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Enzymatic and structural aspects on glutamate decarboxylase

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This manuscript is dedicated to the memory of late Professor Saburo Fukui for his contributions to the field of pyridoxal enzymes.

Abstract

Glutamate decarboxylase (GAD) is a sole enzyme to synthesize γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter in higher animals. Two distinct enzymes are encoded in the mammalian genomes, namely GAD65 and GAD67. These isozymes resemble in their primary structure except for significant heterogeneity found in the N-terminal 100 residues, which suggests certain differences in the cellular localization and functions. Other than brain cells, GAD is found in pancreatic cells. Pancreatic GAD65 is found to be a target antigen for autoantibody produced in diabetes and stiff man syndrome (SMS) patients. Extensive structural information on GAD helps to develop or improve early diagnostic tools for diabetes. GAD is found in a variety of living organisms. Insect has analogous neuronal system to mammals where GAD is responsible for GABA production. Plant GAD is regulated by Ca^{2+} levels since it has a calmodulin-binding site in the C-terminal region, which is found only in the plant enzymes. *Escherichia coli* GAD, a hexameric enzyme with significant structural differences from mammalian GAD, has two types, GAD-α and GAD-β. In this review, structural similarities of GAD enzymes from various sources are compared and some of the characteristic features are described.

Keywords: Glutamate decarboxylase; GABA; Neurotransmitter; Diabetes; Bacterial expression

1. Introduction

Glutamate decarboxylase (GAD) catalyzes an α-decarboxylation reaction of L-glutamate to produce γ-aminobutyric acid (GABA) (Fig. 1). GAD is particularly important in the central nervous system (CNS) where GABA acts as a major inhibitory neurotransmitter [1]. GAD is considered unique among enzymes involved in the neurotransmitter synthesis because both its substrate and product are neurotransmitters and exhibit opposite actions. In neuronal system, L-glutamate acts as an excitatory and GABA as an inhibitory; each involved in glutaminergic and GABAergic synapses, respectively [2].

As both GABA and GAD are widely distributed in living cells of various organisms from mammalians to single cell organisms [1], the role of GAD other than mammalian neu-

Abbreviations: CNS, central nervous system; DOPA, 3,4-Dihydroxyphenylalanine; GAD, glutamate decarboxylase; GABA, γ-aminobutyrate; HDC, histidine decarboxylase; ICL, isocitrate lyase; PLP, pyridoxal 5′-phosphate; Sf, *Spodoptera frugiperda*; SMS, stiff man syndrome

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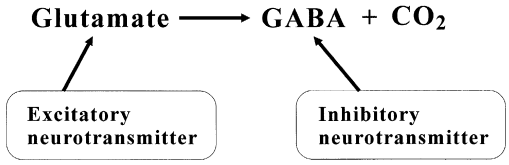

Fig. 1. Reaction catalyzed by GAD.

ronal systems has been explored. The use of genetic engineering techniques has made it possible to clone the genes for GAD and revealed the existence of two isozymes. Structural features of GAD isozymes are increasingly available due to the development of the in vitro expression systems and large-scale purification techniques. Cellular roles of GAD isozymes are becoming clear with the use of knockout mice and transgenic mice with anti-sense DNA.

Reported structural studies on bacterial, plant, insect and mammalian GADs are reviewed, and the differences in the structures of GADs and their relation to chemical reaction and physiological functions are discussed. For bacterial GADs, *Escherichia coli* GAD provides some intriguing information. The cloned *E. coli* GAD has shown that there are two forms of GAD enzymes, which have low sequence identity with the mammalian enzymes. Although decarboxylation reaction for mammalian and *E. coli* GADs is identical, subunit composition and molecular mass are significantly different. Structural information of bacterial GADs has suggested the diversity among the bacterial enzymes. Basic structure of plant GAD is similar to that of *E. coli* enzyme; however, the structural analysis has revealed that an extra segment is attached to the C-terminal segment of some plant GADs. GAD activity is regulated by the Ca^{2+} level via the C-terminal segment. A particular attention is paid to human pancreatic GAD65 as this enzyme has shown antigenicity toward antibody produced in patients with diabetes and stiff man syndrome (SMS). It is of clinical importance as the structural information on GAD65 is vital for developing new and/or improved diagnostic tools.

