in 7–9 chambers of the Rotofor cell, suggesting that some charge isomers focused in two chambers instead of one. Therefore, focusing in the narrow pH ampholyte distorted the seven charge isomer system which is the unique statistical feature

in the hexameric structure of the enzyme. Because the wider pH ampholyte concentrated the GDH to give its characteristic seven charge isomers, IEF was routinely performed in the pH 3–10 range. Also, Fig. 1 shows that the charge resolutions achieved by Rotofor with the pH 3–10 ampholyte were excellent because the isoenzyme patterns were unique and differed from one charge isomer to the other within the same treatment, as well as from one treatment to the other.

Purification of peanut GDH

Ammonium sulphate precipitation of GDH. GDH was purified from the cotyledons of the control peanut. The crude extract of the peanut cotyledons contained 13.8 mg protein per ml, with a specific activity of 11.5 μmol min^{-1} mg^{-1} protein. After $(NH_4)_2SO_4$ precipitation, dialysis, and centrifugation to remove insoluble proteins, the partially purified GDH contained 1.7 mg protein per ml, and had a specific activity of 240 μmol min^{-1} mg^{-1} protein. The $(NH_4)_2SO_4$ precipitation step was therefore effective in the purification of the enzyme.

Rotofor purification of GDH. $(NH_4)_2SO_4$-purified GDH was further purified by Rotofor IEF (Fig. 1). The 20 fractions from the Rotofor purification were dialysed and fractions 7–13 were combined (22 ml total volume) as the Rotofor-purified GDH (2.4 mg protein per ml), with a specific activity of 375 μmol min^{-1} mg^{-1} protein. Therefore, the Rotofor purification step concentrated the enzyme.

Prep cell purification of GDH. Rotofor-purified GDH (24 mg protein) was further purified by Prep Cell SDS-PAGE. A total of 84 fractions was collected. After dialysing the Prep Cell fractions to remove SDS, aliquots (200 μl) were analysed by photometry for GDH activity. Prep Cell fractions 1–30 had no GDH activity, 32–76 contained GDH activity, with fractions 44–56 containing the peak activity.

SDS-PAGE of aliquots (100 μl) of Prep Cell fractions, followed by silver staining of the electrophoresed gels showed that fractions 1–10 contained polypeptides lower than 21 k in M_r; fractions 12–30 contained polypeptides between 24–40 k M_r; fractions 32–40 contained the 45 k polypeptides in conjunction with some 24–40 k polypeptides; fractions 42–64 contained only the 45 k polypeptides (Fig. 2); while fractions 66–84 contained polypeptides higher than 45 k M_r.

Also, aliquots (200 μl) of the Prep Cell fractions were analysed by native PAGE. The electrophoresed gels were stained for GDH activity with tetrazolium bromide reagent. Prep Cell fractions 1–30 gave no GDH isoenzyme bands, but fractions 32–72 gave GDH isoenzyme bands that migrated with the most cathodal GDH size isomers. These results were thus in agreement with those from the photometric assays. In the results (Fig. 3), the GDH contents of fractions 42–64 gave a system of single isoenzyme bands that moved diagonally from the base to the top of the

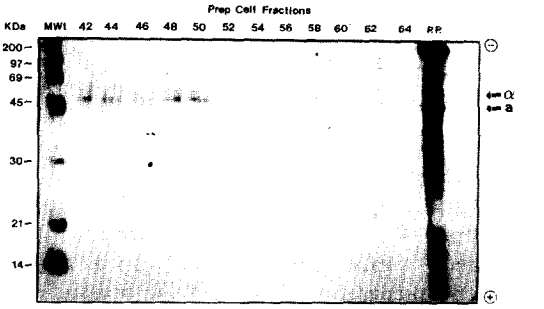

Fig. 2. Protein Profiles of the Prep Cell-Purified Peanut GDH. Equal vols (100 μl) of the Prep Cell fractions containing the GDH activity were electrophoresed (SDS-10% PAG), then the gel was stained with silver. Bands a and α are the acidic polypeptides of peanut GDH. Lane P.P. was Rotofor-purified peanut GDH. The M_r markers were: myosin (200 k), phosphorylase b (97 k), BSA (69 k), ovalbumin (45 k), carbonic anhydrase (30 k), trypsin inhibitor (21 k), and lysozyme (14 k).

