# Pesticide Inactivation of Peanut Glutamate Dehydrogenase: Biochemical Basis of the Enzyme's Isomerization

Godson O. Osuji,*,† Cleantis Braithwaite,† Richard Pointer,‡ and Juanito Reyes†

CARC, Prairie View A&M University, P.O. Box 4079, Prairie View, Texas 77446, and Department of Biochemistry and Molecular Biology, Howard University College of Medicine, Washington, D.C. 20059

Glutamate dehydrogenase (GDH) isomerizes in response to pesticides and environmental chemicals, but the biochemical basis of the isomerization is not known. Clearer understanding of the isomerization would permit expansion of its utility in the diagnosis of the responses of plant tissues to challenged environments. Peanut plants were treated with different rates of Basagran (3-(1-methylethyl)-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide), Bravo 720 (tetrachloroiso-phthalonitrile), and Sevin XLR Plus (1-naphthyl *N*-methylcarbamate). Free solution isoelectric focusing, followed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) fractionated the peanut seed GDH to its constituent subunits and degradation polypeptides. After western transfer to nitrocellulose membrane, the GDH subunits and degradation polypeptides were immunodetected with anti-GDH. The pesticide treatments did not induce increased proteolytic activity, but induced about 50% degradation of the GDH, whereas the GDH of the control peanut suffered only about 25% degradation, thus showing that the degradation rate was about double the rate of de novo synthesis in the pesticide treatments. The heavy displacement of the GDH subunit equilibrium toward degradation explains the biochemical basis of the isomerization reaction.

**Keywords:** *Arachis hypogaea; pesticides; glutamate dehydrogenase; isomerization; degradation; immunoanalysis*

## INTRODUCTION

The ability of glutamate dehydrogenase (GDH, EC 1.4.1.2) to salvage $NH_4^+$ and to isomerize in response to changes in the environment (Srivastava and Singh, 1987; Lauriere and Daussant, 1983; Mazurowa et al., 1980; Barash et al., 1975; Lauriere et al., 1981) makes the enzyme potentially useful for the diagnosis of the response of crops to soil nutrient status (Osuji et al., 1998). The enzyme is multi-isoenzymic in plants, the isoenzymic number varying with plant species (Scheid et al., 1980; Yue, 1969; Fawole, 1977; Lee, 1973) and the concentration of plant nutrients (Osuji and Madu, 1995). Although de novo synthesis of the enzyme was demonstrated (Cammaerts and Jacobs, 1985; Thomas, 1978; Kang and Titus, 1980; Simpson and Dalling, 1981; Kar and Feierabend, 1984), the biochemical basis of the changes in its isoenzyme population distribution patterns in response to environmental chemicals is yet to be explained. Also, although the enzyme is a target site of herbicide action (Osuji, 1997) and the kinetics of the signaling reaction are closely related to those of the amination reaction (Osuji et al., 1998), the exact sites of the signaling reaction are yet to be described. The enzyme is present in all organisms from microbes to higher plants and animals (Srivastava and Singh, 1987). A clearer understanding of the biochemical mechanism of the isomerization reaction would expand the utility of the enzyme in the diagnosis of the responses of plant and animal tissues to challenged environments. To meet increasing demand for food and fiber, agricultural production is being carried out in increasingly challenged environments (Hardy et al., 1998).

GDH is inactivated by formation of enzyme-linked Schiff base between pyridoxal phosphate and the Lys residue in the enzyme's active site (Brown et al., 1973). A similar Schiff base formed between α-ketoglutarate (α-KG) and the $\epsilon$-$NH_2$ group of the Lys residue in the enzyme's active site is one of the intermediate steps in the catalytic mechanism of the enzyme (Smith et al., 1975). Stabilization of the Schiff base intermediate by conversion to an enzyme-linked imine in vivo should inactivate the enzyme and predispose it to degradation because irreversibly inactivated enzymes are generally degraded and replaced by de novo synthesized copies (Davies, 1987; Davies et al., 1987; Desimone et al., 1996). Demonstration of immunochemically positive lower molecular weight bands of GDH would thus be evidence for the inactivation and degradation of the enzyme. Such GDH degradation and de novo replacement would constitute the biochemical basis of the isomerization reaction. The GDH-linked Schiff base intermediate might be the exact site of the action of nucleophiles because the protonation of the Schiff base nitrogen activates it toward nucleophilic attack (Fersht, 1985). Although the catalytic inactivation of GDH was demonstrated (Brown et al., 1973), the degradation of the inactivated enzyme was not demonstrated. Furthermore, the pyridoxal phosphate employed for the in vitro inactivation is not a substrate of the enzyme. In this project, we adopted a different approach by studying the enzyme's degradation that accompanies its isomerization response to the pesticide treatment of crops.