2. Bacterial GAD

E. coli enzyme is probably the best characterized enzyme among the investigated GADs, mainly due to its availability [3–5]. It is induced by glutamate in the growth media and easily produced in a scale of 10 mg/l of media [4,6]. Purified enzyme is constructed with six identical subunits of 53 kDa [7]; each subunit contains one pyridoxal 5′-phosphate (PLP) per active site. Both sedimentation equilibrium and electron microscopic data support the hexameric structure [7–9]. *E. coli* enzyme is dissociable at low temperature, at low protein concentrations, and at pH values of 6.0 and higher [7,10]. The enzyme maintains its optimum activity between pH 4.0 and 4.5 [11,12]. The best substrate for *E. coli* enzyme is L-glutamate but it also decarboxylates other amino acids, such as γ-methylene-DL-glutamate, *threo*-β-hydroxy-DL-glutamate, and L-homocysteine sulfinate. It seems to maintain the strict size and optical activity preferences since D-glutamate and L- or D-aspartate are not substrates [11,13,14]. The physiological role of GAD in *E. coli* is not entirely understood; however, the maintenance of physiological pH under acidic conditions has been proposed [3]. In other bacteria, such as *Bacillus*, the conversion of L-glutamic acid to GABA is thought to play a key role in the germination of bacterial spores [15].

E. coli GAD was a target enzyme for the study of suicide substrate inhibition, i.e., L-serine-*O*-sulfate, *R*-(−)-4-aminohex-5-ynoic acid, and α-fluoromethyl-glutamate [16–19]. Inactivation mechanism of GAD with L-serine-*O*-sulfate was extensively studied, and found to be identical to that of aspartate aminotransferase [19,20]. This finding made significant contribution to the drug development, which utilizes a concept of suicide substrate–enzyme systems [21–26].

The amino acid sequence of *E. coli* GAD was reported for the active site region [27] and only recently for both N- and C-terminal regions [28], then a complete sequence [29]. By

Table 1
Subunit structure and turnover numbers of GAD
(–) Not reported.

Species	Subunit MW, kDa	No of Subunit	Sp. Act., μmol/min/mg	k_{cat}, s^{-1}	K_m, mM	Reference
Mouse	45	2	3.3	2.5	–	[114,115]
Rat, mouse	67	2	3.2	–	–	[116]
Rat	59, 63	2	–	–	–	[117]
Rat	67	2	6.4	7.5	1.1	[118]
Rat	60	2	2.6	2.6	–	[119]
Rat	40, 80	3	2.4	3.2	1.6	[120]
Pig-α	60	2	0.34	4.0	0.17	[121]
Pig-β	60	2	0.73	5.4	0.45	[121]
Pig-γ	60	2	0.70	2.3, 5.8	1.24	[121]
Pig	50	2	47	–	1	[122]
Human	67	2	1.0	1.1	1.3	[123]
Chicken	62, 59	2	2.6	2.6	–	[124]
Feline	59	2	3.25	–	1.38	[103]
Locust (*Schistocerca gregaria*)	51, 44	2	1.06	–	–	[62]
Squash	58	6	95	–	8.3	[56,57,59]
E. coli	50	6	67.9	–	1.0	[125]
L. brevis	60	2	6.0	6.5	9.3	[36]

using a DNA probe based upon this known amino acid sequence, two distinct GAD structural genes, *gadA* and *gadB*, were cloned [30]. Each gene encodes a 466-residue polypeptide and the difference is only by five amino acids. The active site sequence shows high resemblance with mammalian GAD as well as PLP-dependent histidine decarboxylases (HDCs) [29]. Two isozymes of *E. coli* enzyme showed identical kinetic and physico-chemical properties [31]. The crystals suitable for X-ray analysis were prepared from the purified *E. coli* GAD-β [32], which has exhibited space group of $P3_1$ or $P3_2$ with the unit-cell dimensions $a = b = 115.6$ and $c = 206.6$Å.

GAD was also purified from *Streptococcus pneumoniae* [33], *Neurospora crassa* [34,35] and *Lactobacillus brevis* [36]. *S. pneumoniae* GAD was characterized and found to exhibit mammalian GAD-like properties with 54 kDa subunit mass and sequence similarity to human GAD65 (59% similarity, 28% identity) [33]. Unlike *S. pneumoniae* GAD, the subunit mass of *N. crassa* GAD was estimated to be 33 kDa and the similar size was derived from gel filtration study at pH 6 [34]. Kinetic properties of the *N. crassa* enzyme resemble those of *E. coli* GAD with a pH optimum at acidic range and K_m value of 2.2 mM. *L. brevis* GAD has an optimum pH at 4.2 and an optimum temperature at 30°C, similar to *E. coli* enzyme; however, it is a dimer with 60 kDa subunit. Amino-terminal sequence (M–N–K–N–D–Q–E–Q–T) is also distinct from that of the *E. coli* enzyme (M–D–Q–K–L–L–T–D–F–R). Some of the kinetic parameters are shown in Table 1. GABA-containing food, i.e., Gabaron tea and red mold rice, has been reported to have an anti-hypertensive effect for man [37–40]. GABA-producing fermentation bacteria such as *Lactobacillus* may be of interest for food industries as it can be applied to produce health-oriented product such as yogurt with the anti-hypertensive effect.