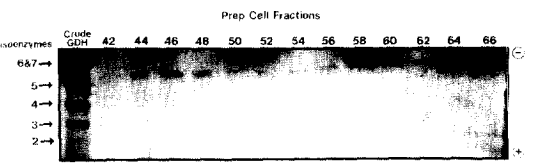

Fig. 3. Native PAGE of Prep Cell-Purified Peanut GDH. Equal volumes (200 μl) of the Prep Cell fractions were dialysed, and then electrophoresed (7.5% native PAG). The electrophoresed gel was stained overnight with tetrazolium bromide reagent before the GDH bands became visible.

most cathodal GDH charge isomer. Thus, the most cathodal charge isomer band of the control GDH (Fig. 1) consists of 2 isoenzymes (rows 6 and 7). The results in Fig. 3 showed that the Prep Cell-purified GDH polypeptides re-associated to produce the hexameric isoenzymes after dialysis to remove the SDS. For each pair of GDH polypeptides per Prep Cell fraction in Fig. 2, there was a corresponding GDH activity band in Fig. 3. Therefore the Prep Cell purification step did not completely denature the polypeptides of the enzyme. The re-assembly of the pair of polypeptides in Prep Cell fractions 42–64 to produce two charge isomers of GDH provided the initial test of identity for the 45 k polypeptides. For Prep Cell fractions 42–64, the native gel was incubated overnight in the tetrazolium bromide reagent before the GDH bands became visible. This was due to the low GDH protein contents of most of the fractions as depicted in Fig. 2.

Based on the results from the SDS-PAGE (Fig. 2), and the native PAGE (Fig. 3), Prep Cell fractions 42–64 contained the purest preparation of the two polypeptides of GDH. The 45, and 46 k M_r polypeptides in fractions 42–58 would correspond to the a-, and α-polypeptides, respectively, of GDH. They were present in progressively changing ratios especially from fractions 42–64, so that when they re-associated to produce hexamers, two kinds of charge isomers were obtained. If only one of the polypeptides

was a GDH polypeptide, only one charge isomer (a homohexamer) would have been produced.

Prep Cell fractions 46–53 which contained the GDH peak amination activity were combined as the pure GDH preparation, and the protein content was salted out with solid $(NH_4)_2SO_4$. The pure GDH (125 μg protein) had a specific activity of 3571 μmol min^{-1} mg^{-1}, and so, was purified 310-fold as compared with the crude extract. The purified GDH was used for antibody preparation. Although the most effective step in the purification process was the Prep Cell electrophoresis, the spread of GDH activity in over 40 of the Prep Cell fractions suggested possible degradation of the enzyme by the 40° treatment prior to Prep Cell electrophoresis. This may explain the low purification (310-fold) achieved.

A comparison of the protein contents (Fig. 2) of the Prep Cell fractions and of the Rotofor-purified GDH shows that the Prep Cell electrophoresis was very effective in the removal of the arachin, and con-arachin storage proteins. Since peanut storage proteins have a wide range of M_rs, and charge distribution [33–35], they will be particularly difficult to remove when peanut enzymes are purified by electrophoresis. This problem was minimized by using the Florunner cv of peanut since it does not possess the 43–47 k arachins [34]. Therefore, the Florunner peanut storage protein polypeptides did not interfere with the purity of the GDH polypeptides.

At least three steps of chromatography were employed in the purification of grapevine [36], maize [37], and pea [28] GDHs. Electrophoretic purification is therefore less complex than the chromatographic purification of GDH.

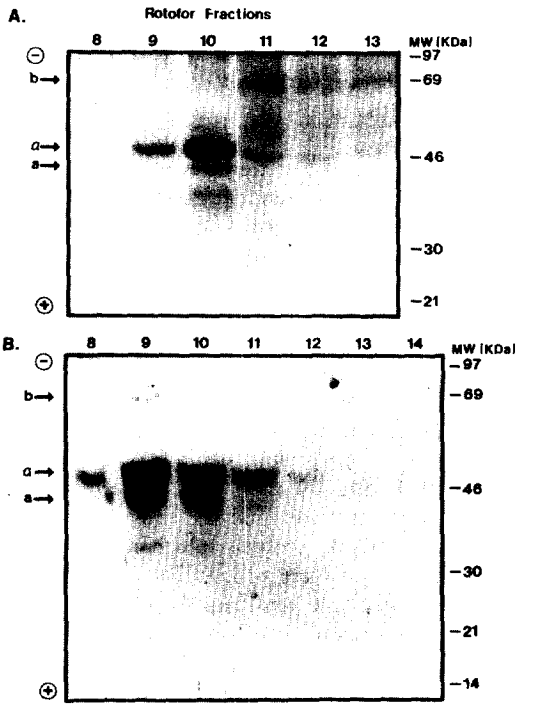

Fig. 4. Immunochemical Characterization of GDH Subunit Polypeptides of (A) the Control and (B) the MSX-Treated Peanuts. Equal vols (50 μl) of the fractions from the Rotofor-purified GDH were electrophoresed (SDS-10% PAG), electro-blotted onto nitrocellulose filter, and then probed with anti-GDH serum, the second antibody being alkaline phosphatase-conjugated. Antibody-antigen complexes were detected by staining the nitrocellulose filter with alkaline phosphatase substrates. The a, α and b protein bands are the GDH subunit polypeptides.

Regulation of the subunit polypeptides of peanut GDH by MSX

This was investigated by immunochemical methods. The GDH antibody was prepared in rabbit. ELISA monitoring of the GDH antisera detected antibodies up to dilutions of 1000, 10 000, and 50 000 in bleeds collected on the 9th, 11th, and 14th weeks, respectively. The 14th week anti-GDH serum was used for all the immunochemical experiments.

