Activation of a Recombinant Petunia Glutamate Decarboxylase by Calcium/Calmodulin or by a Monoclonal Antibody Which Recognizes the Calmodulin Binding Domain

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To date, only plants have been shown to possess a form of glutamate decarboxylase (GAD) that binds calmodulin. In the present study, a recombinant calmodulin-binding 58-kDa petunia GAD produced in Escherichia coli was purified to homogeneity using calmodulin-affinity chromatography, and its responsiveness to calcium and calmodulin was examined in vitro. At pH 7.0–7.5, the purified recombinant enzyme was essentially inactive in the absence of calcium and calmodulin, but it could be stimulated to high levels of activity by the addition of exogenous calmodulin (Kd(0.1) = 15 nM) in the presence of calcium (Km = 0.8 μM). Neither calcium nor calmodulin alone had any effect on GAD activity. Recombinant GAD displayed hyperbolic kinetics at pH 7.3 (Km = 8.2 mM). A monoclonal antibody directed against the carboxy-terminal region, which contains the calmodulin-binding domain of GAD, was able to fully activate GAD in a dose-dependent manner in the absence of calcium and calmodulin, whereas an antibody recognizing an epitope outside of this region was unable to activate GAD. This study provides the first evidence that the activity of the purified 58-kDa GAD polypeptide is essentially calcium/calmodulin-dependent at physiological pH. Furthermore, activation of GAD by two different proteins that interact with the calmodulin-binding domain, a monoclonal antibody or calcium/calmodulin, suggests that this domain plays a major role in the regulation of plant GAD activity.

Calcium ions play a central role in intracellular signal transduction pathways in eukaryotes. The response to various stimuli is mediated by calcium-binding proteins such as calmodulin (CaM)1 which translate a transient calcium signal into a variety of cellular processes. CaM is a highly conserved protein, which upon binding calcium, undergoes a conformational change and is able to recognize and activate a diverse array of specific target proteins (for review, see Refs. 1 and 2). Previously, we cloned a cDNA for a novel 58-kDa CaM-binding protein by screening a petunia petal cDNA expression library with recombinant 35S-labeled CaM and identified it as glutamate decarboxylase (GAD) (3). Plant GAD bears greater sequence similarity to bacterial than to animal GAD (3) and appears to be unique among GADs in its ability to bind CaM. A detailed molecular analysis of the CaM binding domain of petunia GAD identified a 26-amino acid region near the carboxyl terminus with specific residues involved in CaM binding (4). Although the CaM binding domain of petunia GAD displays features similar to those of many animal CaM-binding proteins, it also possesses unique characteristics (4).

GAD catalyzes the decarboxylation of glutamate to yield CO2 and α-aminobutyrate (GABA). GABA is a ubiquitous nonprotein amino acid whose role as an inhibitory neurotransmitter in animals is well known. Several forms of animal GAD have been described previously (5, 6), and GAD appears as a autoantigen in insulin-dependent diabetes mellitus and in the rare neurological disorder, stiff-man syndrome (7, 8). In contrast, the function of GAD and GABA in plants remains unclear (9, 10). Interestingly, GABA accumulates rapidly in plants in response to a variety of environmental stimuli including hypoxia, temperature shock, water stress, and mechanical manipulation (11–16). Prior to the knowledge that plant GAD is a Ca(2+)/CaM-binding protein, it was suggested that GAD activity under stress may help resist cytosolic acidosis (15, 17, 18), given the fact that the decarboxylation of glutamate is a proton-consuming reaction and because plant GAD exhibits an acidic pH optimum (19, 20). Yet, recent results suggest that a decrease in cytosolic pH does not seem to be a prerequisite for GAD activation (15). Moreover, many of the same stresses that induce GABA production also cause increases in cytosolic calcium levels (21–23). It is therefore conceivable that GABA production in plants is regulated at least in part via Ca(2+)/CaM. Consistent with this hypothesis, we and others recently demonstrated a 1.5–9-fold stimulation of activity by Ca(2+)/CaM using partially purified plant GAD (4, 24, 25). However, interpretation of these studies remains limited because of the possible presence of contaminating CaM (25) and different forms of GAD (26).

