# Properties and Interaction of Heterologously Expressed Glutamate Decarboxylase Isoenzymes $GAD_{65kDa}$ and $GAD_{67kDa}$ from Human Brain with Ginkgotoxin and Its 5′-Phosphate

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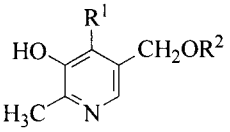
Two isoforms of glutamate decarboxylase ($GAD_{65kDa}$ and $GAD_{67kDa}$) from human brain, which had previously been overexpressed in *Escherichia coli* as fusion proteins containing a glutathione-S-transferase domain, were purified by affinity chromatography on glutathione Sepharose 4B. Both isoforms were also expressed in *Saccharomyces cerevisiae*. After modification of a HPLC based assay, the enzymes were characterized with respect to their biochemical properties. Comparison of kinetic data, pH, and temperature optima as well as of the mode of interaction with pyridoxal phosphate as a cofactor revealed several significant differences between the two isoenzymes reflecting their somewhat different physiological and molecular features. Investigation of the influence of 4′-O-methylpyridoxine (ginkgotoxin) (**1**), a neurotoxin occurring in *Ginkgo biloba* L., on the different isoenzymes, indicates that the phosphorylated form of the toxin, 4′-O-methylpyridoxine-5′-phosphate (**2**), decreases $GAD_{65kDa}$ activity, although in unphysiologically high concentrations, whereas $GAD_{67kDa}$ activity seems to be hardly affected.

## Introduction

Leaf extracts of *Ginkgo biloba* L. (Ginkgoaceae) are well-known components of remedies employed in the therapy of insufficient central and peripheral blood flow.[1,2] This beneficial effect is attributed mainly to ginkgolides and bilobalide which besides flavonoids are the prominent natural products occurring in *Ginkgo* leaves.[3−7] Nevertheless, mainly the seeds of *Ginkgo biloba* L. were shown to accumulate ginkgotoxin (4′-O-methylpyridoxine, **1**, Figure 1), an antivitamin structurally related to vitamin $B_6$.[8,9] This compound is a potent neurotoxin which, when ingested in higher concentrations, causes epileptic convulsions and paralysis of legs as cardinal symptoms.[8,10−12] The toxicity can be alleviated by vitamin $B_6$ indicating that ginkgotoxin is an antimetabolite which might interfere with amino acid metabolism in mammals. The amino acid metabolism is closely connected to the formation of $\gamma$-aminobutyric acid (GABA), a classical inhibitory neurotransmitter of the central nervous system (CNS) involved in several neurological functions. Altered GABA levels are likely to be responsible for a number of pathological conditions including, for example, Parkinson's disease, epilepsy, depression, and general motor disorders.[13] Since 4′-O-methylpyridoxine (**1**) and other convulsant drugs have been shown to reduce the GABA concentration prior to the onset of seizures,[11,14−16] any enzyme involved in the formation of the neurotransmitter might be a possible target for the toxin. Nevertheless, the rate-limiting step in GABA synthesis is the decarboxylation of glutamate catalyzed by glutamate decarboxylase (GAD; L-glutamate 1-carboxy-lyase, E.C. 4.1.1.15). Since this decarboxylation is dependent on pyridoxal-5′-phosphate (PLP, **3**, Figure 1) as a cofactor, and PLP as a $B_6$ vitamer is structurally related to ginkgotoxin, investigations designed to elucidate the biochemical mechanism of GABA decrease, induced by 4′-O-methylpyridoxine, should concentrate initially on glutamate decarboxylase.




| | $R^1$ | $R^2$ |
|---|---|---|
| (**1**) 4'-O-methylpyridoxine (ginkgotoxin) | $OCH_3$ | H |
| (**2**) 4'-O-methylpyridoxine-5'-phosphate (ginkgotoxin phosphate) | $OCH_3$ | $P_i$ |
| (**3**) pyridoxal-5'-phosphate | CHO | $P_i$ |
| (**4**) 4'-deoxypyridoxine | $CH_3$ | H |
| (**5**) 4'-deoxypyridoxine-5'-phosphate | $CH_3$ | $P_i$ |

**Figure 1.** Ginkgotoxin (**1**), pyridoxal-5′-phosphate (**3**), and derivatives.

In the adult human brain, glutamate decarboxylase has been shown to exist in two isoforms of different molecular weight, $GAD_{65kDa}$ and $GAD_{67kDa}$, encoded by two independently regulated genes located on different chromosomes.[13,17] As shown by anatomical studies, the two *gad* genes are coexpressed in most GABA-containing neurons of the CNS.[18] However, there are important differences with respect to the amount and distribution of GAD isotypes observed in the mammalian brain.[19] Although $GAD_{65kDa}$ generally appears to be the major form found in most GABA-containing neurons,[13] the intracellular distribution differs among the two isoenzymes. $GAD_{65kDa}$ is mostly associated with membranes and targeted to nerve endings while $GAD_{67kDa}$ is mainly a cytosolic enzyme widely distributed in cells.[20,21] The fact that a minor amount of $GAD_{67kDa}$ was also detected in neuronal membrane fractions can be explained by the formation of $GAD_{65kDa}$−$GAD_{67kDa}$ heteromers through interactions between the N-terminal domains of the two forms.[22]

In 1992, the cDNAs coding for $GAD_{65kDa}$ and $GAD_{67kDa}$ from human brain were first isolated, sequenced, and

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heterologously expressed in *Escherichia coli*.[17] The amino acid sequences for the two proteins deduced from the DNA data revealed an overall similarity of 81%. They were also shown to contain the tetrapeptide Asn-Pro-His-Lys, identified as the pyridoxal phosphate (**3**) binding site in porcine dihydroxyphenylalanine (DOPA) decarboxylase.[23] Another important feature, especially with respect to the data presented in this paper, is the different response of $GAD_{65kDa}$ and $GAD_{67kDa}$ to the presence of the cofactor pyridoxal-5′-phosphate (**3**). According to experiments with recombinant enzymes, $GAD_{65kDa}$ seems to be more responsive than $GAD_{67kDa}$, but the differences have not as yet been characterized.[24] Furthermore, a number of interesting data concerning the expression and physiological functions of the GAD isoenzymes from mammalian tissues have been published in recent years.[25−28] Other authors describe the influence of certain convulsants[29−31] and anticonvulsants[16,32] on GABA levels in brain tissues. According to an early report, the 4′-substituted vitamin $B_6$ derivative 4′-deoxypyridoxine (**4**, Figure 1) was shown to be a potent inhibitor of GAD.[31] However, this study was performed exclusively with tissue homogenates. Any reports dealing with the influence of putative GAD inhibitors on purified enzymes have been rare so far.

The current work concentrates on the investigation of the influence of ginkgotoxin (4′-*O*-methylpyridoxine **1**, Figure 1) and its 5′-phosphorylated derivative 4′-*O*-methylpyridoxine-5′-phosphate (**2**, Figure 1), respectively, on purified recombinant $GAD_{65kDa}$ and $GAD_{67kDa}$ from human brain. In this paper, the results from enzymological experiments characterizing the two isoenzymes with respect to their differences in kinetic data and pyridoxal phosphate (**3**, Figure 1) interaction are presented.

## Results and Discussion

**Properties of $GAD_{65kDa}$ and $GAD_{67kDa}$ Expressed in *Escherichia coli*.** Glutamate decarboxylase (GAD; L-glutamate 1-carboxy-lyase, E.C. 4.1.1.15) catalyzes the conversion of glutamate to $\gamma$-aminobutyric acid (GABA), the major known inhibitory neurotransmitter in the vertebrate brain. The human GAD was reported to exist in two isoforms, $GAD_{65kDa}$ and $GAD_{67kDa}$, which are 65% identical and share high homology with the corresponding rat and feline enzymes (96% and 97%, respectively).[17] Both enzymes had been expressed in bacterial systems as fusion proteins harboring a glutathione-*S*-transferase (GST) domaine. The fusion proteins were both shown to be enzymatically active.[17] Nevertheless, reports on the biochemical characterization of purified $GAD_{65kDa}$ and $GAD_{67kDa}$, from human or from any other vertebrate tissue, are few.

To compare $GAD_{65kDa}$ and $GAD_{67kDa}$ with respect to their biochemical and kinetic data, two different expression systems were used, the bacterial one published by Bu et al.,[17] and another one based on overexpression of the respective genes in yeast resulting in histidine-tagged fusion proteins (see Experimental Section).