Aliquots (5 μl, containing 5–12 μg protein) of the dialysed Rotofor fractions were prepared and subjected to native PAGE, followed by Western blotting, and probing with the anti-GDH serum. The results (not shown) gave the expected resolution of each GDH charge isomer to its constituent isoenzymes as was observed with the activity staining of the electrophoresed gel (Fig. 1). This immunochemical characterization established the authenticity of the Prep Cell-purified GDH polypeptides.

Aliquots of the Rotoforated GDH were also analysed by SDS-PAGE followed by Western blotting, and probing with the anti-GDH serum. Fig. 4 shows that as expected, the GDH isoenzyme system has three polypeptides: a, α, and b; but their relative distribution in the charge isomers of the MSX-treated peanut [Fig. 4(b)] dramatically differed from the control [Fig. 4(a)]. The b-polypeptide (69 k M_r) which is more basic than the a-, and α-polypeptides was as theoretically expected, more abundant in the basic than in the acidic charge isomers of the control GDH. But in the MSX treatment, the b-polypeptide was suppressed, being just detectable in some of the charge isomers. The predicted suppression of the b-subunit polypeptide by MSX was thereby confirmed. Furthermore, the acidic charge isomers of the GDH of MSX treatment had more of the acidic polypeptides as compared with the control peanut. Therefore, the treatment of peanut with MSX suppressed the b-subunit, and enhanced the a-, and α-polypeptides. This inverse regulation of the expression of the basic and acidic polypeptides of GDH has not been reported before. The difference between the GDH polypeptide distribution of the control peanut and that of the MSX treatment was therefore great (Fig. 1). If this regulation is controlled by the promoter regions of the twin genes [38] of the enzyme, then DNA sequencing of the genes [37] will illuminate the physiological functions of the enzyme.

The native, and SDS-PAGE results for peanut GDH are in agreement with those of the 2-dimen-

sional fractionation of avocado, and grapevine GDHs [7, 30] because native PAGE fractionated the GDHs to their respective isoenzymes; but whereas Rotofor IEF fractionated the GDH isoenzyme population to the different charge isomers, the IEF gels [7, 30] fractionated the isoenzymes straight to their subunit polypeptides.

Maize GDH, like peanut GDH has more than seven isoenzymes. The isomerization of maize GDH was explained on the assumption of the existence of three different polypeptides of the enzyme [20]. The experimental demonstration of these polypeptides (Fig. 4) gives further strength to the binomial isoenzyme population distribution of the enzyme.

Figure 4 shows that the three polypeptides of peanut GDH possess similar antigenic properties. The two polypeptides of grapevine GDH also displayed similar antigenic properties [30]. There is considerable variation in the M_r of GDH polypeptides. The two polypeptides of grapevine GDH have M_r of 43.0, and 42.5 k [30]; the single polypeptide for the GDH of Phycomyces has a M_r of 54 k [39]; while the polypeptides of pea GDH, and *Lemna* GDH have a M_r of 58.5 k [40].

In Fig. 4, the charge isomers in Rotofor tubes 9 and 10 contained additional, but trace amounts of low M_r bands that displayed antigenic similarity to the GDH polypeptides. These could be degradation products of the GDH-polypeptides as suggested above, or other polypeptides that are distantly related to GDH. Similar trace amounts of low M_r GDH polypeptides were detected by Western blot analysis of grapevine GDH [30].

Redox activity of peanut GDH

The inverse regulation of the polypeptides of GDH by the MSX treatment of peanut provided an opportunity for probing the physiological functions of the enzyme. Since the subunit compositions (Fig. 4) of the acidic charge isomers (Rotofor fractions 7–10) differed from those of the basic charge isomers (Rotofor fractions 12–14), the redox activities of the two pools of GDH were determined.

In order to deduce the true kinetic constants, the initial velocities for each pool of GDH charge isomers were used for the construction of Lineweaver–Burk double-reciprocal plots, the 1/V-axis intercepts of which were then used for the construction of the replots [41]. The substrate concentrations used in the GDH assays were those deduced previously for the normal kinetics of the enzyme [1, 26, 27]. The results of the analysis are shown in Table 1.

Figure 5 shows that the acidic charge isomers of the GDH of MSX-treated peanut had a very large Michaelis constant (K_m) for NH_4^+ (50 mM) as compared with the acidic charge isomers of the control peanut (7.7 mM). The enhanced levels of the acidic polypeptides of GDH in the MSX treatment relative to the control, was therefore responsible for the increased K_m value for NH_4^+. Since plant mitochondria contain up to 10 mM NH_4^+ [13], the acidic charge isomers of the control GDH were capable of catalysing amination reaction *in vivo*. On the other hand, the K_m value of 50 mM NH_4^+, being above the normal NH_4^+ concentration of the mitochondria, means that the acidic charge isomers of the MSX-treated peanut would only participate in amination reaction in the presence of an equally high (50 mM) NH_4^+ concentration in the mitochondria. MSX treatment of peanut therefore rendered the acidic charge isomers of GDH ineffective in NH_4^+ salvage.