* Author to whom correspondence should be addressed [e-mail Godson_Osuji@PVAMU.edu; fax (409) 857 2325].

† Prairie View A&M University.

‡ Howard University College of Medicine.

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## MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, unless otherwise stated. Peanut seeds (*Arachis hypogaea*, L. Starr variety) were purchased from a local green grocery. They were cultivated in potted soil and treated with three rates of the pesticides Sevin XLR Plus, Bravo 720, and Basagran as described previously (Osuji and Braithwaite, 1999). The peanut seeds (20 g) were homogenized with three times their volume of ice-cold extraction buffer (0.2 M Tris-HCl (pH 8.5), 14 mM 2-mercaptoethanol, 40 mM $CaCl_2$, 5% PVP, 0.5 mM phenylmethylsulfonyl fluoride), and the homogenate was centrifuged at 5000*g* for 10 min at 4 °C (Osuji and Madu, 1997a). The supernatant was frozen at −70 °C. After the supernatant was thawed at 4 °C, it was recentrifuged at 15 000*g* for 30 min. Proteins that precipitated following the saturation of the supernatant to 65% with solid $(NH_4)_2SO_4$ were pelleted by centrifugation (15000*g* for 20 min, at 4 °C). The protein pellet was dissolved in minimum volume of extraction buffer and dialyzed against 10 mM Tris-HCl buffer (pH 8.5) as described previously (Osuji and Madu, 1995). Proteins which precipitated after dialysis were removed by centrifugation (15000*g* for 20 min, at 4 °C). Free solution isoelectric focusing (IEF) (Rotoforation) of the prepared GDH, followed by dialysis of the resultant 20 Rotofor fractions, were as described previously (Osuji and Braithwaite, 1999).

The volumes of the Rotofor fractions used for the sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) were 50 μL of each Rotofor fraction of the control treatment; 80, 40, and 30 μL of each fraction of the low, recommended, and high treatment rates of Basagran, respectively; 50, 100, and 30 μL of each fraction of the low, recommended, and high treatment rates of Bravo, respectively; and 30, 100, and 50 μL of each fraction of the low, recommended, and high treatment rates of Sevin, respectively. The Rotofor fractions were then denatured by heating at 100 °C with equal volume of SDS−PAGE buffer (Davis et al., 1986) and electrophoresed in SDS−PAG using Bio-Rad Protean II xi cell.

Western transfer followed by immunodetection of the GDH with GDH antibody and alkaline phosphatase-conjugated second antibody were as described previously (Osuji and Madu, 1997a). The peanut GDH antibody used was from the same stock as that used previously for the analysis of Florunner peanut GDH. The antibody was used at 10 000 instead of 30 000 dilution (Osuji and Madu, 1997a). The western analysis of the Rotofor fractions for each rate of pesticide treatment was performed in triplicate but with a different volume of the fractions each time. The results of the GDH subunit patterns presented are those with the neatest resolution of the degraded polypeptides from the undegraded subunits of the enzyme. The GDH band intensities were estimated by densitometry (Osuji and Cuero, 1992).

**Extraction and Assay of Proteolytic Enzymes.** Peanut seeds (2 g) were defatted, and then proteolytic enzymes were extracted from the defatted meal with 25 mM $Na_3PO_4$ buffer pH 7.0 (Osuji and Madu, 1996). Proteins in the extract were precipitated by addition of solid $(NH_4)_2SO_4$ to 50% saturation; were pelleted by centrifugation (15000*g* for 15 min at 4 °C); and the pellet was dissolved in minimum volume of 25 mM $Na_3PO_4$ buffer and dialyzed at 4 °C against the same buffer. Insoluble proteins in the dialyzed enzyme were centrifuged out as described above. The supernatant was brought to a volume of 25 mL with 25 mM $Na_3PO_4$ buffer, pH 7.0. Protein content of the extracts was determined by the method of Lowry et al. (1951).

Proteolytic activity was determined by incubating 2 mL of the dialyzed enzyme with 2 mL of 25 mM $Na_3PO_4$ buffer (pH 7) containing 20 mg azocasein (Tomarelli et al., 1949) for 18 h at 38 °C. The reaction was stopped by addition of 16 mL of 5% trichloroacetic acid solution. Precipitated protein was removed by centrifugation (10000*g* for 15 min at 20 °C). To 2.5 mL of the supernatant, 2.5 mL of 0.5 M NaOH was added, and absorbance of the solution was determined at 440 nm. Each assay was repeated three times. For calculation of the proteolytic activities, 0.5 mg azocasein in 5 mL of solution was used as the reference standard.

