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Functional roles of the hexamer organization of plant glutamate decarboxylase☆

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ABSTRACT

Glutamate decarboxylase (GAD) is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes the α-decarboxylation of glutamate to γ-aminobutyrate. A unique feature of plant GAD is the presence of a calmodulin (CaM)-binding domain at its C-terminus. In plants, transient elevation of cytosolic Ca^{2+} in response to different types of stress is responsible for GAD activation via CaM. The crystal structure of GAD isoform 1 from *Arabidopsis thaliana* (AtGAD1) shows that the enzyme is a hexamer composed of a trimer of dimers. Herein, we show that in solution AtGAD1 is in a dimer–hexamer equilibrium and estimate the dissociation constant (K_d) for the hexamer under different conditions. The association of dimers into hexamers is promoted by several conditions, including high protein concentrations and low pH. Notably, binding of Ca^{2+}/CaM1 abolishes the dissociation of the AtGAD1 oligomer. The AtGAD1 N-terminal domain is critical for maintaining the oligomeric state as removal of the first 24 N-terminal residues dramatically affects oligomerization by producing a dimeric enzyme. The deleted mutant retains decarboxylase activity, highlighting the dimeric nature of the basic structural unit of AtGAD1. Site-directed mutagenesis identified Arg24 in the N-terminal domain as a key residue since its mutation to Ala prevents hexamer formation in solution. Both dimeric mutant enzymes form a stable hexamer in the presence of Ca^{2+}/CaM1. Our data clearly reveal that the oligomeric state of AtGAD1 is highly responsive to a number of experimental parameters and may have functional relevance *in vivo* in the light of the biphasic regulation of AtGAD1 activity by pH and Ca^{2+}/CaM1 in plant cells. This article is part of a special issue titled "Cofactor-Dependent Proteins: Evolution, Chemical Diversity and Bio-applications."

1. Introduction

GAD (glutamate decarboxylase, EC 4.1.1.15) is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes the irreversible α-decarboxylation of L-glutamic acid to γ-aminobutyric acid (GABA) and carbon dioxide, which is the final step of the GABA shunt. GAD and GABA are present in virtually all organisms but exhibit different regulatory mechanisms and biological functions. They are involved in resistance to cytosolic acidosis in bacteria [1], whereas GABA is an important inhibitory neurotransmitter in animals [2,3]. The role of GABA in plants remains unclear. In addition to the well-documented roles of GABA as a buffering mechanism in C and N metabolism and in regulation of cytosolic pH [4], it has a significant role in protection against oxidative stress [5] and defense against herbivorous pests [6]. In this regard, because of the rapid increases in GABA concentration in response to stress, it has sometimes been inferred that GABA might act as an intracellular signaling molecule in plants [7–9].

Compared to GADs from other organisms, plant GADs possess a unique feature, namely, the presence of a C-terminal calmodulin binding site (CaMBD). This characteristic confers plant GADs an additional regulatory mechanism by making them responsive to cytosolic calcium (Ca^{2+}), thus revealing that at least two mechanisms exist, by which GAD activity can be stimulated *in vitro* and *in vivo*, namely, acidic pH and Ca^{2+}/CaM [10–12].

The sequence of the completed *Arabidopsis* genome indicates that five isoforms of GAD exist in *A. thaliana* (AtGAD1-AtGAD5) [13]. Isoform 1 (AtGAD1) was recently characterized both biochemically and structurally by Gut et al. [14]. The crystal structure revealed that the enzyme belongs to the fold Type I family of PLP-enzymes and is a compact homohexamer of 342 kDa similar to the bacterial enzyme GadB [15].

Abbreviations: GAD, Glutamate decarboxylase; CaM1, calmodulin isoform 1 from *Arabidopsis thaliana*; GABA, γ-aminobutyric acid; PLP, pyridoxal 5′-phosphate; PDB, Protein Data Bank; K_d, dissociation constant; SEC, size exclusion chromatography; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; SAXS, small-angle X-ray scattering; CaMBD, calmodulin binding domain; T_{50}, thermal inactivation midpoint.