In the bacterial system, overexpression of the fused genes was induced by isopropyl-$\beta$-D-galactopyranoside (IPTG), and protein was extracted from the recombinant strains. As shown by SDS polyacrylamide gel electrophoresis (SDS−PAGE), after disruption of the bacterial

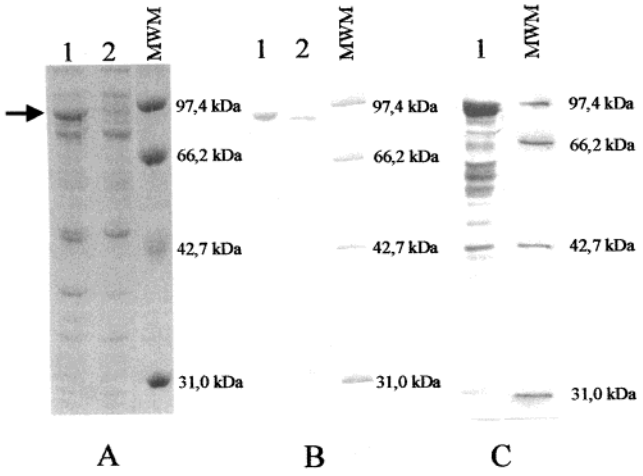


**Figure 2.** Expression of GST-GAD isoforms in *Escherichia coli*. (A) SDS−PAGE of membrane fraction (lane 1) and soluble protein fraction (lane 2) from recombinant strain JM101 (pGEX-3X-$gad_{65kDa}$) grown under standard conditions (LB-medium, 37 °C). As indicated, most of the GST-GAD fusion protein is associated with the membrane fraction (see arrow). (B) Immune reaction of crude extracts from strain JM101 containing GST-$GAD_{67kDa}$ (molecular weight of the fusion protein: 93 kDa) (lane 1) or GST-$GAD_{65kDa}$ (molecular weight of the fusion protein: 91 kDa) (lane 2) with GAD specific antibodies. The *E. coli* strain was grown under modified conditions (see Experimental Section). (C) Immune reaction of a protein fraction (20 $\mu$g) obtained after affinity chromatography, containing the GST-$GAD_{65kDa}$ fusion protein, with GAD specific antibodies (lane 1). The fraction contains several truncated proteins generated during the purification procedure. Molecular weights of standard proteins (MWM): phosphorylase b, 97 kD; albumin, 66.2 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 31 kDa.

cells grown under standard conditions (LB-medium; 37 °C), most part of GST- $GAD_{65kDa}$ (Figure 2 A, lane 1) and also of GST-$GAD_{67kDa}$ (data not shown) appeared in the membrane fraction, indicating that both isoforms, as partially eucyaryotic proteins, might form inclusion bodies in a prokaryotic system.[33] The fact that only ∼10% of a $GAD_{65kDa}$-thioredoxin fusion protein were soluble in a bacterial expression system had already been reported by Papouchado et al.[34] Thus, to improve the solubility, especially of $GAD_{65kDa}$, which in any case was shown to be associated with membranes in its native system, the growth conditions previously described by Bu et al.[17] were modified by addition of sorbitol (1 M) and betaine (2.5 mM) to the growth medium (see Experimental Section). Additionally, during growth the incubation temperature was lowered to 28 °C. With this modified protocol, appreciable levels of GAD activity could be detected in the soluble fraction of the crude cell extract. The identity of the corresponding isoforms was confirmed by Western blot analysis (Figure 2B, lanes 1 and 2).

Both GST-fusion proteins were purified by one-step affinity chromatography on glutathione agarose yielding a plain enrichment of both isoforms [250 $\mu$g/L (∼15 g cells) for GST- $GAD_{65kDa}$ and 800 $\mu$g/L (∼15 g cells) for GST-$GAD_{67kDa}$ ]. This yield is within the range described for a $GAD_{65kDa/67kDa}$ hybrid protein purified from a yeast system,[28] although it is clearly less when compared to the amount of enzyme isolated from a large scale fermentation, which yielded 360−490 mg GAD from 1000 g of cells.[33]

**Table 1.** Biochemical Properties of Recombinant GAD Isoforms Expressed in *Escherichia coli* and *Saccharomyces cerevisiae*

| | optimum pH | $pK_{S1}$ | $pK_{S2}$ | optimum temp [°C] | $K_M$ [mM] | $V_{max}$ [nmol/min] | $k_{cat}$ [$s^{-1}$] | activity without exogenous PLP [%] |
|---|---|---|---|---|---|---|---|---|
| | | | | Expression in *E. coli* | | | | |
| GST-$GAD_{65kDa}$ | 7.5 | 6.0 ± 0.4 | 8.2 ± 0.4 | 37−42 | 2.9 ± 0.21 | 17.4 | 1.8 ± 0.10 | nearly zero |
| GST-$GAD_{67kDa}$ | 6.5 | 5.5 ± 0.4 | 7.2 ± 0.3 | 43−50 | 2.5 ± 0.27 | 74.6 | 7.7 ± 0.85 | 20 |
| $GAD_{65kDa}$[a] | 7.0 | 6.1 ± 0.3 | 7.7 ± 0.4 | 39−43 | 1.3 ± 0.21 | 5.8 | 0.41 ± 0.062 | nearly zero |
| $GAD_{67kDa}$[a] | 6.5−7.0 | 6.1 ± 0.4 | 8.0 ± 0.3 | 45−50 | 1.4 ± 0.19 | 83.3 | 6.2 ± 0.87 | 32 |
| | | | | Expression in *S. cerevisiae* | | | | |
| 6xHis-$GAD_{65kDa}$ | 7.5 | 6.8 ± 0.1 | 8.2 ± 0.4 | 39−43 | 1.4 ± 0.29 | 25.7 | 1.8 ± 0.39 | nearly zero |
| 6xHis-$GAD_{67kDa}$[b] | 6.5 | 5.9 ± 0.4 | 7.3 ± 0.4 | 43−50 | 1.3 ± 0.36 | 8.8 | nd | 30 |

[a] After cleavage with protease factor Xa. [b] Properties were determined in crude extract.

However, as shown by Western blot analysis (Figure 2C, lane 1), in some cases not only one single protein of the predicted molecular weight (91 kDa for GST-$GAD_{65kDa}$ and 93 kDa for GST-$GAD_{67kDa}$) reacted with GAD specific antibodies but also a number of truncated proteins of molecular weights ranging from 26 to 67 kDa. This is again in agreement with previous attempts to purify recombinant mammalian GAD from yeast and from *E. coli* and nonrecombinant GAD from porcine brain which revealed a similar range of obviously truncated proteins,[28,33,35] indicating on one hand the occurrence of proteolysis during purification and on the other hand the coexpression of a 48-kDa byproduct from an internal translation site.[36] Both fusion proteins were then cleaved with protease factor Xa, yielding the corresponding authentic GAD-isoforms of the predicted molecular weights (65 and 67 kDa, respectively), and a smaller protein of about 26 kDa representing the GST-domain. These fractions were further employed in the studies described below.

GAD activity of crude extracts and purified enzymes was monitored by HPLC after precolumn derivatization of GABA, which was derived from decarboxylation of glutamate as substrate. Quantification of GABA was performed by relating the peak areas of GABA and of $\delta$-aminovalerianic acid (DAVA), which was added as internal standard (see Experimental Section). In some cases, GAD activity was determined by the spectrophotometric enzyme assay described below. In each case, GST fusion proteins and authentic GAD-isoforms cleaved off from the GST-domain were found to be enzymatically active after a one-step affinity chromatography on glutathione. However, a significant decrease of enzyme activity (40 to 80%) was observed after storage of the proteins for several days at −20 °C, making their characterization more difficult. This is in contrast to the finding of Davis et al., who under certain storage conditions (argon atmosphere), kept GAD enzymes for up to six months with negligible loss of activity.[33]

**A. Interaction with Pyridoxal-5′-phosphate.** Although $GAD_{65kDa}$ had been reported to be much more responsive toward the addition of pyridoxal-5′-phosphate (PLP) as a cofactor,[24] a detailed comparison of the kinetics of cofactor interaction with purified GAD isoforms is to date unpublished. To evaluate optimal assay conditions, investigation of GST- $GAD_{65kDa}$ and GST-$GAD_{67kDa}$ was initiated by determining the kinetics of GABA formation dependent on increasing amounts of PLP (**3**) (Figure 3). In contrast to GST- $GAD_{65kDa}$ which shows nearly no activity without addition of PLP, GST-$GAD_{67kDa}$ unequivocally catalyzes the conversion of glutamate to GABA under these conditions (Figure 3,

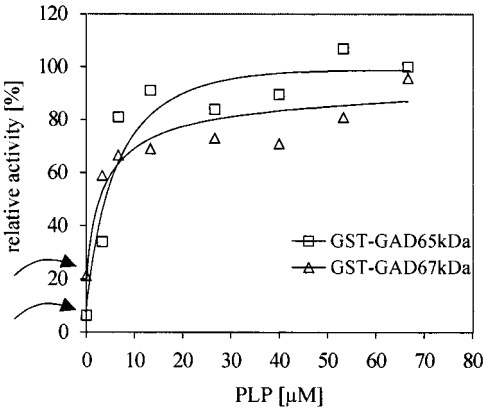


**Figure 3.** Interaction of GST-$GAD_{65kDa}$ and GST-$GAD_{67kDa}$ fusion enzymes with the cofactor pyridoxal-5′-phosphate (PLP, **3**). Arrows indicate the difference of activity between the two glutamate decarboxylase isoforms before addition of the cofactor. Each plot represents the average of three independent determinations.