Expression of recombinant CaM-binding proteins in bacteria offers the advantages of a typically high yield in the absence of endogenous CaM, other plant CaM-binding proteins, or other putative GAD isoforms. In our initial studies on recombinant GAD, we experienced difficulty in obtaining the 58-kDa recombinant GAD in a soluble form (3). Consequently, in the present study, our objective was to purify the soluble 58-kDa recombinant CaM-binding petunia GAD and examine its Ca(2+)/CaM responsiveness in vitro.

1 The abbreviations used are: CaM, calmodulin; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

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Experimental Procedures

Expression and Purification of the Recombinant GAD—Expression of the full-length petunia GAD in Escherichia coli strain BL21(DE3)pLyS using a PET12 expression vector (Novagen) was performed essentially as described previously (3), except that a 100-ml culture of bacterial cells was routinely used, and expression time was 6 h at 30°C. Proteins were extracted as described previously (3), with the exceptions that the extraction buffer (10 ml/100 ml of bacterial culture) consisted of 50 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 10% glycerol, 1 mM diethiothreitol, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Following extraction, soluble protein was immediately frozen in liquid nitrogen and stored at −70°C. To a 4-ml aliquot of thawed extract, CaCl2 and PMSF were added to final concentrations of 10 and 1 mM, respectively. The sample was passed through a 0.45-μm filter and then loaded onto a CaM-afferose (Sigma) affinity column (approximately 300 μl bed volume) pre-equilibrated with CaM binding buffer (50 mM Tris-HCl, pH 7.5, 1 mM CaCl2, 150 mM NaCl, 10% glycerol, and 1 mM freshly prepared PMSF). The first column-volume of effluent was reloaded onto the column and then eluent containing non-adsorbed proteins was collected.

The column was washed with 20-column-volumes of washing buffer lacking added calcium (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM PMSF) in order to remove weakly adsorbed bacterial proteins. Adsorbed GAD was eluted with elution buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EGTA, 10% glycerol, 1 mM PMSF), and fractions were either assayed immediately for GAD activity or were frozen in liquid nitrogen and stored at −70°C. From a 100-ml culture of transformed bacteria, typically about 50 μM of purified soluble GAD were recovered. Purity and intactness of the protein were assessed by SDS-PAGE followed by Coomassie Brilliant Blue staining or by Western blot analysis with a monoclonal anti-GAD antibody (designated as mAb-GAD-107.1).

In Vitro Assay of GAD Activity—GAD assays were performed using a radiometric method based upon [L-14C]glutamate or [L-15N, L-14C]glutamate-dependent CO2 production (25). Reactions were performed using 15 × 100-mm disposable culture tubes containing a CO2 trap of 0.5 ml of freshly prepared 0.1 N NaOH. Unless indicated otherwise, the reaction media consisted of 100 mM 1,3-bis-Tris-HCl buffer (Sigma), pH 7.0, or 100 mM 1,3-bis-Tris-propane-HCl buffer (Sigma), pH 7.3, containing 1 mM diethiothreitol (Sigma), 5 mM l-glutamate containing l-[1,14C]glutamate (0.1 μCi/ml, Amersham Corp.), 0.1 mM pyridoxal phosphate, and 10% glycerol (v/v) in a final volume of 0.5 ml. Calcium (as CaCl2) and bovine brain CaM (Sigma) were included in the reaction mixture at concentrations indicated in figure legends. In experiments where the effect of calcium concentration on GAD activity was examined, estimations of free calcium in the presence of EDTA or EGTA were obtained using a computer program (27).