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The hexamer is a trimer of dimers, consisting of two layers of three subunits each, where the dimers contribute one subunit to each layer (PDB ID: 3HBX) (Fig. 1). Each subunit can be divided into an N-terminal domain (residues 1–57), a large domain (residues 58–347) containing the cofactor binding site, a small domain (residues 348–448), and a C-terminal domain containing a CaMBD that regulates enzyme activity in a CaM-dependent manner.

A low-resolution structure of the AtGAD1 in complex with the calmodulin isoform 1 (CaM1) from *Arabidopsis thaliana* obtained by small-angle X-ray scattering (SAXS) showed that CaM1 activates AtGAD1 by relieving two C-terminal autoinhibition domains of adjacent active sites, thereby forming a 393 kDa AtGAD1-Ca^{2+}/CaM1 complex with an unusual 1:3 stoichiometry. In the complex, one CaM1 molecule attaches to two AtGAD1-CaMBDs of neighboring subunits in the homohexameric enzyme, activating two adjacent active sites by relief of the corresponding autoinhibitory domains [14].

While PLP-enzymes have been extensively investigated, less is known about their quaternary structures in solution and the dynamics of subunit dissociation/association. Herein, we analyze the essential factors governing the stability of AtGAD1 quaternary structure. We show that the oligomeric state of AtGAD1 depends on concentration, revealing an equilibrium between two species in solution, a dimer and a hexamer. The association of dimers into hexamers is promoted by a number of conditions, including high enzyme concentration, presence of Ca^{2+}/CaM1, and acidic pH (pH < 6.5). Guided by sequence analysis of N-terminal domains of plant GADs, by the AtGAD1 crystal structure, and by limited tryptic proteolysis, we have identified a flexible and exposed stretch spanning residues 1–24 as the minimal region required for assembly and stabilization of the hexameric state. This was achieved using a combination of biochemical analyses on a purified recombinant protein where the first 24 N-terminal amino acids had been deleted (AtGAD1-Δ1-24). In addition, site-directed mutagenesis allowed for identification of a crucial hexamerization 'hot spot' in the N-terminal domain, Arg24, which forms a contact network between the dimeric units. Taken together, the results obtained *in vitro* support a model of biphasic regulation of AtGAD1 oligomerization and activity *in vivo*.

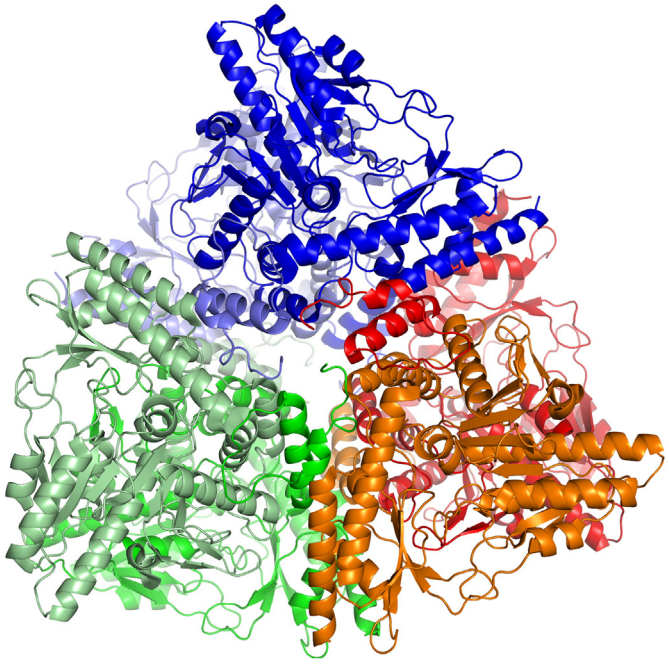

Fig. 1. Cartoon representation of AtGAD1. The six subunits are depicted in different colors. The three dimers are A–B (blue and light blue), C–D (red and orange), and E–F (green and light green) (PDB ID: 3HBX) [14].