Table 1). Nevertheless, since an increase of activity is observed for GST-$GAD_{67kDa}$ with increasing amounts of PLP, this enzyme seems to be still affected by addition of the cofactor. However, as shown by the constant increase of GST- $GAD_{65kDa}$ activity up to a PLP saturation of 14 $\mu$M, the dependence on PLP is much more evident for this isoform. These results are in good agreement with observations on brain extracts from which either $GAD_{65kDa}$ or $GAD_{67kDa}$ was removed by immunoprecipitation.[21] According to these studies, extracts containing $GAD_{67kDa}$ showed only a slight increase in activity when exogenous PLP was added. In contrast, addition of cofactor caused a 2.2-fold increase of activity for extracts containing only $GAD_{65kDa}$. This is in agreement with the assumption of Kaufman et al. that $GAD_{65kDa}$ activity might be regulated by supply of PLP itself, while $GAD_{67kDa}$ activity is not affected in this way.[21] This again is in agreement with results from incubations of brain extracts with $^{32}$P-labeled pyridoxal-5′-phosphate which after protein analysis revealed a strong label of the 65 kDa-isoform of GAD, whereas $GAD_{67kDa}$ was only labeled to a very small extent.[37] From this and the clear reduction of label caused by addition of the pyridoxal-5′-phosphate analogue 4′-deoxypyridoxine-5′-phosphate (**5**), it was concluded that $GAD_{65kDa}$ in contrast to $GAD_{67kDa}$ is present in a rather large amount as the apoenzyme. This apoenzyme may serve as a reservoir of inactive GAD that can be activated by the cofactor when additional GABA synthesis is required.[37]

**B. $K_M$ Values, pH, and Temperature Optima.** The temperature dependence of GST- $GAD_{65kDa}$ and GST-

$GAD_{67kDa}$ activity was studied. Determination of the optimal reaction temperature revealed a rather broad optimum for both fusion enzymes ranging from 37 to 42 °C for GST- $GAD_{65kDa}$ and from 43 to 50 °C for GST- $GAD_{67kDa}$ (Table 1). In contrast, the plots representing the dependence on pH showed a sharp optimum at 7.5 for GST- $GAD_{65kDa}$ and at 6.5 for GST- $GAD_{67kDa}$ (Table 1). The difference between the pH optima for the two isoforms may reflect the different localization of $GAD_{65kDa}$ and $GAD_{67kDa}$ with respect to the cellular compartments as well as their different distribution within the neuronal areas.

Investigations of temperature and pH optima in the fractions containing the authentic GAD isozymes cleaved off from the GST-domain revealed values in the same range as those determined for the fusion enzymes with the exception that the pH optimum of the cleaved $GAD_{67kDa}$ is broader (6.5−7.0) than that of the corresponding fusion protein (6.5) (Table 1). This demonstrates that the rather bulky GST-domain does not drastically influence the properties of the fusion enzyme.

$K_M$ values with respect to glutamate were determined as described later (see Experimental Section). With 2.9 ± 0.21 mM for GST-$GAD_{65kDa}$ and 2.5 ± 0.27 mM for GST-$GAD_{67kDa}$, the $K_M$ values for both fusion proteins were found to be not particularly low (Table 1). This was also the case for the cleaved enzymes (1.3 ± 0.21 mM for $GAD_{65kDa}$ and 1.4 ± 0.19 mM for $GAD_{67kDa}$). Nevertheless, since similar values (1.3−2.1 mM) were reported for recombinant GADα from *Escherichia coli*,[38] for GAD from cowpea (3.2 mM),[39] and for GAD from rat brain (1.59 mM),[40] the $K_M$ values determined in this study seem to be significant. Even higher values were reported for GAD from *Lactobacillus brevis* (9.3 mM)[41] and for GAD determined in rat brain homogenates (21 mM).[42] It seems reasonable that the $K_M$ values with respect to glutamic acid are not particularly low, as GABA concentration in the brain was found to be 4 to 5 times lower than that of glutamic acid, reflecting the low affinity of GAD to the substrate.[43]

**Properties of his-$GAD_{65kDa}$ and his-$GAD_{67kDa}$ Expressed in *Saccharomyces cerevisiae*.** Histidine-tagged GAD-fusion genes were constructed as described in the Experimental Section. Expression of his-$GAD_{65kDa}$ and his-$GAD_{67kDa}$ was proved by Western blot analysis with specific anti-histidine antibodies (Quiagen, Hilden, Germany). As a result of antibody reaction with the histidine residue of the fusion protein, both $GAD_{65kDa}$ and $GAD_{67kDa}$ were identified as single bands of the predicted molecular weight (data not shown). In contrast to the bacterial strain used for overexpression of GST-$GAD_{65kDa}$ and GST- $GAD_{67kDa}$, the yeast system nearly lacked any activity resulting from an internal glutamate decarboxylase. Thus, it was possible to determine enzyme activity and even some biochemical properties of the GAD isoforms without purification of the enzymes to homogeneity.

The isoforms were enriched over a nickel resin (Qiagen, Hilden, Germany), yielding approximately 1.5 mg protein/L. Determination of temperature optima for both histidine-tagged isozymes revealed them to have very similar values compared to those obtained for the GST-fusions and the respective cleaved enzymes (Table 1). In the case of his-$GAD_{65kDa}$, the range of optimal temperature was very broad (39−43 °C) and matches exactly that shown for the cleaved $GAD_{65kDa}$ (Table 1). An exact match was also observed for GST- $GAD_{67kDa}$ and his-$GAD_{67kDa}$ (43−50 °C). pH-Optima of the histidine-tagged GAD-fusions were identical to those of the GST-fusion proteins (Table 1).

The $K_M$ values (1.4 ± 0.29 mM for his-$GAD_{65kDa}$ and 1.3 ± 0.36 mM for his-$GAD_{67kDa}$) were also comparable to those determined for the enzymes obtained after cleavage of the GST- fusion proteins (Table 1).

The similarity or identity of kinetic parameters determined for the fusion proteins expressed in the different systems demonstrate that the different fusion domains do not alter the enzymatic properties of human GAD drastically. Since a protein fused to a 26 kDa GST-domain exhibits characteristics similar to those of the same protein fused to a hexameric histidine-residue, and to the authentic enzyme cleaved off from GST, the molecular weight of a fusion domain plays no important role in influencing the enzymes' properties. Thus, the possibility that a bulky residue might influence the characteristic properties of the native enzyme can be ruled out. The biochemical features of the GAD isoforms also do not seem to depend on whether they are expressed in a procaryotic or in a eucaryotic system. This is noteworthy, as one might expect posttranslational modifications of higher eucaryotic enzymes. In fact, it has been reported that the hydrophobicity of $GAD_{65kDa}$ results from two hydrophobic posttranslational modifications at its $NH_2$-terminal region, one of which consists of the thiopalmitoylation of two cysteine residues.[22] Therefore, since this thiopalmitoylation is not expected to occur in the *E. coli* system and since its possible occurrence in the yeast system still lacks any proof, slightly different kinetic parameters of GAD-isoenzymes purified from their native system, i.e., from human brain, cannot be ruled out. On the other hand, the $K_M$ values determined for several recombinant and native glutamate decarboxylases from different sources[38−42] and those presented in the current work are in the same range of molarity (see earlier). Therefore, our data should not be expected to differ markedly from those which would be derived from studies with GAD purified from human brain.

Further properties (p*K*s, $V_{max}$, and $k_{cat}$) of both isoforms expressed in *E. coli* and *S. cerevisiae* are given in Table 1.