The activity of the recombinant GAD was expressed in bacteria for about 6 h at 30°C with a 100-ml culture of E. coli coli soluble protein (about 1 mg) was loaded onto a CaM-afferose (Sigma) column, and aliquots of total soluble protein (Total, 7 μg of protein), column-effluent (Effluent, 7 μg of protein), and EGTA-eluted fractions (Eluted fractions, 0.3 μg of protein) were separated on SDS-PAGE and either Coomassie stained (panel A) or transferred to a nitrocellulose membrane, and the presence of GAD was detected with an anti-GAD monoclonal antibody (mAb-107.1; 1:20,000 dilution of ascitis, about 4 × 10−4 μg/ml of protein) (panel B). The arrow indicates the position of the recombinant GAD (Rec. GAD). The lane marked kDa contains molecular mass protein markers.

A CaM-afferose column and affinity-chromatography was performed as described above. Eluted proteins were separated by SDS-PAGE, and the major band, that had the same gel mobility as the petunia GAD and that was detected by the anti-petunia-GAD polyclonal antibodies (3), was cut out of the gel and electroeluted from the acrylamide slice with an Elutrap apparatus (Schleicher & Schuell) into SDS-PAGE running buffer. Mouse immunizations and screening for positive hybridomas were performed in the Monoclonal and Polyclonal Antibody Services of the Weizmann Institute. Hybridomas were screened first by enzyme-linked immunosorbent assay using the same antigen as used for mouse immunizations. Positive hybridomas were further tested on Western blots against different recombinant GAD proteins. Specificity of the monoclonal antibodies was determined using either an insoluble recombinant GAD (3) or a recombinant GAD lacking 27 amino acid residues from the carboxyl terminus (4). In addition, antibodies were tested against fusion proteins of glutathione S-transferase with the GAD carboxy-terminal region (amino acids 469–500) containing the CaM binding domain (3), or with deleted forms of the CaM binding domain (4). One monoclonal antibody (designated as mAb-GAD-430.8) recognized a region corresponding to amino acids 475–492 within the CaM binding domain (3), or with deleted forms of the CaM binding domain (4). One monoclonal antibody (designated as mAb-GAD-107.1) recognized an epitope outside of the CaM binding domain (3). Both antibodies recognized the recombinant as well as the plant 58-kDa GAD. Ascitic fluids containing each monoclonal antibody were prepared by injection of the corresponding hybridomas into the peritoneal cavity of pristane (2,6,10,14-tetramethylpentadecane) (Aldrich) CD3 mice (107 cells/mouse).

RESULTS

Purification of the Recombinant 58-kDa Petunia GAD—The recombinant GAD was expressed in bacteria for about 6 h at 30°C after induction by isopropyl-1-thio-β-D-galactopyranoside. A simple, single-step CaM affinity chromatographic method was used to purify the recombinant GAD from the soluble fraction of bacteria. Washing of the CaM affinity column in the absence of added calcium proved useful in removing weakly adsorbed bacterial proteins. SDS-PAGE analysis demonstrated that recombinant GAD is one of the most abundant proteins present in the total E. coli soluble extract, and it was essentially homogeneous after elution from the CaM affinity column (Fig. 1A). Western blotting confirmed that the recom-
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Fig. 2. Response of GAD activity to calcium and calmodulin. Panel A, GAD activity was measured at pH 7.0 as described under “Experimental Procedures” in the absence of calcium and calmodulin (Control), in the presence of 500 μM free calcium (+CaM), in the presence of 50 nM calmodulin without calcium (+CaM, or in the presence of 500 μM free calcium and 50 nM calmodulin (+Ca2+/CaM). EDTA was present in each reaction at a final concentration of 500 μM. Panel B, profile of GAD activity at pH 7.3 in response to increasing calmodulin (CaM) concentration in the presence of 1 mM added calcium. Panel C, profile of GAD activity at pH 7.3 in response to increasing free calcium concentration in the presence of 50 nM calmodulin. Reactions in C were carried out in the presence of 1 mM 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid and 0.05 mM EDTA (filled circles) or in the presence of 0.55 mM EDTA (crosses). In both A and C, estimates of free calcium were determined as described under “Experimental Procedures.” Values represent mean and standard error of three replicate reactions.