The basic charge isomers of the GDHs of MSX-treated, and control peanuts (Fig. 5) had the same very large K_m value (40.0 mM) for NH_4^+ (Table 1). Therefore, the suppression of the b-polypeptide in the MSX-treated peanut did not change the K_m value of the basic charge isomers for NH_4^+. But the accompanying enrichment of the acidic polypeptides in the basic charge isomers of the MSX treatment, increased the V_{max} value about 10 times as was the case in the acidic charge isomers. These results suggest that the acidic polypeptides control the velocity of the GDH amination reaction.

The function of the basic charge isomers was deduced from the assay for their K_m values for 2-ketoglutarate (2-KG). Table 1 shows that whereas the basic charge isomers of both the control and MSX treatment had the same V_{max} value (33.3 μmol min^{-1} mg^{-1}) when 2-KG was the varied substrate, the control peanut had a lower K_m value (0.4 mM 2-KG) than the MSX treatment (7.0 mM 2-KG). The suppression of the b-polypeptide in the MSX treatment relative to the control, could be responsible for the diminished activity of the basic charge isomers for 2-KG. Since their K_m value for 2-KG is much lower than the concentration of 2-KG in plant mitochondria [42], the basic charge isomers of the control GDH were capable of catalysing amination reaction *in vivo*. 2-KG is important in the amination reaction mechanism of crop species because it regulates the catalytic activity of their GDHs [1, 26].

The above results thus far have not explained the possible function of the GDH of MSX-treated peanut. Since deamination by GDH is optimal at pH 9 [43], we considered that it was likely that the basic charge isomers of the MSX treatment might be active in the deamination direction. Table 1 shows that the K_m values of the basic charge isomers of the control, and MSX treatment for L-glu were very high, being 25 mM, and 50 mM, respectively, compared with the GDHs of many plant species [5, 43, 44]. On the other hand, the V_{max} values (10–20 μmol min^{-1} mg^{-1}) for the deamination reaction were low (Table 1). The combination of very high K_m values for L-glu, and low V_{max} values indicates that deamination was not a significant reaction of the GDHs of the control, or the MSX-treated peanuts. Furthermore, since the K_m values of the GDHs for L-glu were much larger than the L-glu concentration of plant mitochondria [42],

Table 1. Effects of MSX treatment of peanut seedlings on the redox activities of GDH

GDH isoenzymes	Kinetic constants*	Varied fixed substrates†	Treatments of peanut	
			Control	MSX-treated
Acidic charge isomers	V_{max}	NH_4^+, 2-KG	25.0±2.0	229±17
	K_m	NH_4^+	7.7±0.5	50.0±6.0
Basic charge isomers	V_{max}	NH_4^+, 2-KG	9.6±0.8	108±12
	K_m	NH_4^+	40.0±2.0	40.0±3.0
	V_{max}	2-KG, NH_4^+	33.3±3.8	33.3±3.8
	K_m	2-KG	0.4±0.03	7.0±0.5
	V_{max}	L-glu, NAD^+	10.4±0.9	20.8±2.0
	K_m	L-glu	25.0±1.8	50.0±3.2

* The paired V_{max} and K_m values are the results of the same experiment. V_{max} is μmol min^{-1} mg^{-1} protein; K_m is mM.
† Under the V_{max} values, the first substrate is the fixed varied, while the second substrate is the varied substrate.

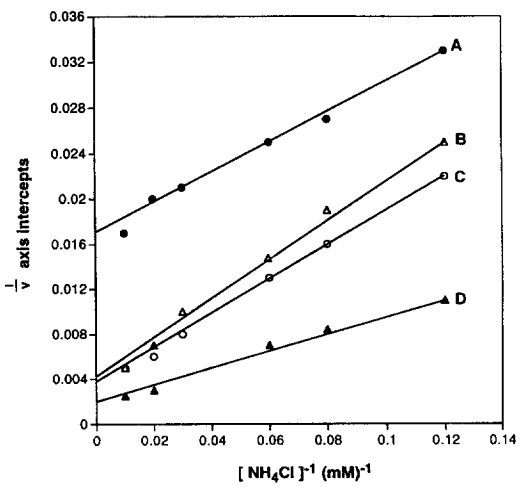

Fig. 5. Amination Activities of the GDH Charge Isomers of Control and MSX-Treated Peanuts. Replots of data from double-reciprocal plots in which the activities of the Rotofor-purified acidic, and basic charge isomers of GDH were assayed at varied 2-KG concs in the presence of constant NADH conc with NH_4Cl held at varied fixed concs, in a total vol of 3 ml per assay. Replots A, and D are for the acidic charge isomers of control, and MSX-treated peanuts, respectively. Replots B, and C are for the basic charge isomers of control, and MSX-treated peanuts respectively. Protein contents of the acidic, and basic charge isomers were 2.6, and 2.3 mg ml^{-1}, respectively.

the GDHs of the control, and MSX-treated peanuts could not catalyse deamination reaction *in vivo*. With the K_m value for 2-KG (0.4 mM) being much lower than that for L-glu (25.0 mM), peanut GDH will bind preferentially to 2-KG and spontaneously catalyse the amination reaction. However, under abnormal conditions (such as when plant tissues are first depleted of carbohydrates, followed by an external supply of L-glu in excess of the high K_m value for the amino acid) then the GDH may participate in the conversion of the L-glu to 2-KG [6, 10–12], provided that the toxic levels of the amino acid do not inhibit the isomerization of the enzyme.