Proteolytic activity was also assayed by PAGE (De Barros and Larkins, 1990) in which 0.2% casein (wt/vol) was copolymerized in native 7.5% PAG. Another proteolysis native PAG (control) was also set up, but without copolymerization with casein. Equal volumes (0.05 mL) of dialyzed protease extracts were prepared, loaded into the gel wells, and electrophoresed at 4 °C, 90 V, until the bromophenol blue tracking dye ran out of the gel. The gel was then incubated overnight at 38 °C in 100 mL of 25 mM $Na_3PO_4$ buffer, but the control gel was incubated at room temperature in the $Na_3PO_4$ buffer. After incubation, gels were stained with Coomassie Blue R.

## RESULTS AND DISCUSSION

**Degradation of GDH.** Figure 1 shows the GDH subunit population distribution patterns of the control and pesticide-treated peanuts. For each peanut, the undegraded a-, α-, and β-subunits of the enzyme had molecular weights of 43, 48, and 69 kD, respectively. These are the molecular weights of peanut GDH (Osuji and Madu, 1997a; Osuji, 1997). All other immunochemically positive polypeptide bands lower in molecular weight than 43 kD (Figure 1) were the degradation products of GDH. Also, the control GDH suffered far less degradation than the GDHs of the pesticide-treated peanuts.

Figure 1A shows that all three of the GDH subunits were present in each charge isomer of the control peanut. This is in agreement with the symmetrical distribution of the GDH isoenzymes of the control peanut (Osuji and Braithwaite, 1999). GDH polypeptide degradation products were also present mainly in the 40 kD molecular weight region, in almost the same regularity and uniformity as the GDH subunits. Densitometry showed that the 40 kD degradation products were about 25% of the undegraded subunits.

Figures 1B−D show qualitatively that increasing application rates of 3-isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide (Basagran) increased the degradation of the peanut's GDH subunits. The subunits of the basic charge isomers (Rotofor fractions 14−18) generally suffered more degradation than those of the acidic isomers. This is in agreement with the positive skewing of the GDH isoenzyme population in the herbicide-treated peanut (Osuji and Braithwaite, 1999). Densitometry showed an approximately 1:1 ratio between the degraded GDH polypeptides and the residual undegraded subunits. Western analysis with up to 80 μL of each Rotofor fraction of the Basagran treatments gave distinct bands of the subunits of the basic charge isomers, but the subunits of the acidic charge isomers were not resolved because of increased protein smearing of the PAG.

Figures 1E−G also show that increasing rates of tetrachloro isophthalonitrile (Bravo) application increased the degradation of the GDH subunits, especially those of the basic charge isomers. This is again in agreement with the positive skewing of the GDH isoenzyme distribution pattern of peanuts treated with the fungicide, with the highest Bravo treatment inducing the highest suppression of the basic isoenzymes (Osuji and Braithwaite, 1999). Densitometry showed that the acidic GDH charge isomers of the high Bravo treatment (Figure 1G) were richer in the β-subunit as compared with those of the recommended rate (Figure 1F), which were in turn richer in that subunit as compared with those of the low treatment rate (Figure