Recombinant GAD was completely bound by the CaM affinity column and that the purified protein was intact (Fig. 1B). The specific activities of GAD in eluted fractions (cf. Fig. 1) was typically about 12-fold higher than in total extracts. GAD activity increased linearly as a function of either time (for at least 15 min) or protein concentration, and an analysis by thin-layer chromatography confirmed that [14C]GABA was the only other reaction product (data not shown). Previously we demonstrated that under our experimental conditions, E. coli GAD does not bind CaM nor display detectable activity (3).

Recombinant GAD is inactive in the Absence of Calcium and Calmodulin at Physiological pH—When purified GAD was assayed at physiological pH (7.0–7.5) in the absence of calcium and CaM, activity was less than 1% of that observed under saturating CaM concentrations (Fig. 2A). Addition of CaM or calcium alone was insufficient to activate GAD, whereas in the presence of both calcium and CaM, GAD displayed high specific activity (Fig. 2A). In addition, GAD was activated by two different isoforms of recombinant petunia calmodulin (GenBank accession numbers M80836 and M80832, respectively) (data not shown).

The effects of increasing exogenous CaM concentrations on GAD activity at pH 7.3 in the presence of 1 mM calcium was also investigated. Stimulation of GAD activity was most pronounced at CaM concentrations between 3 and 50 nM with half-maximal activation at about 15 nM (Fig. 2B). When GAD activity at pH 7.3 was examined as a function of free calcium concentration in the presence of saturating CaM (50 nM), a steep rise in activity was observed at about 0.5 μM free calcium with half-maximal activity estimated at approximately 0.8 μM free calcium (Fig. 2C).

pH-Dependent Responsiveness of Recombinant GAD to Ca2+/CaM—GAD activity was examined over the pH range of 4.5–8.0 using an overlapping system of buffers in either the presence of 1 mM added calcium and 50 nM CaM or in the absence of Ca2+/CaM. When Ca2+/CaM was not included in the assays, GAD displayed a sharp response to pH with maximal activity observed between pH 5.0 and 6.0, consistent with previous estimations of the pH optimum for plant GAD (10). In the presence of Ca2+/CaM, GAD activity was similar to that observed in the absence of Ca2+/CaM except at pH values around neutrality. Only a slight stimulation of GAD activity by Ca2+/CaM (1.2–1.6-fold) was observed between pH 4.5–6.0 (Fig. 3). In contrast, between pH 7.0 and 7.5, GAD activity was virtually Ca2+/CaM-dependent with a peak in response observed at pH 7.5. Between pH 7.5 and 8.0, GAD activity and responsiveness to Ca2+/CaM dropped steeply. Plant GAD is cytosolic (30, 31) and estimates of cytosolic pH in plants range from pH 7.0 to 7.5 (32). Consequently, subsequent analysis of recombinant GAD in this study was performed at pH 7.3.