2. Materials and methods

2.1. Cloning, expression, and purification of recombinant GAD1 mutants and CaM1

Construction of the N-terminally truncated AtGAD1 mutant AtGAD1-Δ1-24, where the first 24 residues of the mature polypeptide chain are absent, was performed by PCR amplification of the plasmid pET12b carrying the gene encoding AtGAD1. Two primers were used: the first annealed from the 25th codon and contained an additional upstream nucleotide sequence used to introduce an *Nde*I restriction site at the 5′-end, which provided the necessary ATG start codon (forward primer 5′-GCTCTAGACATATGACTTCACTTCCTAGG-3′; *Nde*I *site* is underlined), and the second primer annealed over the C-terminus of AtGAD1 (reverse primer 5′-CGGGATCCTTAGCAGATACCACTCG-3′; *Bam*HI is underlined).

The resulting DNA fragment was inserted into pET12b at the *Nde*I/*Bam*HI sites for directional cloning, replacing the wild-type gene to yield the construct pET12b-AtGAD1-Δ1-24. The newly generated plasmid pET12b-AtGAD1-Δ1-24 was fully sequenced to confirm the deletion and used to transform the *Escherichia coli* strain BL21(DE3)pLysS.

The N-terminal point mutants were made on the wild-type (wt) construct pET12b-AtGAD1 using the QuikChange™ site-directed mutagenesis kit (Stratagene, CA, USA) following the manufacturer's protocol. For each mutant, two synthetic oligonucleotide primers were designed (Invitrogen), each complementary to opposite strands of the plasmid and containing the desired mutation. The coding region of all mutated plasmids was verified by DNA sequencing. *E. coli* strain BL21(DE3)pLysS cells were transformed and used for expression.

The conditions for expression and purification of the mutants were essentially as described previously for AtGAD1 wt [14]. The PLP content of all preparations was determined by treating proteins with 0.1 M NaOH and measuring absorbance at 388 nm ($\varepsilon_{388} = 6550\ M^{-1}\ cm^{-1}$). Enzyme concentration was determined as described for the wt enzyme [14].

CaM1 from *A. thaliana* was expressed in *E. coli* strain BL21(DE3) containing plasmids encoding for CaM1 (pET12b-CaM1). The cell culture was grown to exponential phase in LB medium containing ampicillin (100 μg/mL) at 37 °C, and induction was done with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4–5 h at 30 °C. CaM1 was purified essentially as described by Liao and Zielinski [16]. Protein concentration was determined by the Bradford assay [17].

2.2. AtGAD1 activity assay

Enzyme assays were performed as described [18]. To test the effect of temperature on decarboxylase activity of wt and mutant proteins, a temperature profile was obtained. The protein (0.2 mg/mL) in an aqueous buffer containing 25 mM MES, 25 mM MOPS, and 150 mM NaCl at pH 5.8 was incubated for 15 min at temperatures between 30 °C and 90 °C. Samples were removed, cooled on ice for 3 min, and diluted to the concentrations used for assay of enzymatic activity. Residual enzymatic activity was determined as described above.

2.3. Spectroscopic measurements

Absorption measurements were carried out on a JASCO-V560 UV-Visible spectrophotometer. Absorbance data were recorded at 415 nm and at 338 nm in a solution containing 25 mM MES, 25 mM MOPS, and 150 mM NaCl and fitted to Eqs. (1) and (2), respectively:

$$A = \frac{(A_1 - A_2)}{\left(1 + 10^{(pH - pKa)}\right)} + A_2 \quad (1)$$

$$A = \frac{(A_1 - A_2)}{\left(1 + 10^{(pKa - pH)}\right)} + A_2 \quad (2)$$

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