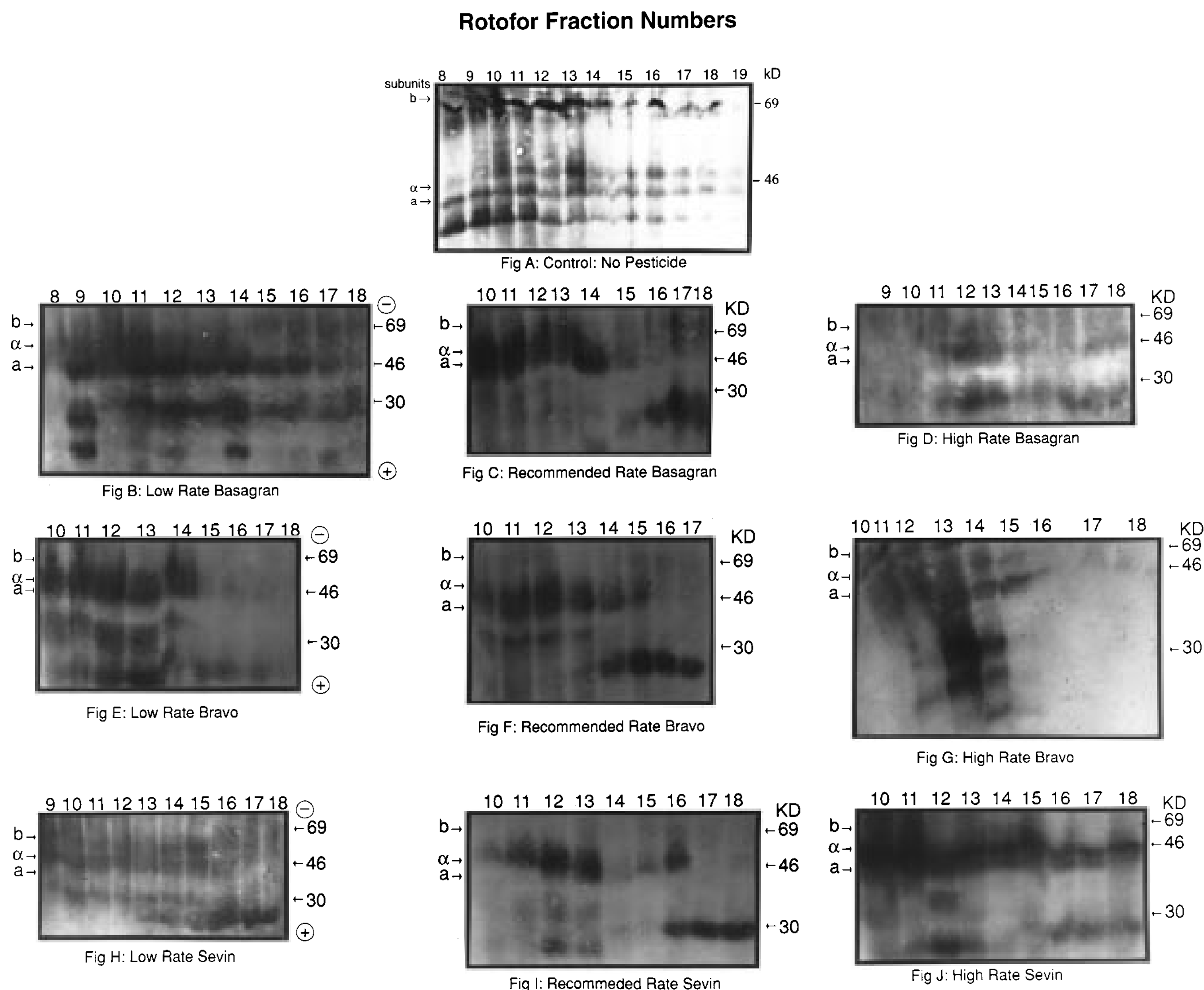


**Figure 1.** Degradation of the GDH of pesticide-treated peanut. Each GDH preparation was Rotoforated to its charge isomers, which were then fractionated to their GDH subunit compositions by SDS−11% PAGE for the control GDH, but SDS−10% PAGE for the pesticide-treated peanuts. For the PAGE, 50 $\mu$L of each Rotofor fraction of the control treatment; 80, 40, and 30 $\mu$L of each fraction of the low, recommended, and high treatment rates of Basagran, respectively; 50, 100, and 30 $\mu$L of each fraction of the low, recommended, and high treatment rates of Bravo, respectively; and 30, 100, and 50 $\mu$L of each fraction of the low, recommended, and high treatment rates of Sevin, respectively, were used. After western transfer to nitrocellulose membrane, the GDH subunits and degradation polypeptides were immunodetected with anti-GDH.

1E). Densitometry also showed that the degraded polypeptides and the residual undegraded GDH subunits were present in approximately 1:1 ratio. Western analysis with up to 100 $\mu$L of each Rotofor fraction of the Bravo treatments gave distinct bands of the subunits of the basic charge isomers and the $\beta$-subunit of the acidic charge isomers, but the bands of the polypeptide degradation products were not resolved because of increased protein smearing of the PAG. The poor resolution means that in addition to the major degradation polypeptides in the 20−40 kD range, there were many others of low abundance. Proteolysis was therefore part of the process for the degradation of the GDH subunits.

