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The critical structural role of a highly conserved histidine residue in group II amino acid decarboxylases

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Abstract Glutamate decarboxylase is a pyridoxal 5′-phosphate (PLP)-dependent enzyme, belonging to the subset of PLP-dependent decarboxylases classified as group II. Site-directed mutagenesis of *Escherichia coli* glutamate decarboxylase, combined with analysis of the crystal structure, shows that a histidine residue buried in the protein core is critical for correct folding. This histidine is strictly conserved in the PF00282 PFAM family, which includes the group II decarboxylases. A similar role is proposed for residue Ser269, also highly conserved in this group of enzymes, as it provides one of the interactions stabilising His241.
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Key words: Glutamate decarboxylase; Histidine residue; Pyridoxal 5′-phosphate; Stabilising interaction

1. Introduction

Most amino acid decarboxylases are pyridoxal 5′-phosphate (PLP)-dependent enzymes. The main reaction they catalyse consists of a simple and irreversible one-substrate reaction, which, depending on the decarboxylase under consideration, fulfills either biosynthetic or biodegradative cellular functions [1]. Comparison of the amino acid sequences led to the proposal that PLP-dependent decarboxylases can be divided into four groups of independent evolutionary origin [2]. Three of these groups (I, II and III) were later suggested to belong to the same family of PLP-dependent enzymes, classified on the basis of the predicted fold as fold type I [3]. Fold type I encompasses PLP-dependent enzymes of the α- and γ-families, i.e. enzymes that act on the amino acid substrates at the level of the α- and γ-carbon atoms, respectively [4]. Thus, evolution has clearly played a major role in providing each enzyme with a different catalytic competence, though retaining a similar overall fold. In the case of the PLP-dependent enzymes belonging to the fold type I, the dimeric assembly is the minimal oligomeric assembly required to generate a functional enzyme, with each subunit consisting of a small (N-terminal) and a large (C-terminal) domain. The large domain contains the PLP-binding site and has an α/β fold consisting of a central seven-stranded mixed β-sheet surrounded by eight α-helices [5]. In fold type I enzymes, a lysine residue forming an internal aldimine with the cofactor, an aspartate interacting with the pyridinium nitrogen of the cofactor and a glycine-rich loop interacting with the cofactor phosphate group are conserved. Amino acid conservation within each class belonging to fold type I is even more significant. The role of conserved histidine residues, in particular, has been investigated in decarboxylases because of the possible role of their side chains in reprotonation after decarboxylation of the substrate [6,7]. Three highly conserved histidine residues could be identified in decarboxylases [8]. Two of these histidines were mutagenised in *Escherichia coli* glutamate decarboxylase, isoform B (GadB; E.C. 4.1.1.15), a member of group II decarboxylases, and shown not to be involved in catalysis [9]. The recently solved crystal structure of *E. coli* GadB [10], coupled with site-directed mutagenesis studies, shows that the third conserved histidine residue (His241 in GadB) has a critical structural role in GadB and, likely, in all group II decarboxylases.

2. Materials and methods

2.1. Materials

Vent polymerase was from New England Biolabs. Restriction enzymes and the agarose gel DNA extraction kit were from Roche. The ligase kit, the T7 sequencing kit and DEAE–Sepharose were from Amersham. [α-^{35}S]dATP (1000 Ci/mmol) was from New England Nuclear. Ingredients for bacterial growth were from Difco. Oligonucleotides were from MWG. Gabase was from Sigma. Other chemicals were from Merck.

2.2. Site-directed mutagenesis

Site-directed mutagenesis was performed by overlap extension polymerase chain reaction [11]. External primers annealing over the N- and C-terminal sequences were those used in the construction of the expression plasmid containing the *gadB* gene, pQ*gadB* [12]. Mutagenic primers were 5′-GACATGGCCATCGACGC-3′ for the GadB-H241A mutant, 5′-GACATGAACATCGACGC-3′ for GadB-H241N, 5′-GACATGCTGATCGACGC-3′ for GadB-H241L, and their complementary sequences. The underlined triplet refers to the mutagenised His241 codon (CAC in wild-type *gadB*). Plasmid pQ*gadB* was used as template [12]. The products from the first polymerase chain reaction, carried out using *Vent* polymerase with denaturation at 95°C for 1 min, annealing at 48°C for 1 min and extension at 74°C for 2 min, were used in the second polymerase chain reaction

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Abbreviations: Gad, glutamate decarboxylase; PLP, pyridoxal 5′-phosphate

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as templates with the external primers to generate the complete coding sequence of *gadB*. The 770-bp fragments *Nco*I/*Eco*RV, generated by digesting the amplicons from the second polymerase chain reaction, were subcloned into pQ*gadB*, digested with the same restriction enzymes [12]. The newly inserted fragments of plasmids pQ*gadB*H241A, pQ*gadB*H241N and pQ*gadB*H241L were sequenced on both strands and the plasmids were subsequently used to transform the *E. coli* strain JM109(pREP4), known to produce low levels of endogenous GadA/B [12].