3. Plant GAD

GAD is widely distributed in a variety of plants, and some have been cloned from tomato, petunia, and soybean [41–43]. In plant, GAD activity is thought to be associated with senescence, seed germination and ripening [41,44]. Barley enzyme is demonstrated to have two distinct forms. The enzyme derived from em-

bryo exhibits high K_m value, 22 mM, for L-glutamate while the enzyme from root shows a K_m of 3.1 mM [44]. Molecular mass of the root enzyme is 310 kDa, more like *E. coli* GAD,

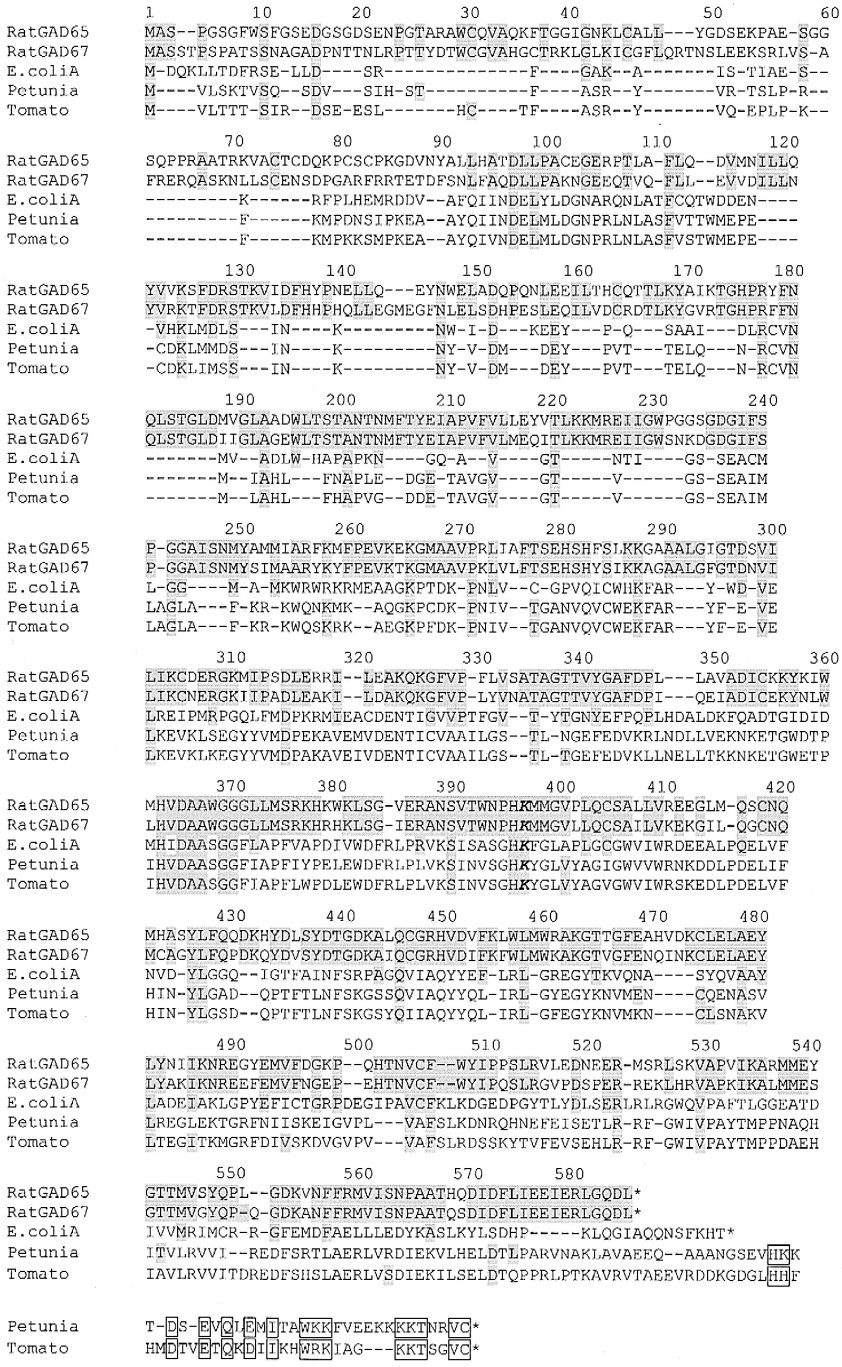

Fig. 2. Comparison of primary sequence of GADs of rat GAD65, rat GAD67, *E. coli* GAD-α, petunia GAD, and tomato GAD. Those shaded residues indicate identical amino acid residues to rat GAD65. Asterisk indicates the C-terminal end. Boxed amino acid residues are calmodulin-binding sites.