There is increasing experimental evidence [13, 14, 20, 31, 45] which question the proposed deamination function of GDH. Since the presence of malate in undialysed crude extracts of plant tissues leads to false high deamination activities of GDH [14, 45], all the peanut GDH extracts were exhausively dialysed after each step of purification to ensure complete removal of interfering metabolites. Therefore, the low deamination activity of peanut GDH (Table 1) is in agreement with the low GDH deamination activities of wheat cells [14], grapevine callus [45], and maize seeds [20, 31].

The above results show that the GDH isoenzymes of the control peanut were capable of catalysing the amination reaction fully *in vivo* because of their very low K_m values for NH_4^+, and 2-KG. However, in the MSX-treated peanut, the GDH isoenzymes were probably dormant in the redox reaction because of their very large K_m values for L-glu, NH_4^+, and 2-KG. The suppression of the b-polypeptide, and enhancement of the a-, and α-polypeptides in the MSX treatment relative to the control peanut, with consequent inhibition of the isomerization of the enzyme in the MSX treatment, could have caused the dormancy of the enzyme. These effects of MSX on GDH show that it is no longer acceptable to assume that MSX has no effect on the enzyme, especially when the herbicide is utilized for the inhibition of GS during NH_4^+ assimilation experimentation.

Great caution is requested when comparing the activities of different enzymes based on their K_m values [24], especially when the enzymes are also localized in different cellular compartments. However, in the case of the acidic and basic charge isomers of GDH, the comparison is valid because they are merely different isoforms of the same enzyme.

The GDHs of many plant tissues are known to possess very high K_m values for NH_4^+ [2]. The suppressible and inducible nature of plant GDHs explain the observed variable and high K_m values for NH_4^+ because plant tissues are normally propagated in media rich in metabolites including NO_3^-, NO_2^-, NH_4^-, amino acids, carbohydrates, and growth regu-

lators, some of which suppress while others enhance the expression of one or more of the subunit polypeptides. There is therefore the need to fractionate the enzyme to its population of hexameric isoenzymes in studies pertaining to its physiological function. The effects of many different metabolites and xenobiotics on the isoenzyme population distribution suggest that the enzyme also performs a redox monitoring function in plant cells, tissues, and organs. In this function, it utilizes its amination activity to monitor the oxidative stress status of the tissue.

Part of the current interest in the mechanisms of GDH centre on the finding that the abilities of crop plants to assimilate NH_4^+ and to synthesize storage proteins depend on the amination activity of the enzyme [1, 2, 20, 26, 46, 47]. Storage protein may be a product of the defence response of plant species to stressful accumulation of NH_4^+ because storage protein is deposited during plant senescence [48], and phytochemical defence response [31].

From the foregoing results, the b-subunit polypeptide confers the reductive amination property on GDH. Although its existence has long been predicted [29, 49], it has not been hitherto identified. The storage organs of protein deficient crops (potatoes, yam tuber, cocoyam, and sweet potato) so far analysed have trace amounts of the b-polypeptide of GDH [50], but the storage organs of protein-rich crops possess high level expression of the polypeptide. The b-polypeptide of GDH is therefore a potentially important enzyme for biotechnological manipulation. In support of the direct relationship between protein content and GDH animation is the finding that the GDHs of protein-deficient crop species are inefficient in NH_4^+ salvage, whereas the GDHs of protein-rich crops are efficient in NH_4^+ salvage [1]. Because of this, GDH has been referred to as the storage protein-related enzyme [2].

EXPERIMENTAL

Germination of peanut seeds. Peanut seeds (*Arachis hypogaea*, L. Florunner cv.) were soaked overnight in H_2O, 1 mM MSX soln, or in 5, 25, 50, 100, and 150 mM NH_4Cl solns. They were then germinated between filter papers in 9 cm diam. Petri dishes (29°, 12 hr light/night cycle). The filter papers were moistened daily with H_2O, MSX soln, or the appropriate NH_4Cl soln. At 108 hr, at least 85% of the seeds in each treatment had germinated. Only the germinating seeds were selected; their testas were removed, and germination stopped by freezing the seedlings on dry ice. Cotyledons were dissected out, and immediately stored at −70°.

Extraction of GDH. Frozen cotyledons were homogenized with × 3 their vol. of ice-cold extraction buffer (0.2 M Tris–HCl (pH 8.5), 14 mM 2-mercaptoethanol (2-ME), 40 mM $CaCl_2$, 5% PVP, 0.5 mM PMSF). The homogenate was centrifuged at 5000 *g* for 10 min at 4°. The supernatant was frozen at −70°. After thawing at 4°, it was re-centrifuged at 15 000 *g* for 30 min. The supernatant was dialysed against 10 mM Tris–HCl buffer (pH 8.2) × 3 at 4°, each change being 3.5 l of the buffer. The dialysed extract was designated as the crude GDH.