2.3. Analysis of mutant forms of GadB

10-ml cultures of *E. coli* JM109(pREP4) containing plasmids pQ*gadB*H241A, pQ*gadB*H241N and pQ*gadB*H241L, respectively, were grown in LB or SB medium containing 200 μg/ml ampicillin and 25 μg/ml kanamycin. Growth was carried out at 37°C or room temperature to an $OD_{600} \leq 0.7$, at which point cultures were induced with 2 mM isopropyl thiogalactose (IPTG) for 1, 2 or 3 h. Bacterial pellets were recovered by centrifugation at 3500 rpm for 20 min and resuspended in 0.5 ml of 1 mM PLP, 1 mM DTT unbuffered solution. After sonication, cell debris was separated from the soluble material by centrifugation at 10 000 rpm and resuspended in 0.5 ml of water. Aliquots of soluble and insoluble material were analysed on 10% SDS–PAGE [13].

Purification and assay of mutant enzyme GadB-H241N were as described for wild-type GadB [12].

3. Results and discussion

According to the multiple sequence alignment computed by Sandmeier [2], three histidine residues (His167, His241 and His275 in *E. coli* GadB) are conserved in group II PLP-dependent decarboxylases, a large family of proteins with members in Eukaryotes, Bacteria and Archaea. Out of these, only the histidine residue corresponding to GadB His241 is present in all group II members and is one of the eight residues being strictly conserved in the alignment of 26 sequences [2]. These strictly conserved residues (in GadB: Gly210, His241, Asp243, Ala244, Ala245, Gly248, Ser269 and Lys276) are inserted into a very small portion of the polypeptide chain (15%, 70 amino acid residues), which includes three active site residues involved in cofactor interactions and activity (Asp243, Ala245 and Lys276) [10,14], whereas the other conserved residues have a yet unidentified role. This region is the core of the GadB large domain (residues 58–346) and comprises the βαβαββ motif which is part of the seven-stranded mixed β-sheet surrounded by eight α-helices, typical of fold type I PLP-dependent enzymes [5]. Analysis of the PF00282 PFAM family, which corresponds to group II decarboxylases and encompasses 582 sequences, confirms that, if one excludes fragment entries, His241 is completely conserved. Structural and sequence alignments reveal that conservation of this residue is not strict in group III decarboxylases, also belonging to fold type I (Table 1), thus suggesting that only in group II decarboxylases this His residue plays a specific role. Interestingly, in group II decarboxylases conservation of the histidine residue equivalent to GadB His275 is high but, unlike that of His241, not complete (Table 1). No three-dimensional structure is available for group I decarboxylases (glycine cleavage system P-protein), which are also assigned to fold type I. However, sequence alignments reveal that also for this group His241 is not conserved [3].

Amongst the amino acids naturally occurring in proteins, histidine is the most versatile. The hydrophilic nature of its side chain accounts for its observed distribution on exposed surfaces (protein surface and/or active site). Since the pK_a of the imidazole ring is close to the physiological pH, histidine residues are able, depending on the protonation state, to coordinate metals, form hydrogen bonds and perform general acid–base catalysis. The role of buried histidine residues in contributing to protein stability has been reported in a number of proteins, such as vigilin [15], *Bacillus circulans* xylanase [16] and class Mu glutathione transferase [17]. In those cases, the unfavourable hydrophobic environment of the protein core is counterbalanced by deprotonation of the ring and hydrogen bond formation. For vigilin, protonation was proven to favour surface exposure and partial or complete unfolding [15]. In *B. circulans* xylanase, the hydroxyl group of a serine residue (Ser130), again localised in the protein interior, is found to interact with the ND1 nitrogen of His149, a residue completely conserved in all family G xylanases [16]. The pK_a of the imidazole group of His149, measured by NMR, was found to be lower than 2.3 [16]. The imidazole ring of His149 is therefore deprotonated also at acidic pH, so that it is neutral in the folded protein over a wide pH range. The role of His241 in *E. coli* GadB appears to be similar.