Kinetic Analysis of Recombinant GAD—The activity of the purified recombinant GAD as a function of substrate concentration at pH 7.3 in the presence of 1 mM added calcium and 50 nM CaM followed Michaelis-Menten kinetics. Estimates of and Vmax were 8.2 mM and 30.6 μmol CO2 min−1 mg of protein−1, respectively, as determined from Lineweaver-Burk plot analysis (Fig. 4). Analysis using half-reciprocal or Eadie-Hofstie plots gave similar values (data not shown). Calcium and CaM-independent GAD activity was consistently less than 1.5% of the activity observed in the presence of Ca2+/CaM over the range of substrate concentrations examined (data not shown).
Calcium- and Calmodulin-independent Activation of GAD by a Monoclonal Antibody Recognizing the Calmodulin-Binding Domain—The CaM binding domain presented in Fig. 5 represents a region (petunia GAD amino acids 470–495), which contains residues previously determined to be involved in CaM binding by at least one of three criteria: chemical cross-linking, enzyme-linked immunosorbent assay (4), and 35S-labeled CaM overlay assays (4). To further elucidate the role of the CaM binding domain in regulating GAD activity, we examined whether a monoclonal antibody, which recognizes an epitope within an 18-residue span of this domain (mAb-430.8, Fig. 5), could activate GAD in the absence of Ca²⁺/CaM. Indeed, GAD was activated by this antibody, and this activation was independent of Ca²⁺/CaM (Fig. 6A), was dose-dependent (Fig. 6B), and was inhibited by 0.5 mM EDTA (Fig. 6A), and was comparable in magnitude with that induced by saturating Ca²⁺/CaM alone (Fig. 6A). In addition, incubation of GAD with both Ca²⁺/CaM and this antibody (mAb-430.8 + Ca²⁺/CaM) yielded similar activation as was observed in the separate treatments, indicating there was no synergism between these two factors. No effect on GAD activity was observed in the presence of an antibody, which recognizes an epitope outside of the CaM binding domain (mAb-107.1) (Fig. 5) even at concentrations that were 20-fold higher than used for immunodetection of GAD by Western blotting (Fig. 1B) or enzyme-linked immunosorbent assay (not shown). The addition of exogenous Ca²⁺/CaM together with this antibody (mAb-107.1 + Ca²⁺/CaM) resulted in full activation of GAD (Fig. 6A). In addition, a nonrelevant monoclonal antibody, which recognizes plant CaM (mAb-17.28), was used as a control and did not affect GAD activity (Fig. 6A).

**DISCUSSION**

Previous work showing in vitro CaM stimulation of plant GAD activity utilized partially purified preparations and thus could not unequivocally exclude the involvement of other proteins in this stimulation (4, 24, 25), including contaminating CaM or other putative subunits of a GAD complex. Moreover, in these earlier studies calmodulin-independent GAD activity ranged from 10% to as high as 50% of Ca²⁺/CaM-stimulated GAD activity (4, 24, 25). In the present study, we purified a soluble recombinant petunia GAD from E. coli cells and studied its activation by Ca²⁺/CaM. Our study did not address the question of whether GAD functions as a multi- or monomeric enzyme under these conditions. It is noteworthy, however, that previous findings suggest that GAD purified from plants is a multimeric enzyme (19, 26, 33).

Prior to the knowledge of GAD regulation by Ca²⁺/CaM, studies of GAD from different plant species demonstrated that the optimal pH for GAD activity in vitro is about 5.8 and that at pH 7.0 the activity ranges from 12 to 30% of maximal activity (11, 19, 20). GAD in plants has been shown to be a cytosolic enzyme (30, 31), and in addition, it was often proposed that activation of GAD is a consequence of cytosolic acidification. Our studies, while confirming the fact that GAD exhibits maximal activity in vitro at acidic pH, establish that over a pH range of 7.0–7.5, which corresponds to the cytosolic pH of a typical plant cell (32), GAD activity is essentially dependent upon Ca²⁺/calmodulin. Similar to our findings, the CaM activation of myosin light chain kinase (34), phosphodiesterase (35), and phosphorylase kinase (36) has also been demonstrated to be pH-responsive. GAD activity becomes nearly CaM-independent below pH 6.5 (Fig. 3). However, plant cytosolic pH is carefully maintained at levels slightly above neutrality (32), and nonlethal perturbations generally do not cause a decline of more than 0.5 pH units (15, 37). Taken together, our results imply that an acidic pH is not essential for GAD activation. Therefore, GAD may be activated in plants by signals that do not involve pH changes.

