Site-directed mutagenesis of K396R of the 65 kDa glutamic acid decarboxylase active site obliterates enzyme activity but not antibody binding

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Abstract The role of K396 in the enzymatic catalysis and the antigenicity of the 65 kDa isoform of glutamate decarboxylase (GAD65) was analyzed using the K396R GAD65 mutant. GAD65 is a major autoantigen in Type 1 diabetes and autoantibodies directed to GAD65 are widely used markers for this disease. We found that (1) recombinant human GAD65 is fully enzymatically active; (2) the K396R mutation abolished GAD65 activity; and (3) the K396R mutant retained full antigenicity to GAD65 autoantibodies in serum from Type 1 diabetes patients, but not to polyclonal antibodies raised to the catalytic domain.

Key words: Glutamate decarboxylase; Autoimmunity; Pyridoxal 5-phosphate; Diabetes

1. Introduction

The decarboxylase reaction of glutamate to generate γ-aminobutyric acid (GABA) and CO_2 is catalyzed by glutamate decarboxylase (GAD) and is assumed to depend on a Schiff base formed between cofactor pyridoxal 5-phosphate (PLP) and a lysine residue (K396) in the active site of the enzyme [1,2]. The importance of the PLP binding lysine residue in the active site of other PLP-dependent enzymes was demonstrated by site-directed mutagenesis studies of aspartate aminotransferase [3], tryptophan synthase [4], D-amino acid aminotransferase [5], serine hydroxymethyltransferase [6], ornithine decarboxylase [7], and aromatic L-amino acid decarboxylase [8].

65 kDa GAD (GAD65) appears to be a major autoantigen in Type 1 diabetes [9–11]. Detection of autoantibodies to the molecule is an essential part of predicting Type 1 diabetes, as the diagnostic sensitivity of these autoantibodies is 75–85% [12,13] and GAD65 autoantibodies (GAD65Ab) enable the discrimination of autoimmune cases in adult-onset diabetes [14].

Human GAD65 cDNA is used to generate radiolabeled GAD65 for studies of the importance of this enzyme in Type 1 diabetes etiology and pathogenesis [15,16]. Immunoprecipitation of in vitro transcribed and translated radiolabeled GAD65 is one of the most widely used approaches to detect GAD65Ab in human serum [17]. However, little is known to what extent the in vitro transcribed and translated GAD65 is enzymatically active and if K396 is critical to enzyme activity.

It was reported that GAD65 administration or antisense expression [18–21] in the NOD mouse prevents or delays the onset of Type 1 diabetes indicating potential use of GAD65 in prevention of Type 1 diabetes. Production of recombinant GAD65 is necessary to provide sufficient quantities for trials. Whole-length human GAD65 was expressed in yeast strain *Pichia methanolica* [22], Sf9 insect cells [23,24], bacteria [25–27], and transgenic plants [28] and reported to be enzymatically active.

2. Materials and methods

2.1. Recombinant GAD65

Wild-type human GAD65 cDNA and the GAD65 cDNA carrying the K396R mutation were inserted into the pcDNAII plasmid (Invitrogen, San Diego, CA, USA). Recombinant [^{35}S]GAD65 antigens were produced by in-vitro-coupled transcription/translation of the respective cDNA with SP6 RNA polymerase and nuclease-treated rabbit reticulocyte lysate (Promega, Madison, WI, USA) as described previously [29,30]. Equal amounts of protein were verified by densitometric analysis of SDS–PAGE. Recombinant human GAD65 (rhGAD65) produced in yeast was kindly donated by ZymoGenetics, Seattle, WA, USA.

2.2. Site-directed mutagenesis

Site-directed mutagenesis of the PLP binding site of GAD65 was performed using a commercially available system (Chameleon™ double-stranded, site-directed mutagenesis kit, Stratagene, La Jolla, CA, USA). Briefly, the p65 plasmid containing the cDNA for human GAD65 was extended using mutagenesis primer (5′-CGTGGAATCCACACCGCATGATGGGAGTCCC-3′), corresponding to nucleotides 1172–1202 of the GAD65 cDNA, containing the codon CGC for arginine, and a kinase selection primer (5′-CTGTGACTGGTGACGCGTCAACCAAGTC-3′). Transformed colonies were screened for the desired mutation and the correct DNA sequence confirmed using an ABI prism DNA sequencer.