**Influence of 4′-Substituted Derivatives of Pyridoxal Phosphate on $GAD_{65kDa}$ and $GAD_{67kDa}$ Isoenzymes.** The influence of 4′-substituted derivatives of pyridoxal-5′-phosphate (**3**) on $GAD_{65kDa}$ and $GAD_{67kDa}$ isozymes was determined by adding increasing amounts of 4′-deoxypyridoxine (DPN) (**4**), 4′-deoxypyridoxine-5′-phosphate (DPNP) (**5**), 4′-*O*-methylpyridoxine (MPN) (**1**), or 4′-*O*-methylpyridoxine-5′-phosphate (MPNP) (**2**), respectively, to the incubation mixtures described in the Experimental Section. As shown in Figure 4 B, the decrease of enzyme activity caused by addition of one of the compounds was generally low for GST- $GAD_{67kDa}$. The same was observed for cleaved $GAD_{67kDa}$ (data not shown). This reflects the high cofactor saturation of the 67 kDa-isoform of glutamate decarboxylase. However, when comparing the effect of the different compounds in detail, slight differences in the maximum decrease

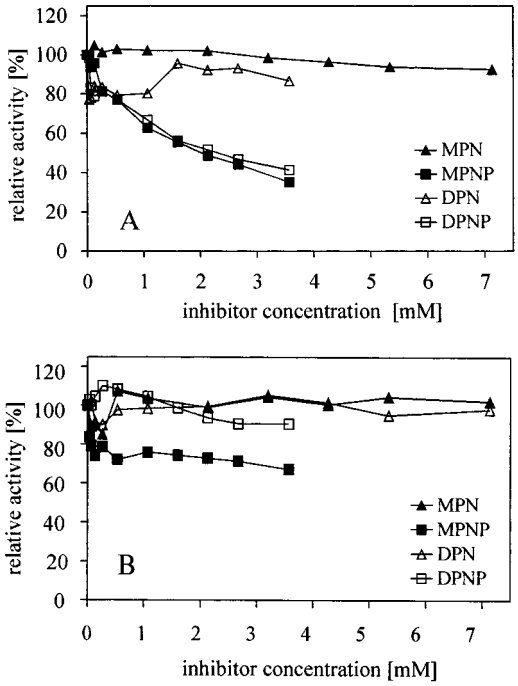


**Figure 4.** Influence of compounds structurally related to pyridoxal-5′-phosphate (**3**) on glutamate decarboxylase isoforms GST-$GAD_{65kDa}$ (A) and GST-$GAD_{67kDa}$ (B). MPN: 4′-*O*-methylpyridoxine (**1**); MPNP: 4′-O-methylpyridoxine-5′-phosphate (**2**); DPN: 4′-deoxypyridoxine (**4**); DPNP: 4′-deoxypyridoxine-5′-phosphate (**5**). Each plot represents the average of three independent determinations.

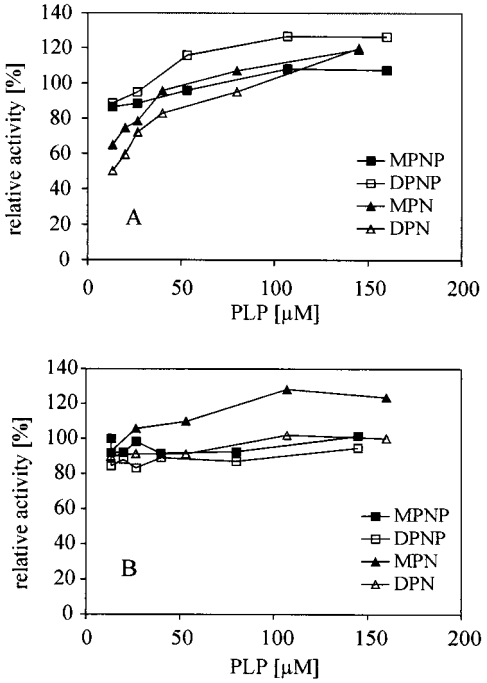


**Figure 5.** Reconstitution of GST-$GAD_{65kDa}$ (A) and GST-$GAD_{67kDa}$ (B) activity by replacement of 4′-O-methylpyridoxine (-5′-phosphate) [MPN(P), **1**, **2**] and 4′-deoxypyridoxine(−5′-phosphate) [DPN(P), **4**, **5**] with pyridoxal-5′-phosphate (PLP, **3**). Inhibitor concentrations were 1.6 mM for MPNP and DPNP and 3.2 mM for MPN and DPN. Each plot represents the average of three independent determinations.

of activity can be noted, and range from approximately 10% (for DPN, **5**) to about 30% (for MPNP, **2**) (Figure 4B). This variation is in agreement with the observation that addition of exogenous pyridoxal phosphate still causes an increase of $GAD_{67kDa}$ activity (Figure 3) and demonstrates that the enzyme is not completely saturated with cofactor.

While the effect caused by the nonphosphorylated vitamin $B_6$ analogues was generally low, an unambiguous decrease of activity was observed when 4′-deoxypyridoxine-5′-phosphate (**5**) or 4′-*O*-methylpyridoxine-5′-phosphate (**2**) were added to the incubation mixtures containing $GAD_{65kDa}$ or GST-$GAD_{65kDa}$-isoforms. GST-$GAD_{65kDa}$ activity was diminished down to about 40% of the initial activity (which was set to 100%) by DPNP (**5**) (3.5 mM) and down to about 35% of the initial activity by MPNP (**2**) (3.5 mM) (Figure 4A). The $IC_{50}$ values for inhibition of GST-$GAD_{65kDa}$ were calculated to be 2.4 mM for DPNP (**5**) and 2.7 mM for MPNP (**2**). Enzyme activity could be restored by subsequent addition of pyridoxal phosphate (**3**) and even increased up to 120%, when high PLP (**3**) concentrations were applied to either GST-$GAD_{65kDa}$ or GST-$GAD_{67kDa}$ treated with the different inhibitors (Figure 5A,B). According to these results the phosphorylated forms of 4′-substituted pyridoxal derivatives seem to decrease the activity of $GAD_{65kDa}$ or GST-$GAD_{65kDa}$-isoforms, at least in high concentrations, while the nonphosphorylated forms do not inhibit the enzymes significantly.

Since bilobalide, one of the well-known constituents of *Ginkgo biloba* extracts, has been reported to possess inhibitory effect against 4′-*O*-methylpyridoxine-induced convulsions in vivo,[16] this sesquiterpene was employed in the investigations concerning the effect of 4′-substituted pyridoxine derivatives on glutamate decarboxylase. At low concentrations it did not show any effect on GAD isoforms treated with the inhibitors, at unphysiologically high concentrations (90 mM) it even inhibited GAD activity (data not shown). This finding coincides with results from previous investigations which found that, after administration of bilobalide, a potentiation of glutamate decarboxylase activity was observed in vivo, but, as shown by negative results of in vitro experiments with brain homogenates, this appeared to be a secondary effect rather than a direct action of bilobalide on the enzyme.[32]

Summarizing the results from the inhibition studies discussed above: The phosphorylated form of ginkgotoxin (**1**), 4′-*O*-methylpyridoxine-5′-phosphate (**2**), inhibits the activity of especially $GAD_{65kDa}$. Since the decrease of GAD activity is reversible by addition of the coenzyme pyridoxal-5′-phosphate (**3**) (Figure 5A), the type of inhibition is best described as being competitive. However, the concentrations of MPNP (**2**), which caused a clear inhibition, were in the range 0.5−3.5 mM. It seems very unlikely that these high concentrations are physiologically significant, for the MPN (**1**) concentration determined in the serum of a patient 8.5 h after ingestion of about 50 *Ginkgo* seeds was only 0.09 $\mu$g/mL (i.e., about 0.5 $\mu$M)[10] and may be even lower in the different compartments of the brain. Thus, a direct inhibition of glutamate decarboxylase by MPN (**1**) or MPNP (**2**) causing a decrease in the cerebral inhibitor GABA, and insofar triggering the typical effects of MPN (**1**) intoxification like epileptic convulsions, can most likely be ruled out. In this context it is remarkable that the GABA content of the brain might not be the decisive factor in the development of seizures induced by struc-

tural analogues of vitamin $B_6$, because convulsions have been found to occur with decreased as well as with increased concentrations of brain GABA.[43] More likely is an altered turnover of the brain specific glutamate-GABA shunt, which participates to about 40% in the oxidative metabolism of the brain, as the cause of diminished oxygen consumption in the brain leading to the symptoms of induced vitamin $B_6$ deficiency. This alteration can be caused not only by inhibition of GAD isoenzymes but also by inhibition of another pyridoxal-5-phosphate dependent enzyme of the brain, GABA transaminase, which catalyzes the transamination between GABA and α-ketoglutaric acid to form succinic semialdehyde, and in so doing also participates in the regulation of the shunt.[43]

Whereas the latter consideration is pure speculation, it is more likely that enzymes involved directly in the biogenesis of pyridoxal-5′-phosphate (**3**) are possible targets for MPN (**1**) or MPNP (**2**). An inhibition of pyridoxine/pyridoxamine-5′-phosphate oxidase or of pyridoxal kinase, both of which catalyze the formation of pyridoxal-5′-phosphate (**3**) in the brain, would lead to a diminished level of cofactor and to a reduction of GAD activity or GABA transaminase activity. An inhibition by 4′-deoxypyridoxine-5′-phosphate (**5**) in higher concentrations (200 $\mu$M−1 mM) has already been shown for pyridoxine-5′-phosphate oxidase from rabbit liver,[44] and by 4′-deoxypyridoxine (**4**) and 4′-*O*-methylpyridoxine (**1**) for pyridoxal kinase from mouse brain,[11] as well as by 4′-deoxypyridoxine (**4**) for human pyridoxal kinase.[45] These enzymes may be useful subjects for further investigating the influence of ginkgotoxin (**1**) in the amino acid metabolism of man. Interestingly, the failure of deoxypyridoxine to reduce pyridoxal phosphate concentrations in several mammalian tissues significantly suggests that the in vivo mechanism of at least this compound may involve more than simple competitive inhibition of enzymes.[46] A comparison of these data with those which will be obtained from further enzymatic in vitro studies with ginkgotoxin should contribute to clear up the role of pyridoxal phosphate analogues in the mammalian metabolism.