Figures 1H−J show that the recommended 1-naphthyl *N*-methyl carbamate (Sevin) rate induced greater degradation of the subunits of the basic GDH charge isomers as compared with the low and high rates. This is also in agreement with the positive skewing of the isoenzyme pattern induced by the recommended rate of Sevin, while those induced by the low and high rates were symmetrical in distribution (Osuji and Braithwaite, 1999). The degraded polypeptides and the residual undegraded GDH subunits were also present in roughly 1:1 ratio in each Sevin treatment. When up to 100 $\mu$L of each Rotofor fraction of the Sevin treatments was used for western analysis, the bands of the acidic degradation products became so pronounced that they did not separate from the bands of the a- and $\alpha$-subunits. Results in Figure 1 show that whereas all three of the subunits were present and were symmetrically distributed in each of the charge isomers of the control peanut, they were nonsymmetrical and in many cases only two subunits were detectable per charge isomer of the GDHs from pesticide-treated peanuts. Also, whereas the degradation products were uniformly present in each Rotofor fraction of the control GDH, they were not uniformly distributed for the pesticide-treated peanuts. The immunopositive bands in Figure 1 were due to the specific binding of the GDH antibody with the peanut GDH because no GDH antibody was detected in rabbit's preimmune serum (Osuji and Madu, 1997a).

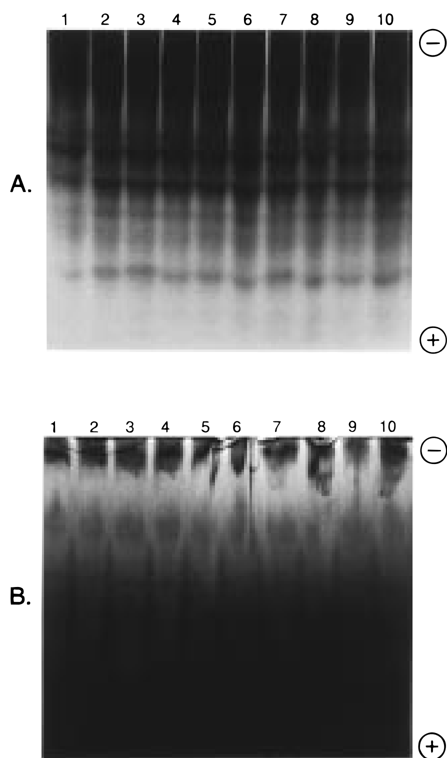


**Figure 2.** Proteolytic activities of extracts from peanut seeds. Extracts from equal weights (4 mg) of the seeds of (1) control, and treatments with (2) low rate of Basagran, (3) recommended rate Basagran, (4) high rate of Basagran, (5) low rate of Bravo, (6) recommended rate of Bravo, (7) high rate of Bravo, (8) low rate of Sevin, (9) recommended rate of Sevin, and (10) high rate of Sevin were electrophoresed through native PAG (A) without copolymerized casein and (B) with copolymerized casein. Gel (A) was incubated at room temperature, but gel (B) was incubated at 38 °C, overnight for proteolysis to occur. Gels were then stained with Coomassie Blue.

From the foregoing discussion, each immunoanalysis result of the GDH has four sections, viz., residual undegraded subunits of the acidic and basic charge isomers and the acidic and basic degradation polypeptides of the enzyme. The internal relationships among the four sections changed from one pesticide rate to the other. This was the reason an empirical procedure was adopted in the selection of the volumes of the Rotofor fractions used for the western analysis. Furthermore, the immunoanalysis results did not suggest whether the acidic degradation polypeptides were the direct products of the subunits of the acidic charge isomers or of the basic charge isomers, and whether the basic degradation polypeptides were the direct products of the subunits of the basic charge isomers or of the acidic charge isomers. In view of these limitations, it was not possible to quantitatively compare the effects of increasing concentrations of the pesticides on the inactivation of the enzyme. However, the immunoassay results illuminated the isomerization mechanism of the enzyme.

**Proteolysis of GDH.** The results in Figure 1 suggested possible induction of proteolytic activity by the pesticides. The results in Figure 2A show that the peanut extracts contained identical protein bands irrespective of the pesticide treatments. The protein bands did not indicate any differential proteolysis despite the incubation of the gel overnight at room temperature. On the other hand, the results in Figure 2B show that the peanut proteins were degraded when the gel was incubated overnight at 38 °C. The proteolysis was uniform irrespective of the pesticide treatment of the peanut. Some proteins at the cathode end (origin of the electrophoresis) were resistant to the proteolysis. However, their fractionation pattern differed from the isoenzyme pattern characteristic of peanut GDH. Figure 2B also shows that the proteins of the control peanut were as degraded as those of the pesticide-treated peanuts.