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Abbreviations: GAD65, 65 kDa glutamate decarboxylase; PLP, pyridoxal 5-phosphate; rhGAD65, recombinant human GAD65; GAD65Ab, antibodies to GAD65; RIA, radioimmunoassay; ICA, islet cell antibodies

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2.3. GAD65 activity assay

GAD activity was measured by the $^{14}CO_2$-trapping method described previously [31] with major modifications. rhGAD65 (100 ng) was incubated with reaction buffer (50 mM K_2HPO_4, 0.03 mM PLP, 0.56 mM L-glutamate, 0.018 μCi ^{14}C-glutamate (Amersham Life Science Inc, Arlington Heights, IL, USA), 0.1 mM DTT, pH 6.8) for 1 h at 37°C with gentle agitation. During incubation, released $^{14}CO_2$ was captured on filter paper (Kontes, Vineland, NJ, USA) soaked in 50 μl 1 M NaOH. After 1 h incubation, the tubes were removed and placed on ice for 10 min. The filter paper was then placed in 4 ml of Optifluor scintillation fluid (Packard Biosciences, Torrance, CA, USA) and the radioactivity was allowed to absorb overnight. The amount of $^{14}CO_2$ radioactivity released was counted in a Beckman scintillation counter. The GAD activity was calibrated with respect to both incubation time and protein concentration, and expressed as U/mg protein (1 unit is equal to 1 μmol of product formed per min at 37°C).

2.4. GAD65Ab radioimmunoassay (RIA)

GAD65Ab were determined in a RIA, as described previously [29,30]. Serum samples were tested at a final serum dilution of 1/25, unless indicated otherwise. rhGAD65 (ZymoGenetics, Seattle, WA, USA) was used in the GAD65Ab competitive RIA at the indicated concentrations.

Antibody positive and negative samples were included in every assay to correct for inter-assay variation. Antibody levels were expressed as a relative index as described previously [29,30]: GAD65Ab index = (cpm of tested sample − cpm of negative standard)/(cpm of positive standard − cpm of negative standard). The Juvenile Diabetes Foundation islet cell autoantibody standard [32], which is also GAD65Ab positive as verified by immunoprecipitation, was used as the GAD65Ab positive standard [32,33]. A randomly selected control serum from a healthy volunteer was used as the negative standard. All samples were tested in duplicate and the coefficient of variations was determined for each sample. The average intra-assay coefficient of variation was 9.5 with the highest value of 18 and the lowest being 0.2. The upper limit (index of 0.04) of the normal range was established as the 99th percentile of the levels of 182 healthy control subjects.

2.5. Antisera

Two groups of sera from newly diagnosed Type 1 diabetes patients were used. Group I represents 10 children who were diagnosed with diabetes at age 7–12 (median 10) and subjected to plasmapheresis [34]. These samples were used in all Immunology of Diabetes Workshops to standardize islet cell antibodies (ICA) and GAD65Ab [32]. One sample from this set of 10 samples is serving as the worldwide standard for expression of ICA levels in JDF units [32] and of GAD65Ab as a GAD65Ab index [33]. Group II ($n = 27$) consists of randomly selected sera from 1–16 year old (median 9) Type 1 diabetes patients. The subjects were diagnosed at the St. Görans Hospital, Sweden, Stockholm, between 1986 and 1992 [35]. All serum samples were kept frozen at −80°C as small aliquots. The study was approved by the Ethic Committee of the Karolinska Institute, Sweden. All individuals gave their informed consent to participate in the study.

Rabbit antisera were raised by immunization with synthetic peptides corresponding to the PLP binding site of GAD65 [36]. Antisera 5551, 5545, and 5565 were raised towards the amino acid residues 390–404, antisera 5581, 5576, and 5052 were raised towards the amino acid residues 390–403 of human GAD65.