## Conclusions

Former in vivo experiments and in vitro studies with brain homogenates demonstrated an induction of central nervous seizures by 4′-*O*-methylpyridoxine (**1**) with a simultaneous decrease of GABA concentration in the examined brain compartments.[47] These findings led to the hypothesis that pyridoxal-5′-phosphate (**3**) dependent glutamate decarboxylase isoenzymes may be possible targets for the toxin. Since 4′-*O*-methylpyridoxine (**1**) and the corresponding 5′-phosphorylated form (**2**) are structurally related to pyridoxal phosphate (**3**), a competitive inhibition of the GAD isoforms seemed reasonable. Indeed, in the current study with purified enzymes inhibition was shown, mainly of $GAD_{65kDa}$ by the phosphorylated form of ginkgotoxin (**1**). However, since this inhibition was only observed with nonphysiologically high concentrations, a direct influence of the toxin on glutamate decarboxylase can probably be excluded. Possibly other enzymes involved in the GABA shunt of the brain or enzymes directly involved in pyridoxal phosphate (**3**) biosynthesis may be influenced by ginkgotoxin or its phosphate.

**Table 2.** $^1$H ($D_2O$ with 5% $CD_3OD$, 300 MHz [ref. $D_2O$: $\delta$ 4.79]) and $^{13}$C NMR ($D_2O$ with 5% $CD_3OD$, 75.5 MHz [ref. $CD_3OD$: $\delta$ 49.0]) Data for 4′-*O*-Methylpyridoxine-5′-phosphate[a]

| position | $\delta$ $^{13}$C ppm | $\delta$ $^1$H ppm |
|---|---|---|
| 2 | 144.4 (s)[b] | |
| 3 | 153.3 (s) | |
| 4 | 139.5 (s) | |
| 5 | 136.0 (ds)[c] $J_{^{13}C-^{31}P} = 7.5$ Hz | |
| 6 | 131.2 (d) | 8.17 (1H, s) |
| *O*-methyl | 59.7 (q) | 3.43 (3H, s) |
| 2′ | 15.5 (q) | 2.59 (3H, s) |
| 4′ | 67.0 (t) | 4.80 (2H, d, $J = 8.1$ Hz) |
| 5′ | 62.4 (dt[d] $J_{^{13}C-^{31}P} = 4.6$ Hz) | 5.01 (2H, d, $J = 7.3$ Hz) |

[a] All assignments are based on extensive 1D and 2D NMR experiments (HMQC, HMBC). [b] Implied multiplicities by DEPT (C = s, CH = d, $CH_2$ = t, $CH_3$ = q). [c] d from coupling with $^{31}$P, s from coupling with $^1$H. [d] d from coupling with $^{31}$P, t from coupling with $^1$H.

## Experimental Section

**Analytical Material and Methods.** $^1$H, $^{13}$C, and $^{31}$P spectra were recorded on a Bruker DPX-NMR-spectrometer (300 MHz) in appropriately deuterated solvents as indicated in the procedure (see later). The melting point of 4′-*O*-methylpyridoxine-5′-phosphate (**2**) is uncorrected. HPLC separations were conducted using a Merck-Hitachi L-6200A HPLC apparatus (Merck-Darmstadt), connected to a fluorescence detector [Shimadzu RF551 (Shimadzu, Kyoto, Japan) set to 330 nm (EX) and 440 nm (EM)]. Samples were separated on a reversed phase column (Nucleosil 100 $C_{18}$ AB; 250 mm × 4 mm; Macherey-Nagel, Düren, Germany) with a guard column (Nucleosil 100 $C_{18}$ AB; 30 × 4 mm; Macherey-Nagel, Düren, Germany ) at a flow rate of 1 mL/min. As eluent 78−80% of solvent A (0.1 M sodium phosphate buffer, pH 5.5; 100 mg/L EDTA) were mixed with 20−22% of solvent B (100% acetonitrile). Samples were evaluated with Nelson 3000, model 2000, revision 5.1 (Perkin-Elmer, Norwalk, USA) and Eurochrom 2000 Integration (Knauer, Berlin, Germany) software packages. Spectrophotometric assays were performed on a UV single beam mini-spectrophotometer (Shimadzu, Kyoto, Japan).

**Synthesis of 4′-*O*-Methylpyridoxine-5′-Phosphate (2).** The synthesis of 4′-*O*-methylpyridoxine-5′-phosphate (**2**) modified after a previously reported procedure[11] was carried out starting from 300 mg of 4′-*O*-methylpyridoxine (ginkgotoxin, **1**) in a mixture (w/w) of one part phosphorus pentoxide (6.5 g) and 1.3 parts of 85% phosphoric acid (5 mL). After stirring for 3.5 h at 60 °C, 25 mL of water were added. This mixture was then refluxed for 35 min. The progress of the reaction was controlled by thin-layer chromatography (TLC). After cooling, the solution was mixed with 5 g of charcoal and stirred for a further 12 h. The charcoal was removed by filtration and washed with 50% ethanol until the eluent was devoid of any phosphate. When phosphate was present, the violet color of a spot of eluent sprayed with sulfosalicyl-$FeCl_3$ reagent on a TLC plate should fade. Subsequently the product was eluted from the charcoal with ethanol (100%). After freeze-drying, the ethanol fraction was obtained as a white crystalline material. The identity of the product was confirmed by $^1$H, $^{13}$C (in $D_2O$, 10% deuteriomethanol), and $^{31}$P (in DMSO) NMR spectra as well as by elemental analysis (found: C 41.94, H 5.59, N 5.55%; calculated for $C_9H_{14}NO_6P$: C 41.07, H 5.36, N 5.34%). The melting point was determined to be 216−218 °C as is in agreement with literature values (215−217 °C).[11] NMR data were not given previously and are listed in Table 2.

**Growth of Recombinant *Escherichia coli* Strains.** For overexpression of *gad* isogenes, *E. coli* strain JM101 [*F′ traD36 lacI$^q$ Δ (lac Z)M15 pro A$^+$ B$^+$/supE thi Δ(lac-proAB)*] (New England Biolabs, Beverly, MA) was transformed with vector

pGEX-3X (Amersham-Pharmacia, Freiburg, Germany) containing the coding regions of either $gad_{65kDa}$ or $gad_{67kDa}$, each fused to a gene coding for glutathione-S-transferase.[17] For growth of each recombinant strain, 1 L of LB-medium (10.0 g tryptone, 10.0 g NaCl, 5.0 g yeast extract) supplemented with sorbitol (1 M), betaine (2.5 mM), and ampicillin (100 $\mu$g/mL) was inoculated with 5 mL of a stationary preculture grown in LB/ampicillin(100 $\mu$g/mL). Incubation of the main cultures was carried out at 28 °C in an Infors shaker (180 rpm) until the $OD_{600}$ reached a value of 0.2. Isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) (final concentration 0.2 mM) was then added to induce the expression of *gst-gad* fusion genes. After further 12−18 h cells were collected by centrifugation. Cell pellets (15 g wet weight) were resuspended in 5−10 mL of cold PBS buffer (0.14 M NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3) containing 1% (v/v) Triton X-100 and 10 mM 2-aminoethylisothiouronium bromide (AET) and subsequently frozen at −20 °C for at least 2 h.