The azocaseinolytic assays showed that the control and the pesticide treatments had the same activity (3.0 $\pm$ 0.32 $\mu$g azocasein/mg peanut protease/h). There were no regular trends in the azocaseinolytic activities because some of the low rates of pesticide treatments displayed more activities than the high rates, whereas some of the high rates of pesticide treatments had the same activities as the control peanut.

These results therefore showed that the GDH degradation was not due to a pesticide-activated proteolytic activity, but was rather due to the normal housekeeping proteases of the peanut. The increased degradation rate and the differential susceptibilities of the acidic and basic subunits of GDH to degradation in the pesticide-treated peanuts relative to the control were therefore pesticide-induced on the GDH.

Increased proteolytic activity is a cellular response reaction to stress (Hochstrasser, 1995; Desimone et al., 1996). The fact that the proteolytic induction had not occurred by the time the GDH isomerization had occurred in response to the pesticide treatments is evidence that the GDH response occurs very early in the cascade of cellular reactions to environmental stress. But the origins (chloroplastic, cytosol, or mitochondrial) of the proteolytic activity observed in the peanut extracts were not investigated.

The degradation of GDH could have proceeded via proteolysis and/or nonenzymatic fragmentation (Desimone et al., 1996; Stadtman, 1993). Nonenzymatic fragmentation is possible because in the case of Basagran-treated peanut, the herbicide enhancement of the peroxidation of the peanut's lipids may increase the level of the protein cross-linker, malondialdehyde (Kunert and Dodge, 1989; Dupont et al., 1982; Gardner, 1979), thereby increasing the polymerization and fragmentation of the enzyme. In support of the nonenzymatic fragmentation as a step in the degradation is the preponderance of the 40, 30, and 21 kD polypeptides (Figure 1). For the control peanut, the GDH degradation product was virtually the 40 kD polypeptide (Figure 1A). The persistence of a few major degradation polypeptides of about the same molecular weights suggests that the initial step in the degradation was the nonenzymatic production of the few major polypeptides, followed subsequently by their slow proteolysis to polypeptides of lower abundance. Degradation by proteolysis without a nonenzymatic step would have generated many GDH polypeptides of diverse molecular weight ranges. Degradation of the inactivated large subunit of Rubisco to a major polypeptide of 36 kD was also attributed to a chloroplastic proteolytic system (Desimone et al., 1996).

**GDH Site of Pesticide Action.** The catalytic mechanism of GDH involves formation of an enzyme-linked Schiff base intermediate (Figure 3) between the carbonyl group (S) of $\alpha$-KG and the $\epsilon$-amino group (E) of the Lys residue in the active site of the enzyme (Smith et al., 1975). The Schiff base nitrogen is protonated at neutral pH (Fersht, 1985). The strong electron withdrawing property of the protonated nitrogen so formed predis-

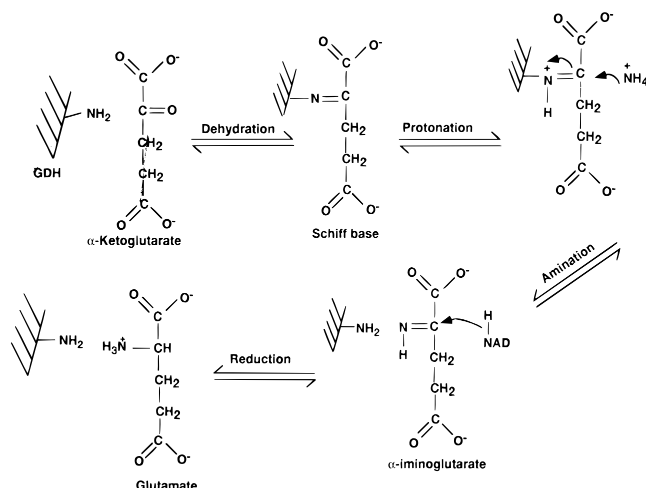


**Figure 3.** Catalytic reaction steps of GDH. The $\epsilon$-$NH_2$ group of the Lys residue in the active site of GDH is E; the carbonyl group of α-KG is S; Shiff base formation is between the Lys $\epsilon$-$NH_2$ in the active site of GDH and the carbonyl group of α-KG; EI is GDH-linked pesticide-substituted imine complex.

poses the site to nucleophilic attack (Figure 3). Therefore, the Schiff base readily undergoes alternative nucleophilic reactions depending on the nucleophilicities and the concentrations of the prevalent nucleophiles. Attack of ammonia on the Schiff base yields GDH-bound α-iminoglutarate which is then reduced by NADH to yield the product, glutamate, the $\epsilon$-$NH_2$ group of the active site Lys residue (Figure 3) being regenerated as a consequence (Brown et al., 1973). Attack of water molecule on the Schiff base reverses the reaction by regenerating α-KG and also the $\epsilon$-$NH_2$ group of the active site Lys of the enzyme. In both of these cases, because water molecule and ammonia are weak nucleophiles, they are able to break the Schiff bond thereby liberating the GDH without inactivating much of it. In the control peanut, water molecule and ammonia were also the most abundant nucleophiles. Therefore, the probability of the reaction of the Schiff base with other nucleophiles was low, and accordingly, the extent of the inactivation of the enzyme was equally low (Figure 1A).