whereas the embryo enzyme is 120–256 kDa. Moreover, the embryo enzyme is sensitive to thiol reagents, whereas the root enzyme is not. It is therefore speculated that plant GAD has functions specific for the different developmental stages.

The primary structure of petunia GAD was determined to have 500 amino acid residues with subunit mass of 58 kDa, which resembles to that of *E. coli* enzyme (Figs. 2 and 3). A startling finding was that it had an extended C-terminal sequence with a typical calmodulin-binding domain [42]. In vitro binding assay has shown that recombinant petunia enzyme can bind to calmodulin from either petunia or mammalian apparently via C-terminal domain, Val-469 through Cys-500; however, activity was not affected by the Ca^{2+} concentration [42]. The calmodulin-binding domain is probably to form an α-helical structure [45]. Recombinant petunia GAD showed no activity, whereas the activity was recovered when both Ca^{2+} and calmodulin were added [46]. Besides petunia enzyme, GADs isolated from four other plant species, Arabidopsis, fava bean, tomato, and soybean, are reported to exhibit having calmodulin-binding domain in their C-terminal segment [42,47–51].

Both the presence of GABA-synthesizing enzyme in plant and calmodulin-binding ability of GAD seem to be of biological significance. Although neither the role of GABA nor physiological function regarding the relationship between Ca^{2+} and GAD has been established, it is probable to suggest that GABA may be involved in the response to stresses, i.e., hypoxia, mechanical stress, cold shock, heat shock, and water stress [52–55]. Moreover, such stresses are known to induce intracellular transient Ca^{2+} signals, and, therefore, the participation of Ca^{2+} in the regulation of GABA synthesis is suggested [53].

The investigations on other plant GADs are in progress, however, limited information is available. For example, squash GAD is isolated and purified to homogeneity [56–58], and the optimum pH of squash enzyme is determined to be 5.8, 1 pH unit higher than that of the *E. coli* enzyme. Squash GAD appears to have a hexameric subunit structure according to the sedimentation equilibrium experiments [59]. The presence of calmodulin-binding domain in squash enzyme is not yet demonstrated.

4. Insect GAD

It has been known that insect has brain and neuronal networks since late 17th century. Modern analysis has revealed that insect brain is analogous to mammalian brain [60] and, indeed, large GAD activity has been located in the insect brain [61–63]. The GAD activity is found in a number of insect species including cockroach [64], grasshopper [62,65], moth [66], honeybee [67], and fly [68]. Isolated grasshopper GAD has a dimeric structure of closely related subunits of 44 and 51 kDa. Cockroach enzyme was inhibited with Cl^- ion [64]. The properties of insect GADs and chemical transmitters utilized in insect are analogous to mammals; thus, insect GAD has potential to serve as a model enzyme for mammalian GAD studies. Since much of the insect behavior has been characterized, i.e., mating, hunting, and sleeping

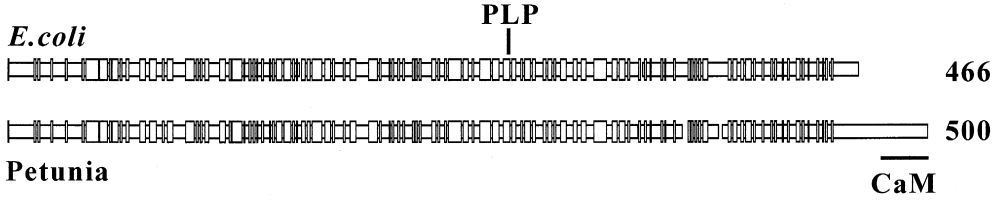

Fig. 3. Sequence comparison between *E. coli* and petunia GAD. PLP indicates PLP binding site. CaM indicates the location of calmodulin-binding site. Thick and thin boxes denote identical amino acid residues and non-identical amino acid residues, respectively.

(circadian), effects of regulatory elements to GAD activity could be conveniently observed and interpreted with less complexity than the mammalian system.