$(NH_4)_2SO_4$ precipitation. The crude GDH was treated with solid $(NH_4)_2SO_4$, and the protein which pptd between 20–65% satn was collected by centrifugation (15 000 *g* for 20 min, at 4°). The protein pellet was dissolved in minimum vol. of extraction buffer, and dialysed against 10 mM Tris–HCl buffer (pH 8.5) as described above. Protein ppt. which appeared after dialysis was removed by centrifugation (15 000 g for 20 min, at 4°). The GDH prepn was further purified by Rotofor IEF technique.

Rotofor IEF of GDH. $(NH_4)_2SO_4$-pptd GDH containing 0.1–0.2 *g* total protein was made 4 M with deionized urea, and 2% with Bio-Lyte ampholyte (pH 3–10, 40%). This soln (50 ml) was applied to the Rotofor cell [51], and focused for 4 hr at 4°. The 20 Rotofor frs were then harvested; their pH values determined; and their ampholyte contents were removed by dialysis at 4°.

Native PAGE of GDH. Equal vol. (100 μl) of GDH solns were electrophoresed at 4° through 7.5% acrylamide separating gel. Gels were stained for GDH activity with phenazine methosulphate-L-glutamate-NAD^+-tetrazolium bromide reagent [49], or were used for Western blot analysis.

SDS-PAGE. SDS-PAGE [52] was with 10% acrylamide separating gel. Gels were either stained with Ag [53], or were used for Western blot analysis.

Prep cell purification of GDH. The seven Rotofor frs containing the GDH activities were pooled, and then satd to 65% with solid $(NH_4)_2SO_4$. The protein ppt was pelleted by centrifugation (15 000 *g* for 20 min at 4°), the pellet was redissolved in minimum vol. of 42 mM Tris–HCl buffer (pH 6.8), and dialysed against 3.5 l of same buffer overnight at 4°. The dialysed enzyme (6 ml, about 50 mg total protein) was divided into two; and one part (3 ml) was made 0.1% with SDS, 8% with glycerol, 3% with 2-ME. and 0.01% with bromophenol blue. After incubation at 40° for 15 min, the sample (24 mg total protein) was loaded on the PAG in the Bio-Rad model 491 Prep Cell [54]. The SDS-10% separating gel in the Prep Cell was 9 cm high, and was polymerized in the 37 mm diam. tube of the apparatus. The stacking gel was 3 cm high.

The gel was electrophoresed at 4°, and at 100 V (constant). After the bromophenol blue dye had migrated out of the separating gel, the running buffer (50 mM Tris, 384 mM gly, and 0.1% SDS) was pumped through the elution chamber at 0.15 ml min^{-1}. Frs 84 × 3 ml were collected. Frn number 2 was the test one that contained visible amounts of bromophenol blue dye. All the 84 frs were dialysed against 4 changes of 3.5 l 10 mM Tris-HCl buffer (pH 8.5) overnight, at 4° to remove the SDS.

In order to locate the frs containing GDH activity, equal vols (100 μl) of even number frs were analysed for amination activity by photometry at 340 nm as

described below. Once the elution position of the enzyme was located, equal vols (200 μl) of the even number frs within the zone of activity were analysed by 7.5% native PAGE. The electrophoresed gels were stained for GDH activity with the phenazine methosulphate–tetrazolium bromide reagent as described above. Also, equal vols (100 μl) of the frs containing GDH activity were analysed by SDS-10% PAGE; the electrophoresed gel was then stained with Ag. The best frs with respect to purity of the GDH polypeptides were pooled. Solid $(NH_4)_2SO_4$ was added to make 65% satn in order to ppt all the protein in the pooled frs. Protein ppt was pelleted by centrifugation (15 000 *g* for 20 min at 4°); the protein redissolved in minimum vol. of 10 mM Tris–HCl buffer (pH 8.5); followed by dialysis in the same buffer at 4° to remove $(NH_4)_2SO_4$. The dialysed GDH was used as antigen for antibody production.

Antibody production. Primary antibody against the purified GDH was prepd in rabbits (Alpha Diagnostic, San Antonio, Texas, USA). Pre-immune serum was taken before the rabbits were immunized. Bleeds were collected on the 9th, 11th and 14th weeks. Antisera titre was monitored by ELISA.

Western blot analysis. Proteins fractionated by PAGE (native or SDS) were electrophoretically transferred to nitrocellulose filters using Bio-Rad's Trans-Blot Transfer Cell. The transfer buffer for native gels was 25 mM Tris, 192 mM gly (pH 8.3); but for SDS gels, it was 25 mM Tris, 192 mM gly, and 20% MeOH [55]. Electrophoretic transfer was done at 30 V (constant) overnight.