But the Schiff base could also be attacked by strong nucleophiles (e.g., nitrile, amine, and carboxyl) to form GDH-linked substituted imine complexes (EI) that are stable (Fersht, 1985; Brown et al., 1973; Cross and Fisher, 1970; Cross, 1972). Ultimately, such modified GDH subunits (dead-end complexes) are degraded because inactivated enzymes are removed by degradation (Davies, 1987; Davies et al., 1987). The extensive degradation of the GDH of pesticide-treated peanut (Figure 1) may therefore be a consequence of the inactivation of the enzyme via GDH-linked pesticide-substituted imine complexes. This is the biochemical basis of the differential responses of the enzyme to nucleophiles (Osuji et al., 1998; Osuji, 1997; Osuji and Braithwaite, 1999).

The GDHs of the pesticide-treated peanuts behaved as the $NH_4^+$-inhibited variants of the control GDH (Osuji, 1997). The pesticide-dependent modification of the Schiff base in the active site of the enzyme therefore explains the noncompetitive inhibition by Bravo and Sevin, the uncompetitive inhibition by Basagran (Osuji and Braithwaite, 1999), and also the noncompetitive inhibition of corn GDH by phosphate fertilization of corn (Osuji et al., 1998), because the substituted Schiff base is a GDH-inhibitor (EI) complex that was derived by modification of the GDH-linked Schiff base (Figure 3). The chemical structures of the pesticides Bravo 720, Sevin XLR Plus, and Basagran show that they possess functional groups that are strongly nucleophilic. The $V_{max}$ and/or $K_m$ values decreased because the EI complex became completely removed from the system, thereby altering the equilibria between the enzyme and its substrates and thus inducing more synthesis of the GDH-linked Schiff base intermediate (Figure 3). These are some of the altered kinetics of plant GDHs that result in the repeatedly observed large values of its Michaelis constants for $NH_4^+$ (Lea and Miflin, 1974). Thus, the GDH site of pesticide action is the Schiff base that is linked to the active site. The validity of this reaction mechanism was verified in the design of the in vitro assay of GDH activity in the presence of pesticides (Osuji and Braithwaite, 1999). The assay was also successful because the control GDH (possessing all three of the subunits) was used rather than the GDH (lacking some of the subunits) of pesticide-treated peanut.

**Biochemical Basis of GDH Isomerization.** Figure 1 shows that about 50% of the GDH was degraded under each pesticide-treated peanut. Therefore, 50% of the GDH-linked Schiff base intermediate needed replacement, and consequently, 50% of the enzyme also needed to be synthesized de novo. But the GDH subunit distribution patterns of the pesticide-treated peanuts deviated from that of the control. If the rate of de novo synthesis was approximately equal to the rate of GDH inactivation, the pesticide-induced GDH subunit distribution patterns would have been similar to that of the control GDH. The 1:1 ratio between the subunits and the degradation polypeptides means that the rate of GDH inactivation was double the rate of de novo synthesis. In view of the binomial assembly of the three subunits in the hexameric isoenzymes, the degradation of such a high percentage of the subunits explains the biochemical basis of the enzyme's isomerization. Although GDH isomerization was observed repeatedly in plant extracts (Hartmann, 1973; Hartmann et al., 1973; Kanamori et al., 1972; Loulakakis and Roubelakis-Angelakis, 1991; Osuji and Madu, 1995), its biochemical basis remained unexplained.