5. Mammalian GAD

Mammalian GAD catalyzes the decarboxylation reaction of L-glutamate to synthesize GABA whose biological functions is not limited to be an intercellular signaling molecule in both CNS and non-CNS. GABA is also involved in development and differentiation of cells [69–72]. Only recently, it became clear that vertebrate has two distinct genes for GAD, for instance, human has one on chromosome 2 and the other on chromosome 10, where each produces GAD67 and GAD65, respectively [73–75]. The functional roles of GAD isozymes in various tissues are being questioned in relation to physiological significance of GABA. Recent studies using gene-targeting technique have made a clear distinction between the isozymes; *GAD67 −/−* mice are born with cleft palate and die within a day after birth while *GAD65 −/−* mice survive with a slightly increased tendency in seizures [76–79]. A similar study was carried out on fruit fly, *Drosophila melanogaster*, in which mutations introduced on *Gad* gene were found to be lethal [80]. Although detailed study of mapping the isozymes in different regions of brain or other tissues has been conducted, how the isozymes being involved in the synthesis of GABA and their regulatory roles are still in question.

Pancreatic β cells produce GABA and exhibit high level of GAD activity [81,82]. The function of both GABA and GAD in pancreatic β cells is still obscure, but GABA is considered to have a primary role as a signaling molecule in the pancreatic islets [82–85]. Although other functions are still undeniable, a regulator of protein biosynthesis [86] and an alternative energy source for the β-cell are also proposed [87,88]. Recently, pancreatic GAD65 was identified as a target antigen for autoantibody found in blood circulation of patients with insulin-dependent diabetes [89]. The antibody is made in T-cells, which is responsible for destroying the entire β cells; thus, understanding the process of the antibody formation is crucial to prevent the diabetic symptoms. Recently, the mechanism of how GAD65 ends up causing antibody formation was studied with transgenic mice having anti-sense DNA toward GAD [90]. Diabetic patients appear to express autoantibody as early as 10 years prior to developing their clinical symptoms; therefore, purified GAD65 protein has a potential for the diagnostic tool for early detection of diabetes [91]. For this purpose, detailed information concerning protein structure and stability of GAD65 are highly desired.

Autoantibody toward GAD analogous to above described diabetes was also found in patients with SMS, a rare neurological disease [92,93]. It turns out that both anti-sera from diabetic and SMS patients show cross-reactivity toward GAD65 and is suggested that there may be a common cause between two diseases.

5.1. Structural aspect of mammalian GAD

Primary structure of mammalian GAD has not been determined at protein level; however, amino acid sequences of GAD isozymes from various mammalian species have been predicted based upon corresponding DNA sequences (consult GenBank or SwissProt databases). There is a high sequence homology among the same isozymes from different species; typically, 90% identity is found for either GAD67 or GAD65 (Figs. 2 and 4). Within the same species, sequence homology between GAD67 and GAD65 is rather low; typically 60% identity (Figs. 2 and 4). Sequence homology of mammalian GAD with non-mammalian species is significantly lower; for example, mammalian GAD vs. *E. coli* GAD shows around 24% identity. Active site of GAD, where PLP cova-

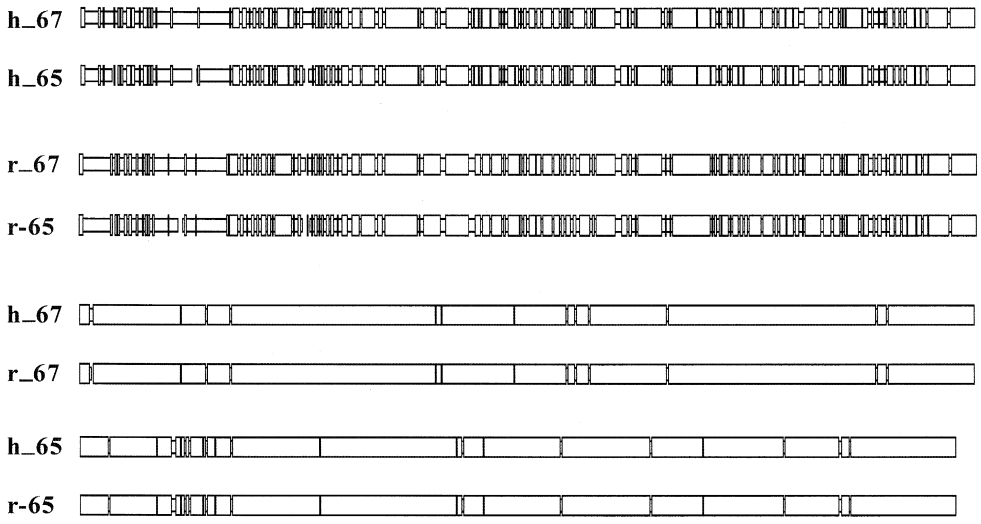

Fig. 4. Sequence comparison between isozymes from the same species and the isozyme from different species. Animal species are indicated as h_ and r_ for human and rat, respectively. Isozymes are shown as 65 and 67. Thick and thin boxes are as explained in Fig. 3 legend.