**Purification of GST-$GAD_{65kDa}$ and GST-$GAD_{67kDa}$ Fusion Proteins.** The frozen bacterial cells harboring either GST- $GAD_{65kDa}$ or GST- $GAD_{67kDa}$ were thawed in a cold water bath before ultrasonic treatment (Branson sonifier, Danbury, USA, 10 times, 10 s, 50% output at stage 5). After sedimentation of the cell debris (30 min, 11000*g*, 4 °C) supernatant was passed twice over a column (15 cm × 1 cm; 1 mL/min) packed with 3 mL gluthathione-agarose (Pharmacia, Freiburg, Germany) equilibrated with 8 column volumes of PBS buffer. The column was washed with 10−15 column volumes of PBS buffer (specified above), and the fusion proteins were eluted with 50 mM Tris-HCl (pH 8.0) buffer containing 10 mM reduced glutathione. The eluent containing the fusion proteins was optionally concentrated over an Amicon cell ('8050'-cell, Amicon, Witten, Germany, with filter 'Omega Filter 15 K', Filtron) to a final concentration of 0.3 $\mu$g/$\mu$L (for GST-$GAD_{65kDa}$) or 1 $\mu$g/$\mu$L (for GST-$GAD_{67kDa}$). Enrichment of fusion protein was controlled by SDS−PAGE.[48]

To obtain GAD isoenzymes separated from the glutathione S-transferase domain, GST- $GAD_{65kDa}$ and GST- $GAD_{67kDa}$ fusion proteins were cleaved with 1 $\mu$g of protease factor Xa per 50 $\mu$g fusion protein supplemented with 0.1 volumes of factor Xa-buffer (10 mM Tris-HCl, 5 mM $CaCl_2$, 10 mM NaCl, pH 8.0) for 2−4 h at 4 °C. After determination of the protein concentration,[49] and confirmation by SDS−PAGE[48] that the cleavage was complete, the mixture was employed in further incubations.

**Construction and Growth of Recombinant *Saccharomyces cerevisiae* Strains.** The BamHI fragments of vector pGEX-3X[17] containing the reading frames for either $GAD_{65kDa}$ or $GAD_{67kDa}$ were isolated and treated with Klenow fragment (Roche, Mannheim, Germany) to generate blunt ends. Both fragments subsequently were each ligated into vector pQE (Qiagen, Hilden, Germany) cleaved with SmaI (New England Biolabs, Schwalbach, Germany) and treated with shrimp alkaline phosphatase (United States Biochemicals, Bad Homburg, Germany). The recombinant vectors pQEKBA (containing $GAD_{65kDa}$ ) and pQEKBB (containing $GAD_{67kDa}$) were checked for correct construction by restriction analysis. For transformation of a *Saccharomyces cerevisiae* strain (Mat α, leu2−3, 112 ura3−52 his3-trp1-lys2−801 suc2−9) (Qiagen, Hilden, Germany) yeast cells were incubated overnight in YP-glucose-medium (20 g glucose, 10 g bacto yeast extract 20 g bacto peptone/L). 0.5 mL of the overnight culture were transferred into 50 mL of fresh warm medium and were further grown up to an $OD_{600}$ of 0.5−0.7. After sedimentation of the cells by centrifugation, they were washed with sterile water. After further sedimentation, they were resuspended in 1 mL of sterile distilled water, transferred to a microtube and again centrifuged for 5 min. The cell pellet was then washed with 1 mL of TE-buffer (100 mM Tris/HCl, pH 7.5; 1 mM EDTA) containing LiAc (100 mM). After sedimentation, the cells were resuspended in 1 mL of the same buffer. After addition of 100 $\mu$L glycerol (100%), the cell suspension was divided into aliquots of 50 $\mu$L and frozen at −70 °C. For transformation, the cells were thawed on ice and mixed with 1 $\mu$g DNA and 50 $\mu$g herring sperm DNA, 300 $\mu$L of sterile PEG 4000 (40%) in TE-buffer were then added. The resultant suspension was mixed and incubated for 30 min at 30 °C. Addition of 35 $\mu$L of DMSO prior to heat shock increases the number of transformants. Heatshock was performed for 6 min at 42 °C. The cells were subsequently chilled on ice for 1 min and centrifuged for 5 s. The pellet was resuspended in 0.2−1 mL of TE-buffer, and 200 $\mu$L of the cell suspension was distributed on selective agar plates.

For expression, 50 mL of YP-glucose-medium was inoculated with 1 mL of a stationary preculture of the recombinant yeast strain. After vigorous shaking for 48 h at 30 °C, the glucose concentration was determined. If glucose concentration was 0.2 mM or less, cells were centrifuged at 2500*g* at room temperature. The cell pellet was resuspended in 200 mL of YP galactose medium (20 g galactose, 10 g bacto yeast extract, 20 g bacto peptone/L) and further shaken for 48 h at 30 °C. After harvest of the cells by centrifugation, the pellets may be stored at −20 °C or −70 °C.

**Purification of his- $GAD_{65kDa}$ and his-$GAD_{67kDa}$ Fusion Proteins.** Preparation of cell free crude extracts from cell pellets obtained from a 50 mL culture of the recombinant *Saccharomyces cerevisiae* strain harboring the his-GAD fusion genes was performed according to the supplier's recommendation for protein purification with Ni−NTA-agarose (Dr. F. Schäfer, Qiagen, Hilden, Germany, personal communication). Cells were disrupted enzymatically with zymolyase (Lyticase, Sigma-Aldrich, Deisenhofen, Germany) (1 mg/g fresh weight) and osmolysis of the resulting spheroplasts. The crude extract was incubated for 1 h under smooth shaking with 1−2 mL of Ni−NTA-agarose (Qiagen, Hilden, Germany). The suspension was filled into a small column cartridge (5 mm × 10 cm) and was washed 4 times with washbuffer (50 mM sodium phosphate, pH 7.5, 300 mM NaCl, 50 mM imidazole) containing 50 mM imidazole. Subsequently, the bound protein was eluted in 4 fractions with 1 mL of the same buffer containing 250 mM imidazole each, and the protein content was determined.[49] The eluent was further concentrated by ultrafiltration in centricon tubes (Centriplus 50, Amicon, Inc., Beverly, MA, USA; 50 kDa exclusion mass), if the protein concentration was below 0.2 $\mu$g/$\mu$L.

**Immuno Blotting.** Immuno blotting was performed according to the manufacturer's recommendation using a Mini Trans Blot Electrophoretic Transfer Cell (Biorad, Muenchen). After equilibration of the SDS gel in transfer buffer (25 mM Tris, 192 mM glycine, 20% [v/v] MeOH) the proteins were transferred overnight onto a polyvinylidene fluoride membrane (Roti-PVDF, Roth, Karlsruhe, Germany) at 30 V. The membrane was then washed twice for 10 min with TBS-buffer (10 mM Tris/HCl pH 7.5; 150 mM NaCl) and was subsequently incubated in blocking solution (TBS-buffer pH 7.5 containing 3% bovine serum albumine) for at least 1 h. After washing of the membrane twice for 10 min with TBS buffer, a dilution (1: 5000) of the polyclonal anti-hGAD-antibody from rabbit (1−2 mg/mL) (Chemicon, Hofheim, Germany) or of the monoclonal anti-penta-his-antibody from mice (1−2 mg/mL) (Qiagen, Hilden, Germany) was added to the blocking solution. After incubation for at least 1 h and subsequent washing with TBS/Triton-X100- buffer (twice for 10 min) and with TBS-buffer (10 min), the membrane was incubated for 1 h with anti-rabbit-alkaline-phosphatase conjugate (Roche, Mannheim, Germany) or with anti-mouse-alkaline-phosphatase conjugate (Chemicon, Hofheim, Germany), respectively. The alkaline-phosphatase conjugates were diluted 1:10000 in 15 mL of blocking solution. The membrane was then washed 4 times for 10 min with TBS/Triton-X100-buffer and subsequently equilibrated in detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl). Finally 10 mL of detection buffer mixed with 80 $\mu$L of a solution containing 4-nitroblue-tetrazolium-chloride/5-bromo-4-chloro-3-indolyle-phosphate (NBT/BCIP) (Roche, Mannheim, Germany) was added to the membrane, which was incubated for 10 to 20 min in the dark. After complete development of the alkaline-phosphatase reaction, the membrane was rinsed in water and dried at 40 °C

**Enzyme Incubations.** All enzyme incubations carried out in order to convert glutamate to γ-aminobutyric acid were performed in 0.5 M sodium-phosphate buffer. The pH was adjusted to the pH-optimum of the respective isoenzyme (Table 1). The total volume of the incubation samples was 150 μL containing 3.5 (for inhibition studies) to 4 mM glutamate (Fluka, Buchs, Switzerland), 13.3 μM pyridoxal-5′-phosphate (**3**) (Fluka, Buchs, Switzerland), and 10 μg of the respective fusion protein. $K_M$ values were determined according to Lineweaver−Burk plots with concentrations of glutamate ranging from 0.07 to 7 mM. Regression lines were calculated using software "Microsoft Excel 1997". To guarantee optimal stability, incubations were performed at the lowest optimal temperature within the range of the broad temperature optima determined for both isoenzymes. The incubation period varied between 10 and 20 min depending on the concentration of substrate, but in any case the turnover of enzyme per minute was proved to be constant. Incubations were stopped by denaturing the enzymes for 10 min in a boiling water bath. All incubations were generally performed in triplicate.