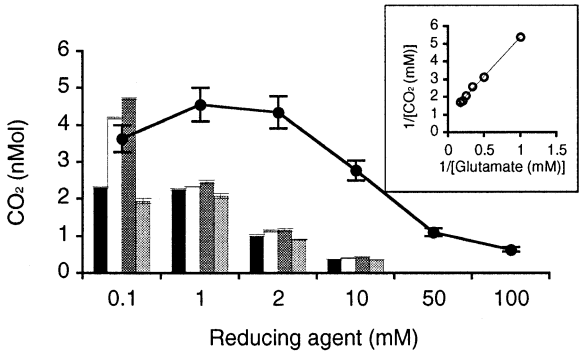

Fig. 1. Effects of PLP and reducing agents on rhGAD65 enzyme activity. The optimal PLP-cofactor concentration (0.01 mM: light gray bar; 0.03 mM: dark gray bar; 0.3 mM: white bar; 1.2 mM PLP: black bar) was determined at different DTT concentrations (0.1–10 mM). The effect of BME was tested at different concentrations (0.1–100 mM) (inserted line plot). Standard errors are indicated. Insert: Lineweaver–Burk plot of rhGAD65.

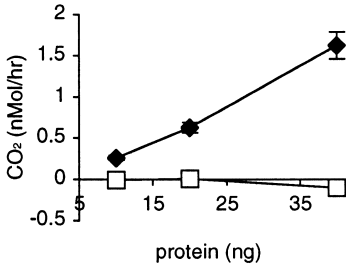

Fig. 2. Enzyme activity of K396R mutant GAD65. K396R GAD65 (□) and wild-type GAD65 (♦) were tested for their enzymatic activity at the indicated concentrations. Standard errors are indicated.

3. Results

3.1. pH optimum and K_m of rhGAD65

K_m and pH optimum were determined for rhGAD65 produced in yeast cells. The pH optimum was observed at 6.8, K_m was calculated at 3.6 mM (Fig. 1, insert). Specific enzyme activity was found to be 1 U/mg.

3.2. Effect of reducing agents and titration of cofactor PLP

DTT and BME were employed to test the effect of reducing agents on GAD65 enzymatic activity (Fig. 1). Low concentrations of DTT and BME (0.1 and 1 mM, respectively), enhanced the enzymatic activity (40 and 20%, respectively). At higher concentrations the enzymatic activity decreased and was completely inhibited at 5 and 100 mM, respectively. The optimal concentration of the PLP cofactor, titrated at different DTT concentrations, was found to be 0.03 mM (Fig. 1).

3.3. rhGAD65 produced in a cell-free system is enzymatically active

The enzymatic activity of GAD65 produced by cell-free transcription/translation was measured at estimated GAD65 concentrations of 55–225 μg/ml. The pH, cofactor and DTT concentrations were optimized as above. The specific activity of GAD65 was found to be 0.68 U/mg (Fig. 2).

3.4. K396R mutant is enzymatically inactive

We substituted K396 with arginine to test the influence of this amino acid substitution on enzyme activity (Fig. 2). While the wild-type GAD65 showed linear increase of enzyme activity in relation to protein, no enzymatic activity was detected for the K396R mutant.

3.5. The antigenicity of the active site is changed by the amino acid substitution

The possible structural perturbation caused by the K396R amino acid substitution was probed with polyclonal antibodies raised to 20-mer peptides encompassing the active site [36]. The polyclonal antibodies were tested at different concentra-