lently binds, is highly conserved. A consensus sequence is –RXXSXXXXPHKMMXVX-LXC–.

When sequences of two isozymes were compared, a significant heterogeneity was found for the first 100 amino acid residues at the N-terminal region, whereas considerably high similarity is maintained at the C-terminal region. Different cellular localization of GAD isozymes may be explained by the site specificity of the isozymes originating the heterogeneity of the N-terminal sequences. GAD65 contains more Cys residues within the N-terminal region than GAD67. Three of such Cys residues of GAD65 were found to be palmitoylated, which further suggests some membrane affinity of this isozyme [94,95]. Since GABA molecules are packed into synaptic vesicles in neurons and microvesicles in pancreatic β cells, it is reasonable to assume that GAD molecules associate membrane components of the vesicles. To this extent, N-terminal segment, whether or not it is palmitoylated, is likely to be involved in anchoring mechanism to vesicles [94,95]. It is also reported that several serine residues in the N-terminal segment are susceptible for phosphorylation, where physiological significance of the phosphorylation was examined [96]. It is suggested that phosphorylation may be involved in the membrane anchoring or regulation of GAD activity [96,97].

The heterogeneity of N-terminal segment of GAD is considered in connection to other decarboxylases. GAD exhibits primary sequence homology with other PLP-dependent decarboxylases including 3,4-Dihydroxyphenylalanine (DOPA, also named aromatic amino acid) decarboxylase and HDC. It is of interests to point out that these three decarboxylases maintain significant sequence homology and apparently belong to the same family [98]. Fig. 5 shows diagrammatically how similar in their sequences among DOPA, glutamate, and histidine decarboxylases, where each proteins has total amino acid residues of 480, 593, and 656, respectively. It is clear that GAD has an extra N-terminal segment (about 100 residues) and HDC has an extra C-terminal segment (about 170 residues) as compared with DOPA decarboxylase [99]. DOPA decarboxylase may be the simplest form within this class of decarboxylase proteins [98]. In case of HDC, the extra C-terminal segment is cleaved off by an elastase-like protease to become a fully active mature protein [100]. It is also evident that C-terminal segment of HDC

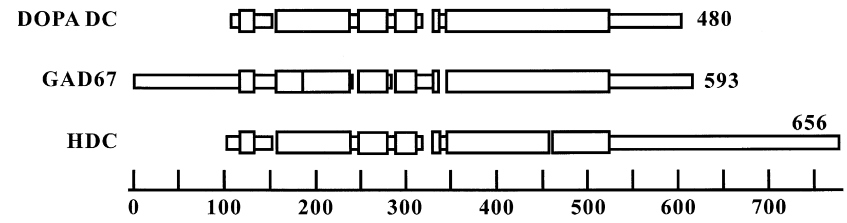

Fig. 5. Diagrammatic representation of homology comparison of three decarboxylases. DOPADC, GAD67, and HDC stand for rat DOPA (aromatic amino acid) decarboxylase, rat GAD67, and rat histidine decarboxylase, respectively. Numbers indicate the length of amino acid residues. Thick and thin boxes are as explained in Fig. 3 legend.

serves as a membrane anchor [101,102]. Extra segment at N-terminal region of GAD shows no homology with any other decarboxylases. Feline GAD67, expressed in *E. coli* and purified as a form of its N-terminal 90 residues being truncated, is fully active, which suggests that N-terminal segment may not be required for GAD activity [103]. It is further speculated that C-terminal segment of each decarboxylases may have a role for substrate recognition since very little homology is identified among the C-terminal segments of three decarboxylases (Fig. 5).

It is of interest to consider that whether or not the N-terminal segment of GAD is processed under in vivo conditions. Susceptibility of the in vitro expressed enzyme to protease digestion suggests this possibility [103]; however, in vivo processing is still uncertain. Previous attempts to purify the enzyme from brain tissues showed the subunit molecular weight is inconsistent with the expected molecular weight from DNA sequences (Table 1), which suggests a possibility of in vivo processing. Confirming the N-terminal processing may be a task since it is believed that N-terminal of GAD is blocked, probably having some post-translational modification such as acetylation. Performing protein sequencing of GAD is critically important for clarifying the N-terminal processing.