Nitrocellulose blots of native gels were blocked with 2% soln of milk protein; incubated with GDH antibody at 30 000 dilution for 1 hr at room temp; washed $\times 3$ with TBST buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20), 5 min each wash; incubated with peroxidase-labelled antibody conjugate for 1 hr at room temp.; and then washed $\times 5$ with TBST buffer, 5–10 min each wash. The filter was then incubated in NuGlo chemiluminescent substrates according to ref. [56]. Antibody–antigen complexes were visualized by exposure of the chemiluminescent nitrocellulose filter to X-ray films for 1–2 min, followed by processing of the films.

Nitrocellulose blots of SDS gels were blocked with 1% BSA in TBST buffer; incubated with GDH antibody; washed with TBST buffer; incubated with alkaline phosphatase-labelled antibody conjugate; and then washed $\times 5$ with TBST buffer. Antibody–antigen complexes were visualized by incubating the nitrocellulose filter in alkaline phosphatase substrate soln (16.5 mg nitro blue tetrazolium and 8.25 mg 5-bromo-4-chloro-3-indolylphosphate in 50 ml of 100 mM Tris–HCl pH 9.5 containing 100 mM NaCl, and 5 mM $MgCl_2$) according to ref. [57].

Assay of GDH activity. GDH activities [25, 26] were determined by photometry at 340 nm. In the amination direction, 0.3–35.0 mM 2-KG, 3.0–530.0 mM NH_4Cl, 0.16 mM NADH, 1.3 mM $CaCl_2$, and 0.2 ml of GDH soln were used. In the deamination direction, 10–330 mM L-glu, 0.2–2.0 mM NAD^+, 0.6 mM $CaCl_2$, and 0.2 ml of GDH soln were used. Substrates for the amination reaction were prepd in 0.1 M Tris–HCl buffer (pH 8.0), while the substrates for the deamination reaction were prepd in 0.1 M Tris–HCl buffer (pH 9). Final vol. of each reaction was 3 ml.

Protein concns were determined by the method of ref. [58]. Enzyme and protein assays were done in triplicates, the values reported being the averages of the triplicate assays.

REFERENCES

1. Osuji, G. O., Cuero, R. G. and Washington, A. C., *Journal of Agriculture and Food Chemistry*, 1991, **39**, 1590.
2. Srivastava, H. S. and Singh, R. P., *Phytochemistry*, 1987, **26**, 597.
3. Pahlich, E., Ott, W. and Schad, B. *Journal of Experimental Botany*, 1980, **31**, 419.
4. Sukalovic, V. H., *Physiologia Plantarum*, 1990, **80**, 238.
5. Garland, W. J. and Dennis, D. T., *Archives of Biochemistry and Biophysics*, 1977, **182**, 614.
6. Stewart, G. R., Shatilov, V. R., Turnbull, M. H., Robinson, S. A. and Goodall, R., *Australian Journal of Plant Physiology*, 1995, **22**, 805.
7. Loulakakis, K. A., Roubelakis-Angelakis, K. A. and Kanellis, A. K., *Plant Physiology*, 1994, **106**, 217.
8. Nauen, W. and Hartmann, T., *Planta*, 1980, **148**, 7.
9. Chou, K. and Splittstoesser, W., *Plant Physiology*, 1972, **49**, 550.
10. Fox, G. G., Ratcliffe, R. G., Robinson, S. A. and Stewart, G. R., *Canadian Journal of Botany*, 1995, **73**, 1112.
11. Robinson, S. A., Slade, A. P., Fox, G. G., Phillips, R., Ratcliffe, R. G. and Stewart, G. R., *Plant Physiology*, 1991, **95**, 509.
12. Robinson, S. A., Stewart, G. R. and Phillips, R., *Plant Physiology*, 1992, **98**, 1190.
13. Yamaya, T., Oaks, A. and Matsumoto, H., *Plant Physiology*, 1984, **76**, 1009.
14. Fricke, W. and Pahlich, E., *Journal of Experimental Botany*, 1992, **43**, 1515.
15. Engel, P. C. and Dalziel, K., *Biochemical Journal*, 1967, **105**, 691.
16. Genetet, I., Martin, F. and Stewart, G. R., *Plant Physiology*, 1984, **76**, 395.
17. Pate, J. S., in *Plant Nitrogen Metabolism* Vol. 23, ed. J. E. Poulton, J. T. Romeo and E. E. Conn. Plenum Press, London, 1989, p. 65.
18. Rhodes, D., Brunk, D. G. and Magalhaes, J. R., in *Plant Nitrogen Metabolism* Vol. 23, ed. J. E. Poulton, J. T. Romeo and E. E. Conn. Plenum Press, London, 1989, p. 191.
19. Shaner, D. L., in *Plant Nitrogen Metabolism* Vol.