**Determination of GAD Activity. HPLC Assay.** The activity of GST-GAD fusion and GAD enzymes was mainly determined by high performance liquid chromatography (HPLC) following a modified method of the one described by Lasley et al.[50] and by Chakraborty.[51] This method was based on the precolumn derivatization of γ-aminobutyric acid (GABA) which reacts with *o*-phthaldialdehyde (OPA) reagent to form a fluorescent compound. GABA which was derived from enzymatic conversion of glutamate in the preceding incubations, was quantified by adding to the samples an internal standard, δ-aminovalerianic acid (40 nM) (Fluka, Buchs, Switzerland), which also gives a fluorescent compound after derivatization. Precolumn derivatization was performed by addition of 50 μL of OPA reagent (13.5 mg OPA [Fluka, Buchs, Switzerland]) dissolved in 0.5 mL of ethanol and supplied with 25 μL of β-mercaptoethanol (99%) and by addition of 5 mL of 0.1 M sodium-borate buffer, pH 10.5) to the DAVA and GABA containing samples. After incubation for 90 s at room temperature, the samples were injected immediately into the HPLC apparatus. After approximately 20 min, DAVA and GABA were completely eluted. Specification of the HPLC apparatus and solvent composition are described earlier (vide supra).

**Spectrophotometric Assay.** A coupled enzyme assay was employed to confirm the identity of GABA determined by the HPLC-method described above. This assay was based on the conversion of GABA and α-ketoglutarate to glutamate and succinic semialdehyde catalyzed by GABA transaminase and the subsequent oxidation of succinic semialdehyde by NADP-dependent succinic semialdehyde dehydrogenase. In this assay, which was performed according to the published method of Zhang and Bown,[52] GABA values were related to the increase in absorbance due to NADPH production. Although the determination of GABA by HPLC is much more sensitive compared to the spectrophotometric method, because of its high specificity the coupled enzyme assay provided a useful confirmation of the values obtained by HPLC.

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## References

(1) Oberpichler-Schwenk, H.; Krieglstein, J. Pharmakologische Wirkungen von Ginkgo biloba-Extrakt und- Inhaltsstoffen. *Pharm. Unserer Zeit* **1992**, *21* (5), 224−235.
(2) Herrschaft, H. Zur klinischen Anwendung von Ginkgo biloba bei dementiellen Syndromen. *Pharm. Unserer Zeit* **1992**, *21* (6), 266−275.
(3) Tang, W.; Eisenbrand, G. Chinese Drugs of Plant Origin. *Chemistry, Pharmacology, and Use in Traditional and Modern Medicine*; Springer-Verlag: Berlin, 1992; pp 555−565.
(4) Okabe, K.; Yamada, S.; Yanamura, S.; Takada, S. Ginkgolides. *J. Chem. Soc. (London)* **1967**, *(C)*, 2201−2206.
(5) Weinges, K.; Hepp, M.; Yaggy, H. Isolierung und Strukturaufklärung eines neuen Ginkgolids. *Liebigs Ann. Chem.* **1987**, 521−526.
(6) van Beek, T. A.; Lelyveld, G. P. Concentration of Ginkgolides and Bilobalide in Ginkgo biloba Leaves in Relation to The Time of the Year. *Planta Med.* **1992**, *58*, 413−416.
(7) Briancon-Scheid, F.; Lobstein-Guth, A.; Anton, R. HPLC Separation and Quantitative Determination of Biflavones in Leaves from Ginkgo biloba. *Planta Med.* **1983**, *49*, 204−207.
(8) Wada, K.; Ishigaki, S.; Ueda, K.; Sakata, M.; Haga, M. An Antivitamin B6, 4′-O-Methylpyridoxine, from the Seed of *Ginkgo biloba* L. *Chem. Pharm. Bull.* **1985**, *33*, 3555−3557.
(9) Arenz, A.; Klein, M.; Fiehe, K.; Gross, J.; Drewke, C.; Hemscheidt, T.; Leistner, E. Occurrence of Neurotoxic 4′-O-Methylpyridoxine in *Ginkgo biloba* leaves, *Ginkgo* Medications and Japanese *Ginkgo* food. *Planta Med.* **1996**, *62*, 548−551.
(10) Yagi, M.; Wada, K.; Sakata, M.; Kokubo, M.; Haga, M. Studies on the Constituents of Edible and Medicinal Plants, IV, Determination of 4′-O-Methylpyridoxine in Serum of the Patient with Gin-nan Food Poisoning. *Yakugaku Zasshi* **1993**, *113*, 596−599.
(11) Mizuno, N.; Kawakami, K.; Morita, E., J. Competitive Inhibition Between 4′-Substituted Pyridoxine Analogues and Pyridoxal for Pyridoxal Kinase from Mouse Brain. *J. Nutr. Sci. Vitaminol.* **1980**, *26*, 535−543.
(12) Yanai, A.; Minami, M.; Takano, Y.; Endo, T.; Hamaue, M.; Wada, K.; Take, Y.; Haga, M.; Morii, K.; Togashi, H.; Yoshioka, M.; Saito, H. 4′-O-Methylpyridoxine-Induced Convulsion in Guinea Pigs and Rats. *Life Sci. Adv.* **1990**, *107*, 1499−1500.
(13) Soghomonian, J.-J.; Martin, D. L. Two Isoforms of Glutamate Decarboxylase: Why? *Trends Pharm. Sci.* **1998**, *19*, 500−505.
(14) Haug, P.; Nitsch, C. Increase in Taurine Content Before Onset of Seizures Induced by a Glutamate Decarboxylase Inhibitor. *Exp. Brain Res.* **1982**, *48*, 463−466.
(15) Horton, R. W.; Chapman, A. G.; Meldrum, B. S. The Convulsant Action of Hydrazides and Regional Changes in Cerebral γ-Aminobutyric Acid and Pyridoxal Phosphate Concentrations. *J. Neurochem.* **1979**, *33*, 745−749.
(16) Sasaki, K.; Hatta, S.; Wada, K.; Ohshika, H.; Haga, M. Anticonvulsant Activity of Bilobalide, a Sesquiterpene in *Ginkgo biloba* L. Leaves, Against Chemical-Induced and Electroshock-Induced Convulsions in Mice. *Res. Commun. Biol. Psychol. Psychiatry* **1995**, *20* (3, 4), 145−156.
(17) Bu, D.-F.; Erlander, M. G.; Hitz, B. C.; Tillakaratne, N. J. K.; Kaufman, D. L.; Wagner- McPherson, C. B.; Evans, G. A.; Tobin, A. J. Two Human Glutamate Decarboxylases, 65-kDa and 67-kDa GAD, Are Each Encoded by a Single Gene. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 2115−2119.
(18) Esclapez, M.; Tillakaratne, N. J. K.; Kaufman, D. L.; Tobin, A. J.; Houser, C. R. Comparative Localization of Two Forms of Glutamic Acid Decarboxylase and Their mRNAs in Rat Brain Supports the Concept of Functional Differences Between the Forms. *J. Neurosci.* **1994**, *14*, 1834−1855.
(19) Feldblum, S.; Erlander, M. G.; Tobin, A. J. Different Distributions of GAD65 and GAD67 mRNAs Suggest That the Two Glutamate Decarboxylases Play Distinctive Functional Roles. *J. Neurosci. Res.* **1993**, *34*, 689−706.
(20) Namchuk, M.; Lindsay, L.; Turck, C. W.; Kanaani, J.; Baekkeskov, S. Phosphorylation of Serine Residues 2, 6, 10 and 13 Distinguishes Membrane Anchored from Soluble Glutamic Acid Decarboxylase 65 and is Restricted to Glutamic Acid Decarboxylase 65alpha. *J. Biol. Chem.* **1997**, *272*, 1548−1557.
(21) Kaufman, D. L.; Houser, C. R.; Tobin, A. J. Two Forms of the γ-Aminobutyric Acid Synthetic Enzyme Glutamate Decarboxylase Have Distinct Intraneuronal Distributions and Cofactor Interactions. *J. Neurochem.* **1991**, *56*, 720−723.