5.2. Structural consideration in connection with diabetes

As described in the previous section, GAD65 is identified as a major antigen protein targeted by autoantibody formed in patients with diabetes. Although an important question of how and why GAD causing the antibody formation is still under intense investigation, sequence homology study has suggested a possibility of virus infection. It has been known that there is an evidence of Coxsackie virus infection among patients with insulin-dependent diabetes. As a part of the coat protein coded by Coxsackie virus, an amino acid sequence ranging from 28 to 50 was found to have a unique resemblance with a part of the sequence of GAD65, 250–273, whereas corresponding range in GAD67, 258–281, has less similarity than GAD65 (Fig. 6). Whether or not GAD65 is solely required for the antibody formation is still not known. A possibility of both GAD isozymes being participants for the process remains open. Meanwhile, mutant GADs are under investigation in order to determine antibody recognition sites, or common epitopes, which information might be used for diagnostic purpose of diabetes [104,105].

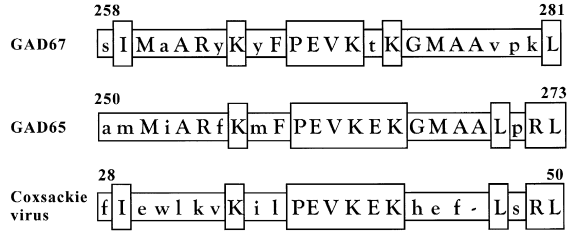

Fig. 6. Potential antigenic region of human GAD, which is homologous to Coxsackie virus sequence. Regions considered homologous to each other are shown in thick boxes and others are in thin boxes. Numbers indicate amino acid residue number.

5.3. In vitro expression of mammalian GAD

Genetic engineering techniques have allowed us to produce a sufficient quantity of mammalian GAD for biochemical characterizations. Most commonly employed was *E. coli* system, in which both isozymes were successfully expressed (Table 2). Earlier studies of recombinant enzyme from *E. coli* were mainly used to study the reactivity toward antisera of diabetic patients; thus, overall structural information was limited (for example [73]).

Spectral data was reported by Chu and Metzler [103] on a feline GAD67 expressed in *E. coli*. After purification, authors found that the subunit molecular mass was 59 kDa, which was considerably smaller than expected. After the amino acid sequencing, it was identified that N-terminal 77- to 84-amino acid residues were deleted, probably due to the proteolysis during the purification. The truncated protein was enzymatically active and subjected to physicochemical studies. The feline enzyme exhibits absorption maxima at 338 and 420 nm, both are due to the bound coenzyme, PLP. These two peaks changed the height as a pH dependent manner, giving pK_a value of 6.92. A possible structural environment around the coenzyme was proposed, where two unidentified nucleophilic residues are drawn to coordinate with 3′-phenolic OH and Schiff base nitrogen groups [103]. *E. coli* expression of GAD65 has been reported (Table 2); however, characterization of the recombinant GAD65 has not been achieved at the level of the truncated GAD67 described by Chu and Metzler.

E. coli system may be the first choice for protein expression because of its easy manipulation and large accumulated knowledge and library of strains and vectors. However, in general, it has some disadvantages: (1) N-terminal amino acid residue starts with methione in *E. coli*, which is usually cleaved off in higher animals by the post-translational processing. (2) Post-translational modification of eukaryotic system is not possible; therefore, carbohydrate attachment or palmitoylation, the latter being reported for GAD65, cannot be reproduced in *E. coli* system. (3) Folding process may be different from eukaryotic system; thus, sometimes produced proteins end up as an inclusion body because of the insolubility. In order to overcome disadvantages of *E. coli* expression system, alternative systems such as yeast and

Table 2
In vitro expression systems for GAD
(U) μmol/min; (–) unclear.