23, ed. J. E. Poulton, J. T. Romeo and E. E. Conn. Plenum Press, London, 1989, p. 227.
20. Osuji, G. O. and Madu, W. C., *Phytochemistry*, 1995, **39**, 495.
21. Moyano, E., Cardenas, J. and Munoz-Blanco, J., *Physiologia Plantarum*, 1995, **94**, 553.
22. Lea, P. J., Blackwell, R. D., Murray, A. J. S. and Joy, K. W., in *Plant Nitrogen Metabolism* Vol. 23, ed. J. E. Poulton, J. T. Romeo and E. E. Conn. Plenum Press, London, 1989, p. 157.
23. Forde, B. G. and Cullimore, J. V., in *Oxford Surveys of Plant Molecular and Cellular Biology*. Vol. 6, ed. B. J. Miflin. Oxford University Press, New York, 1989, p. 247.
24. Oaks, A., *Canadian Journal of Botany*, 1995, **73**, 1116.
25. Loyola-Vargas, V. M., Yanez, A., Caldera, J., Oropeza, C., Robert, M. L., Quiroz, J. and Scorer, K. N., *Journal of Plant Physiology*, 1988, **132**, 289.
26. Osuji, G. O. and Cuero, R. G., *Journal of Agriculture and Food Chemistry*, 1992, **40**, 724.
27. Stone, S. R., Heyde, E. and Copeland, L., *Archives of Biochemistry and Biophysics*, 1980, **199**, 560.
28. Garland, W. J. and Dennis, D. T., *Archives of Biochemistry and Biophysics*, 1977, **182**, 614.
29. Cammaerts, D. and Jacobs, M., *Plant Science Letters*, 1983, **31**, 65.
30. Loulakakis, K. A. and Roubelakis-Angelakis, K. A., *Plant Physiology*, 1991, **97**, 104.
31. Osuji, G. O. and Madu, W. C., *Phytochemistry*, 1996, **42**, 1491.
32. Hedrick, J. L. and Smith, A. J., *Archives of Biochemistry and Biophysics*, 1968, **126**, 155.
33. Neucere, N. and Ory, R. L., *Plant Physiology*, 1970, **45**, 616.
34. Basha, S. M., *Journal of Agriculture and Food Chemistry*, 1990, **38**, 373.
35. Basha, S. M. and Pancholy, S. K., *Peanut Science*, 1981, **8**, 82.
36. Loulakakis, C. A. and Roubelakis-Angelakis, K. A., *Journal of Experimental Botany*, 1990, **41**, 1223.
37. Sakakibara, H., Fujii, K. and Sugiyama, T., *Plant Cell Physiology*, 1995, **36**, 789.
38. Oaks, A., *Canadian Journal of Botany*, 1994, **72**, 739.
39. Laere, A. J. V., *Journal of Genetics and Microbiology*, 1988, **134**, 1597.
40. Scheid, H. W., Ehmke, A. and Hartmann, T., *Zeitschrift für Naturforschung*, 1980, **35**, 213.
41. Segel, I. H., in *Enzyme Kinetics: Behaviour and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*. Wiley, New York, 1975, p. 505.
42. Bowman, E. J., Ikuma, H. and Stein, H. J., *Plant Physiologia*, 1976, **58**, 424.
43. Smith, E. L., Austen, B. M., Blumenthal, K. M. and Nyc, J. F., in *Enzymes* Vol. 2, ed. P. D. Boyer. Academic Press, New York, 1975, p. 293.
44. Stone, S. R., Copeland, L. and Heyde, E., *Archives of Biochemistry and Biophysics*, 1980, **199**, 550.
45. Loulakakis, K. A. and Roubelakis-Angelakis, K. A., *Physiologia Plantarum*, 1996, **96**, 29.
46. Osuji, G. O. and Cuero, R. G., *Food Biotechnology*, 1992, **6**, 105.
47. Osuji, G. O. and Cuero, R. G., in *Sweetpotato for the 21st Century Technology*, ed. W. A. Hill, C. K. Bonsi and P. A. Loretan. Tuskegee University Press, Alabama, 1992, p. 78.
48. Boulter, D., *Philosophical Transactions of the Royal Society of London*, 1984, **B304**, 323.
49. Cammaerts, D. and Jacobs, M., *Planta*, 1985, **163**, 517.
50. Osuji, G. O. and Madu, W. C., *Annual Meeting of the American Society for Biochemistry and Molecular Biology. FASEB Journal*, 1995, **9**, *abstract* 58.
51. Rotofor System Manual, Bio-Rad Laboratories, California, 1990.
52. Laemmli, U. K., *Nature*, 1970, **227**, 680.
53. Sigma Technical Bulletin, Silver Stain Kit for Polyacrylamide Gels P3040; Sigma Chemical Co., St. Louis, 1989.
54. Model 491 Prep Cell Instruction Manual, Bio-Rad Laboratories, California, 1995.
55. Trans-Blot Electrophoretic Transfer Cell Instruction Manual, Bio-Rad Laboratories, California, 1989.
56. NuGlo Chemiluminescent Western Blot Kit for HRP Instruction Manual, no. M-80200. Alpha Diagnostic International, San Antonio, 1995.
57. ProtoBlot Western Blot AP System Technical Manual, Promega, Madison, 1987.
58. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., *Journal of Biological Chemistry*, 1951, **193**, 265.