(22) Dirkx, R.; Thomas, A.; Li, L.; Lernmark, A.; Sherwin, R. S.; De Camilli, P.; Solimena, M. Targeting of the 67-kDa Isoform of Glutamic Acid Decarboxylase to Intracellular Organells is Mediated by its Interaction With the $NH_2$−Terminal Region of the 65-kDa Isoform of Glutamic Acid Decarboxylase. *J. Biol. Chem*. **1995**, *270*, 2241−2246.
(23) Bossa, F.; Martini, F.; Barra, D.; Voltattorni, C.; Minelli, A.; Turano, C. The Chymotryptic Phosphopyridoxyl Peptide of DOPA Decarboxylase from Pig Kidney. *Biochem. Biophys. Res. Commun*. **1977**, *78*, 177−184.
(24) Erlander, M. G.; Tillakaratne, N. J. K.; Feldblum, S.; Patel, N.; Tobin, A. Two Genes Encode Distinct Glutamate Decarboxylases. *Neuron* **1991**, *7*, 91−100.
(25) McCarthy, M. M.; Kaufman, L. C.; Brooks, P. J.; Pfaff, D. W.; Schwartz, Giblin, S. Estrogen Modulation of mRNA Levels for the Lwo Forms of Glutamic Acid Decarboxylase (GAD) in Female Rat Brain. *J. Comput. Neurol*. **1995**, *360*, 685−697.
(26) Mally, M.; Cirulli, V.; Otonkoski, T.; Soto, G.; Hayek, A. Ontogeny and Tissue Distribution of Human GAD Expression. *Diabetes* **1996**, *45*, 496−501.
(27) Williamson, S.; Faulkner-Jones, B. E.; Cram, D. S.; Furness, J. B.; Harrison, L. C. Transcription and Translation of Two Glutamate Decarboxylase Genes in the Ileum of Rat, Mouse and Guinea pig. *J. Auton. Nerv. Syst*. **1995**, *55*, 18−28.
(28) Law, R. H. P.; Rowley, M. J.; Mackay, I. R.; Corner, B. Expression in *Saccharomyces cerevisiae* of Antigenically and Enzymatically Active Recombinant Glutamic Acid Decarboxylase. *J. Biotechnol*. **1998**, *61*, 57−68.
(29) Katoh, J.; Taniguchi, H.; Ogura, M.; Kasuga, M.; Okada, Y. A. Convulsant, 3-Mercaptopropionic Acid, Decreases the Level of GABA and GAD in Rat Pancreatic Islets and Brain. *Experientia* **1995**, *51*, 217−219.
(30) Diaz-Munoz, M.; Tapia, R. Glutamate Decarboxylase Inhibition and Vitamin B6 Metabolism in Brain of Cirrhotic Rats Chronically Treated with Carbon Tetrachloride. *J. Neurosci. Res*. **1988**, *29*, 376−382.
(31) Lindgren, S.; Anden, N.-E. On the Use of Enzyme Inhibitors to Study the Synthesis and Utilization of Brain GABA. *Acta Pharmacol. Toxicol*. **1984**, *55*, 41−49.
(32) Sasaki, K.; Hatta, S.; Haga, M.; Ohshika, H. Effects of Bilobalide on $\gamma$-Aminobutyric Acid Levels and Glutamic Acid Decarboxylase in Mouse Brain. *Eur. J. Pharmacol*. **1999**, *367*, 165−173.
(33) Davis, K. M.; Foos, T.; Bates, C. S.; Tucker, E.; Hsu, C. C.; Chen, W.; Jin, H.; Tyburski, J. B.; Schloss, J. V.; Tobin, A. J.; Wu, J.-Y. A novel method for expression and large-scale production of human brain L-glutamate decarboxylase. *Biochem. Biophys. Res Commun*. **2000**, *267*, 777−782.
(34) Papouchado, M. L.; Valdez, S. N.; Ghiringhelli, D.; Poskus, E.; Ermacora, M. R. Expression of Properly Folded Human Glutamate Decarboxylase 65 as a Fusion Protein in *Escherichia coli*. *Eur. J. Biochem*. **1997**, *246*, 350−359.
(35) Tuomi, T.; Rowley, M. J.; Knowels, W. J.; Chen, Q. Y.; McAnally, T.; Zimmet, P. Z.; Mackay, I. R.. Autoantigenic Properties of Native and Denatured Glutamic Acid Decarboxylase: Evidence for a Conformational Epitope. *Clin. Immunol. Immunopathol*. **1994**, *71*, 53−59.
(36) Santos, J.; Anton, E. A.; Marino-Buslje, C.; Iacono, R.; Maffia, P.; Poskus, E.; Ermacora, M. R. Replacement of Methionine-161 With Threonine Eliminates a Major By-Product of Human Glutamate Decarboxylase 65-kDa Variant Expression in *Escherichia coli*. *Biotechnol. Appl. Biochem*. **2000**, *31*, 205−212.
(37) Martin, D. L.; Martin, S. B.; Wu, S. J.; Espina, N.. Regulatory Properties of Brain Glutamate Decarboxylase (GAD): The Apoenzyme of GAD is Present Principally as the Smaller of Two Molecular Forms of GAD in Brain. *J. Neurosci*. **1991**, *11*, 2725−2731.
(38) Shul'ga, A. A.; Kurbanov, F. T.; Khristoforov, R. R.; Darii, E. L.; Sukhareva, B. S. Glutamate Decarboxylase of *Escherichia coli*: Expression of *gadA* Gene, Purification and Properties of GAD$\alpha$. *Mol. Biol*. **1999**, *33*, 491−497.
(39) Johnson, B. S.; Singh, N. K.; Cherry, J. H.; Locy, R. D. Purification and Characterization of Glutamate Decarboxylase from Cowpea. *Phytochemistry* **1997**, *46*, 39−44.
(40) Denner, L. A.; Wei, S. C.; Lin, M. S.; Lin, C.-T.; Wu, J.-T. Brain L-Glutamate Decarboxylase: Purification and Subunit Structure. *Proc. Natl. Acad. Sci. U.S.A*. **1987**, *84*, 668−672.
(41) Ueno, Y.; Hayakawa, K.; Takahashi, S.; Oda, K.. Purification and Characterization of Glutamate Decarboxylase from *Lactobacillus brevis* IFO 12005. *Biosci. Biotechnol. Biochem*. **1997**, *61*, 1168−1171.
(42) Wingo, W. W.; Awapara, J. Decarboxylation of L-Glutamic Acid by Brain. *J. Biol. Chem*. **1950**, *187*, 267−271.
(43) Holtz, P.; Palm, D. Pharmacological Aspects of Vitamin $B_6$. *Pharmacol. Rev*. **1964**, *16*, 113−178.
(44) Wada, H.; Snell, E. E.. The Enzymatic Oxidation of Pyridoxine and Pyridoxamine Phosphates. *J. Biol. Chem*. **1961**, *236*, 2089−2095.
(45) Hanna, M. C.; Turner, A. J.; Kirkness, E. F. Human Pyridoxal Kinase. cDNA Cloning, Expression, and Modulation by Ligands of the Benzodiazepine Receptor. *J. Biol. Chem*. **1997**, *272*, 10756−10760.
(46) Coburn, S. P.; Mahuren, D.; Schaltenbrand, W. E.; Wostmann, B. S.; Madsen, D. Effects of Vitamin B-6 Deficiency and 4′-Deoxypyridoxine on Pyridoxine Kinase and Other Aspects of Metabolism in the Rat. *J. Nutr*. **1981**, *111*, 391−398.
(47) Nitsch, C. Regulation of GABA Metabolism in Discrete Rabbit Brain Regions Under Methylpyridoxine-Regional Differences in Cofactor Saturation and the Preictal Activation of Glutamate Decarboxylase Activity. *J. Neurochem*. **1980**, *34* (4), 822−830.
(48) Lämmli, U. K. Cleavage of Structural Proteins During Assembly of the Head of the Bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(49) Bradford, M.. A Rapid and Sensitive Method for Quantification of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem*. **1976**, *72*, 248−254.
(50) Lasley, S. M.; Greenland, R. D.; Michaelson, I. A. Determination of Gamma-Aminobutyric and Glutamic Acids in Rat Brain by Liquid Chromatography With Electrochemical Detection. *Life Sci*. **1984**, *35*, 1921−1930.
(51) Chakraborty, M. Use of High-Performance Liquid Chromatography for Assay of Glutamic Acid Decarboxylase. Its Limitation in Use for *Post-Mortem* Brain. *J. Chromatogr*. **1991**, *571*, 235−240.
(52) Zhang, G.; Bown, A. W. The Rapid Determination of $\gamma$-Aminobutyric Acid. *Phytochemistry* **1997**, *44*, 1007−1009.

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