The control peanut, because it was neither treated with pesticides nor with $NH_4Cl$, suffered less GDH degradation (Figure 1A). That lower extent of degradation was likely due to the reaction of the GDH-linked Schiff base intermediate with cellular metabolites, especially the free amino acids: Cys, Phe, Pro, Asn, Gln, Arg, and Tyr, the carboxyl groups of which are good nucleophiles. Amino acids regulate GDH, nitrogen metabolism, and plant growth (Singh and Srivastava, 1983; Barneix and Causin, 1996). Therefore, the rate of de novo synthesis of the control GDH was almost sufficient for the replacement of the inactivated copies of the enzyme. Treatment of the peanuts with the pesticides Basagran, Bravo, and Sevin then increased the degradation of the GDH (Figure 1). This is because the three pesticides are stronger nucleophiles than the free amino acids. Therefore, the effect of the peanut's free amino acids was quickly overridden by that of the pesticide molecules. Signal discrimination and integration by GDH are by nucleophilic displacement (Osuji et al., 1998; Osuji and Braithwaite, 1999). However, nucleophile-dependent inactivation does not rule out other mechanisms of GDH regulation. The slow de novo

synthesis of GDH is evidence that other control points operate at the transcriptional and/or translational levels (Melo-Oliveira et al., 1996). It is plausible that the pesticide-induced stress could have affected the steady-state levels of the GDH mRNA via the induction of the peanut's antioxidant enzymes, similar to the effects of paraquat on pea superoxide dismutase and glutathione reductase (Donahue et al., 1997). But such a possible change in the GDH mRNA level would not account for the inactivation, inhibition, and degradation of the enzyme.

The diversion of about 50% of GDH from its normal aminating function to EI complex formation may be accidental. But many cellular metabolites, for example, reducing sugars, free amino acids, water molecule, glycolytic and Krebs cycle intermediates, bicarbonate, plant nutrients, and pesticides, are nucleophiles, potentially able to react with the GDH-linked Schiff base intermediate. Because the resultant EI complex inhibits the $NH_4^+$ salvage function of the enzyme, thereby regulating plant growth and yield (Osuji and Madu, 1997b; Osuji et al., 1998), the observed large percentage of the enzyme inactivation is possibly an integral aspect of the essential function of the enzyme.

The inactivation of GDH by pesticides may be one of the conjugation reactions (Devine et al., 1993; Kunert and Dodge, 1989) for the suicidal removal of the pesticide from the active cellular pool. Also, the signaling by GDH may enable the plant to maintain an equilibrium between its nitrogen metabolism, growth, and the environment. GDH is a mitochondrial enzyme. Its substrate, α-KG (being one of the tricarboxylic acid cycle intermediates), enables it to influence cellular energy metabolism with its isomerization response reactions. The nucleophile-mediated displacement of the equilibrium between GDH de novo synthesis and degradation decreases the amination activity of the enzyme, thereby altering the cellular energy status, crop yield, and development (Osuji and Madu, 1997b; Osuji et al., 1998). This cellular signaling for the regulation of energy metabolism was initiated by the GDH-linked Schiff base complex. Although glutamine synthetase is isoenzymic and also assimilates $NH_4^+$ (Hirel et al., 1987; Bennett and Cullimore, 1989), its location outside the mitochondrion perhaps excludes it from influencing the cellular energy metabolism. Conversely, transaminases that utilize the Schiff base reaction mechanism are not located in the mitochondrion, and so do not influence nitrogen metabolism (Osuji and Cuero, 1991).

A potential practical application of the signal integrating property of GDH could be in the monitoring of the pesticide levels present in challenged environments, because the inactivation of the enzyme does not immediately result in retardation of crop growth until more than 50% of the enzyme has been inactivated. The peanut plants grew well (figure not shown) even under the high rates of pesticide treatments without morphological signs of toxic response, although up to 50% of the GDH had been degraded. An advantage of this method is its ability to demonstrate the effects of sublethal doses of pesticides on the metabolism of a nontarget crop. The treatments of peanuts with less than the recommended rates of the pesticides induced demonstrable inactivation of the enzyme, but there was no morphological evidence of the effect because the threshold inactivation essential for growth retardation (Osuji et al., 1998; Osuji and Madu, 1997b) was not attained. Peanuts in which more than 90% of the GDH was degraded because of $NH_4^+$ fertilization in combination with pesticide treatment exhibited severe retardation of growth (Osuji and Braithwaite, 1999). The threshold level of inactive GDH was exceeded in those crops. The results presented here suggest that the isomerization of plant GDH could be a sensitive technique for the objective monitoring (Madhun and Freed, 1990) of the spread of a pesticide in the environment, as well as for the monitoring of the environmental fate of a pesticide (Severn and Ballard, 1990). GDH inactivation assay is simple, requiring only leaf samples of the crops in the selected environment. It is not known, however, whether the development of resistance to pesticides in some crop species involves a development of insensitivity by the GDH-linked Schiff base intermediate complex to strong nucleophiles.

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