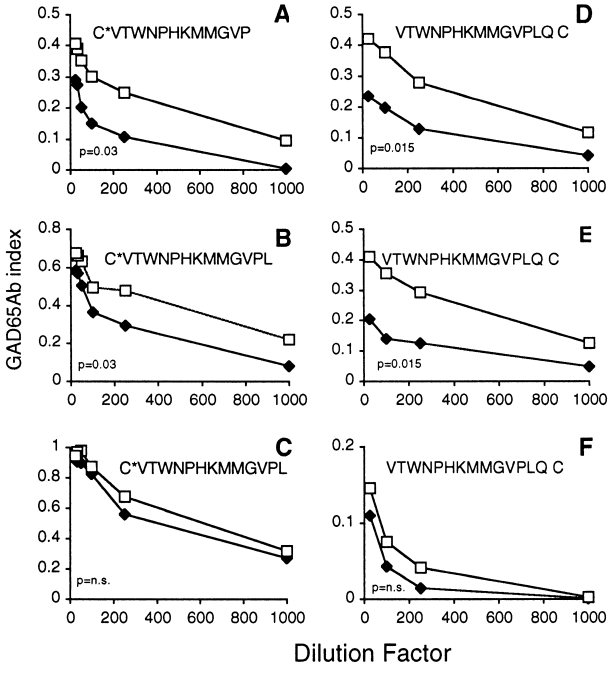

Fig. 3. Polyclonal antibodies raised to peptides representing the active site of GAD65 bind wild-type GAD65 significantly better than the K396R mutant. Six rabbit polyclonal antibodies (A: 5581; B: 5052; C: 5576; D: 5545; E: 5551; F: 5565) raised towards peptides representing the active site of GAD65 were tested for their reactivity to wild-type GAD65 and K396R mutant GAD65 in a RIA. The sera were tested at the indicated dilutions with both wild-type GAD65 (□) and mutant (♦). The peptide used for immunization is indicated for each antibody. Cysteine residues introduced to allow coupling to carrier protein KLH are indicated by an asterisk.

tions in a RIA (Fig. 3). Four of the sera (5581, 5545, 5052, and 5551) show preferred binding to the unmutated molecule as compared to the K396R mutant. Sera 5576 and 5565 showed equal binding to both molecules.

3.6. K396R mutant retains full antigenicity for antibodies from Type 1 diabetes patients

Next the antigenicity of the K396R mutant was analyzed with sera from Type 1 diabetes patients. Sera from 10 Type 1 diabetes patients, used as standards for ICA [37] and GAD-65Ab [33], were compared for binding capacity to wild-type and mutated GAD65 (Fig. 4). No significant differences in binding to the K396R mutant as compared to wild-type GAD65 was observed for any of the GAD65Ab positive sera. Serum samples 898 and 853 were negative for GAD65Ab as previously demonstrated [29]. Similar results were observed with sera from 27 consecutively diagnosed Type 1 diabetes children (Fig. 4, insert B).

3.7. Binding of GAD65Ab from Type 1 diabetes patients to the K396R mutant can be displaced with RhGAD65

The specificity of antibody binding was confirmed by displacement of both wild-type GAD65 and the K396R mutant with unlabeled rhGAD65 (Fig. 4, insert A). Serum sample 622 was incubated with either of the two radiolabeled proteins in the presence of increasing concentrations of unlabeled rhGAD65. Both ^{35}S-labeled proteins were equally well displaced from the serum sample. These results confirm findings that GAD65Ab from Type 1 diabetes patients do not bind to the active site of the molecule [38].

4. Discussion

Radioactive labeled GAD65 is used in RIAs to detect conformation-dependent antibodies directed to this antigen [30]. Hence, it is crucial that the protein is in its native conformation. The active site of GAD65 is located at amino acid residues 393–396 [39]. Site-directed mutagenesis of the conserved K396 residue in the active site of GAD65 was undertaken to confirm that this residue is required for enzyme activity. Arginine was selected to substitute lysine because it is similar in hydrophobicity and size, while being unable to form a Schiff