Host	Expressed form	Promoter/vector	Properties	Reference
E. coli	Feline GAD67	β-gal/λgt11	Fusion protein	[126]
	Human GAD65, GAD67	pmalc2	Fusion protein Western blot analysis	[104]
	Human GAD65	–	–	[127]
	Human GAD67	pET-21b	S.A. = 2.7 U/mg (lysate)	[128]
	Feline GAD67	T7/pET11a	59 kDa spectral studies S.A. = 3.25 U/mg (purified)	[103]
	Petunia GAD	–	S.A. = 30 U/mg (purified) K_m = 8.2 mM	[46]
S. cerevisiae	Rat GAD65	ICL	S.A. = 70 mU/mg (lysate)	[106]
Pichia methanolica	Human GAD65	AUG1/pUC	S.A. = 1.8 mU/mg (lysate)	[110]
Pichia pastoris	Human GAD65	pVL941	Western blot analysis N-terminal deletion	[112]
Sf9 insect cells	Human GAD65, GAD67	pVL1393	20–30 mU/mg (lysate)	[129]
	Human islet GAD65	pB1812	S.A. = 0.45 U/mg (purified)	[130]
	Rat GAD65	–	Spectral studies	[111]
COS-7	Rat GAD65	pSV-SPORT	Western blot analysis	[112]
	Rat GAD65	pSV-SPORT	Mutation studies	[95]
Baby hamster kidney cells	Human islet GAD65	–	S.A. = 0.15–0.18 U/mg (purified)	[113]

other eukaryotic expression systems have been attempted for GAD expression (Table 2).

Rat brain GAD65 cDNA was placed into the shuttle vector, which is under the control of isocitrate lyase (ICL) promoter [106–108]. Transformed *Saccharomyces cerevisiae*, a conventional baker's yeast, has produced soluble GAD65 when an inducer, such as glycerate or acetic acid, was added into the growth media [106]. An expressed GAD65 reacted with GAD-6, an anti-GAD65 monoclonal antibody, and migrated at the same position with GAD-6 reactive band of rat brain homogenate on SDS–PAGE. GAD activity measured for cell lysate was 70 mU/mg, nearly 70-fold increase upon induction. This *S. cerevisiae* system was successfully applied to express full length of human HDC in a soluble fraction [109]. There are several advantages in yeast expression system: (1) Like *E. coli* system, variety of vectors and host strains are becoming available. (2) Higher density of cell population (up to OD = 10–30) can be achieved in the growth media as compared to *E. coli* (OD = 1). (3) Growth conditions and gene manipulation are as simple as *E. coli* and cost effective. (4) Intracellular metabolism is similar to higher eukaryotes. The expression system developed by Kanai et al. [106] is promising for a large-scale production of GAD65, a potential diagnostic agent for diabetes. More recently, *P. methanolica* system was developed to express human GAD65 [110]. Production of GAD65 in *Pichia* is induced by methanol addition and specific activity in cell lysate was 1.8 mU/mg. The advantage of *Pichia* system is that a produced protein can be excreted into media for easy purification.

Insect expression system has been developed for both GAD65 and GAD67 (Table 2). Crude cell lysates gave 20–30 mU/mg of activity, indicating that both yeast and insect systems are comparable in producing GAD proteins. Chen et al. [111] have purified recombinant rat GAD65 from Sf9 insect cells and studied its spectral properties. Similar to feline GAD67 [103], rat GAD65 exhibits two absorption maxima at 420 and 330 nm. Spectral alteration upon PLP addition was observed on both fluorescence and circular dichroism studies of GAD65, to suggest that considerable amount of rat GAD65 exists as an apo-form. Moreover, there is a conformational change during the formation of holoenzyme. Since apo-enzyme lacks enzymatic activity, the conversion between holo- and apoenzymes may have a regulatory role on GAD65 in CNS [111].

In vitro GAD expression system in animal cells, such as COS-7 and baby hamster kidney cells, has provided an excellent model system for studying the physiological role of GAD and GABA [95,112,113]. The system is also useful to demonstrate the cellular location or membrane affinity of GAD. Although it may not be suited for large scale culturing due to the cost efficiency, it gives sufficiently pure GAD protein, 0.15–0.18 U/mg.

6. The next stage

Tertiary structure GADs from various sources should be determined, as the information would give detailed mode of interaction with substrates or substrate analogs to provide intelligent guide for developing inhibitory substances with pharmacological activity. Also, the interaction of GAD with other proteins should be explored. This will address the fundamental questions of where GAD locates, how GAD is regulated by other cellular components, how GAD participates in the GABA-packaging mechanism of microvesicles, what the life span of GAD in cellular levels is, and what components responsible for GAD degradation are. As glutamate is not only a substrate for GAD, but also acts as an excitatory neurotransmitter and for interacting with glutamate receptors, there must be separate pools in CNS. Detailed mapping of glutamate pools in CNS and its relation to GAD distribution should be clarified.

GAD distributes widely among living organisms. Information obtained from the lower or-

ganisms may provide us with some important clues to unanswered questions including physiological roles of GAD and GABA in higher organisms.

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