The concentration of CaM required for half-maximal stimulation of the purified recombinant GAD (Kₐ₅₅ about 15 nM, Fig. 2B) is similar to values published for other CaM-dependent enzymes (38–41) and is lower than a previous estimate using partially pure soybean GAD (Kₐ₅₅ = 25 nM) (25). In the presence of saturating CaM, recombinant GAD was inactive at calcium levels estimated to represent resting physiological values (~0.01–0.1 μM calcium) (42) but was activated at calcium concentrations greater than about 0.3 μM. External stimuli induce calcium fluxes in plant cells (42), and thus GAD activity may be regulated by calcium fluxes in vivo via CaM activation.

**FIG. 5. Diagram of GAD showing the calmodulin-binding domain and anti-GAD monoclonal antibody recognition regions.** The numbers above the diagram refer to amino acid residues as described previously (3, 4). The location of the calmodulin-binding domain, including a critical tryptophan residue (amino acid 485), was determined previously (4). The diagram shows a region (amino acids 1–469) containing an epitope recognized by an anti-GAD monoclonal antibody designated mAb-107.1, and the carboxyl-terminal region (amino acids 469–500), which contains the calmodulin-binding domain (amino acids 470–495), and a domain (amino acids 475–492) recognized by a different anti-GAD monoclonal antibody (mAb-430.8). Determination of monoclonal antibody specificity was performed as described under “Experimental Procedures.”
monodonal antibody recognizing an epitope within the CaM binding domain is reminiscent of a previous report using the CaM-regulated myosin light chain kinase (47). Activation of the kinase by a monodonal antibody that recognizes the CaM binding domain appears to result from a disruption of autoinhibition (47). Autoinhibition by CaM binding domaains seems to be a common feature of CaM-regulated proteins (for review, see Refs. 2 and 48). Several models describing this phenomenon suggest that the CaM binding and autoinhibitory domains either overlap or are at least in close proximity to one another (49–51). It is possible that the C-terminal region of GAD, which contains the CaM binding domain, may be involved in suppressing GAD activity in the absence of Ca$^{2+}$/CaM. Our results suggest that CaM binding induces a conformational change necessary for GAD activation. The monodonal antibody may mimic the action Ca$^{2+}$/CaM in this respect, or it may induce a different conformational change that results in GAD activation. Further research is needed to characterize these phenomena.

Our data support the idea that plant GAD is CaM-regulated but do not exclude the possibility that other mechanisms of post-translational regulation may be involved in vivo. Phosphorylation of brain GAD by protein kinase A results in inhibition of activity, which can be reversed by the calcium-dependent phosphatase calcineurin (52). Similarly, the CaM binding domain of many proteins is often a target for reversible serine/threonine phosphorylation (53–55). Current models suggest that the introduction of this negative charge hinders CaM (an acidic protein) binding and serves to inactivate CaM targets (2, 48). It is not known whether a similar mechanism occurs in plants.

To our knowledge, plant GAD may be unique among eukaryotic GADs in having evolved as a CaM-regulated enzyme. If so, it implies that some adaptive advantage is associated with the regulation of GABA synthesis by calcium signaling in plants. The ability of Ca$^{2+}$/CaM to activate recombinant GAD is consistent with reports on the rapid induction of GABA synthesis in plants under various stress conditions (11–16) as many of these stresses are known to increase the level of cytosolic calcium (Ref. 42 and references therein). However, the role of GABA accumulation under stress remains unresolved.

In summary, our results demonstrate that the catalytic activity of the 58-kDa recombinant petunia GAD is essentially dependent upon Ca$^{2+}$/CaM at physiological pH. Furthermore, activation of GAD by a monodonal antibody that recognizes the CaM binding domain suggests that one function of this domain may be to suppress GAD activity in the absence of a calcium signal and that the role of Ca$^{2+}$/CaM is to relieve this suppression. Further studies are required to elucidate the regulatory features of the carboxyl-terminal region of GAD containing the CaM binding domain.

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