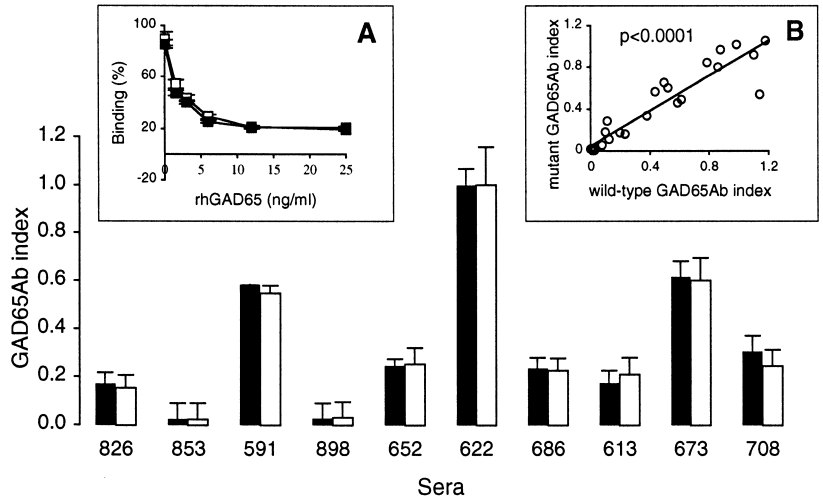

Fig. 4. Binding of mutant GAD65 by sera from Type 1 diabetes patients. Sera from 10 Type 1 diabetes patients were tested for their binding to mutant GAD65 (□) and wild-type GAD65 (■) in a RIA. The respective GAD65Ab index is indicated for each sample. Insert A: Serum sample # 622 was further tested in a competition assay. The sample was incubated with the indicated concentrations of rhGAD65 produced in yeast cells and ^{35}S-labeled mutant GAD65 (□) or wild-type GAD65 (■). The GAD65Ab index observed at no addition of unlabeled antigen was defined as 100% binding. Insert B: Additional samples ($n=27$) of Type 1 diabetes patients were tested for their binding to mutant and wild-type GAD65 in a RIA. Wild-type GAD65Ab index was plotted against mutant GAD65Ab index. P value for the correlation is indicated in the figure.

base. Previous reports of site-directed mutagenesis on PLP-dependent enzymes substituted lysine with arginine [6,7] demonstrating the usefulness of this amino acid substitution for the analysis of the role of lysine. The GAD65 K396R mutation resulted in the total loss of enzymatic activity. However, the molecule retained its full antigenicity to antibodies in sera of Type 1 diabetes patients while the binding capacity to polyclonal antibodies raised towards the active site of the molecule was significantly reduced. Competition studies with rhGAD65 suggested that disease-specific antibody epitopes of both mutant and wild-type GAD65 are identical. The high enzyme specific activity and the immunological characteristics of the rhGAD65 indicate that the molecule is in its native conformation.

In order to substantiate our major findings, we report major modifications in the $^{14}CO_2$ trapping method for measuring the enzymatic activity of GAD65. With this modified protocol we tested the enzymatic characteristics of rhGAD65 produced in yeast and in a cell-free system. The enzymatic activity measured in our assay for yeast GAD65 (1 U/mg) and GAD65 produced in a cell-free system (0.68 U/mg) correlates well with the range of reported specific activities for human GAD65; rhGAD65 expressed in Sf9 insect cells has a specific activity of 0.45 U/mg [23], and rhGAD65 expressed in bacteria has a specific activity of 1.96 U/mg [27]. We found the pH optima and K_m-values of rhGAD65 to be comparable to previously reported values for purified GAD from human brain [40].

The amino acid sequence of GAD65 contains 15 cysteine residues, all of which are conserved between human, mouse and rat, suggesting an important role in the tertiary structure of GAD65. Our data of the effect of reducing agents suggest that some of the cysteine residues are present in a reduced state, while others may form disulfide bridges. The reduction of the latter results in the loss of the native conformation and consequently in the loss of enzymatic activity. This observation is in agreement with recent findings [2,23] that GAD65 contains sulfhydryl groups prone to oxidation.

This work represents the biochemical characterization of full length rhGAD65 expressed in yeast cells and by in vitro transcription and translation. We conclude that rhGAD65 produced in a cell-free transcription and translation system has immunochemical and enzymatic activities similar to those reported for the native molecule. We showed by site-directed mutagenesis that K396 is a crucial amino acid for GAD65 enzymatic activity, however, the structural perturbation of the K396R mutation does not influence the binding of GAD65Ab from Type 1 diabetes patients. This mutant may be useful in future studies to analyze PLP and glutamate binding by GAD65.

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