The crystal structures of *E. coli* GadB [10] and of *Sus scrofa* DOPA decarboxylase [18] show that His241 and its homologues of group II decarboxylases are completely buried. In GadB, the side chain imidazole ring of His241 is hydrogen-bonded to Ser269 with its ND1 nitrogen and to Ser271 with its NE2 nitrogen (Fig. 1). Interestingly, the three-dimensional structure of *E. coli* GadB, determined both at pH 4.6 (PDB entry 1PMM) and at pH 7.6 (PDB entry 1PMO), confirms that the hydrogen bonds to Ser269 and Ser271 are not significantly affected by the pH-dependent structural change involving other portions of the protein [10]. The pK_a of the imidazole ring of His241 should be low, so that the side chain remains neutral also at acidic pH, thereby contributing to the stability of the enzyme to low-pH stress, when acidification of the cytoplasm occurs, especially near the membrane.

Table 1
Conservation (bold) of His241 and His275 (GadB numbering) in some representative decarboxylases

Decarboxylase (organism)	Group							
GadB (*Escherichia coli*)	II	**H241**	S269	S271	W166	G133	M137	**H275**
DOPA decarboxylase (*Sus scrofa*)	II	**H**	S	N	V	A	A	**H**
Histidine decarboxylase (*Lycopersicum esculentum*)	II	**H**	S	S	I	G	G	**H**
Histidine decarboxylase (*Homo sapiens*)	II	**H**	S	T	H	A	A	S
GAD65 (*Homo sapiens*)	II	**H**	S	T	L	A	A	**H**
putative GAD (*Methanosarcina acetivorans*)	II	**H**	S	N	W	G	H	**H**
Arginine decarboxylase (*Escherichia coli*, biodegradative)	III	**H**						**H**
Ornithine decarboxylase (*Lactobacillus* 30a)	III	E						**H**
Ornithine decarboxylase (*Homo sapiens*)	IV	D						A

For group II decarboxylases conservation of the residues around His241 is shown.

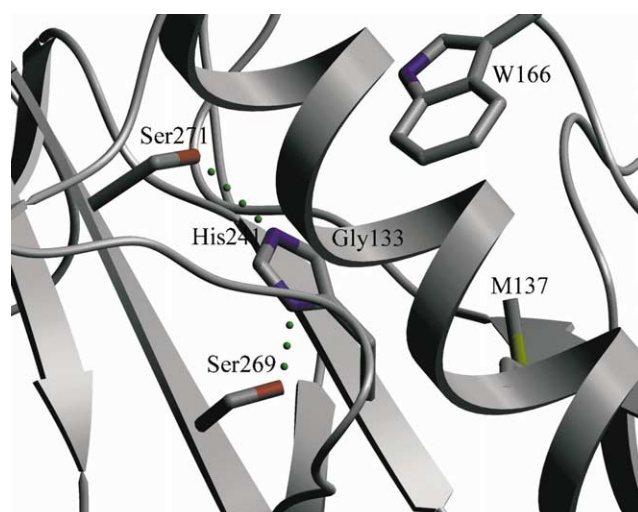

Fig. 1. Position and main interactions of His241 in GadB. The protein backbone appears in cartoon representation, with residue side chains in ball-and-stick representation. Hydrogen bonds are depicted as dotted green lines. Prepared with the programme Setor [19].

Notably, when extracellular pH drops, GadB migrates to the membrane [10], so it needs to be particularly stable against low-pH stress.

Also, Ser269 appears to be conserved in the PF00282 family. This residue is not conserved only in three members of PF00282. In those cases, a Thr residue (likely to interact in the same way with His241) replaces it. Superposition of the GadB and DOPA decarboxylase structures shows that the environment of His241 is structurally very well conserved in the two enzymes at the backbone level; at the side chain level, only Ser269 is conserved and the other residues exhibit moderate variability (Table 1). Multiple sequence alignments and secondary structure prediction support the view that this should be the case for the other members of group II decarboxylases. This points to Ser269 as the most important residue of the environment of His241.

To probe the structural and functional role of His241 in *E. coli* GadB, three mutants were produced: GadB-H241A, GadB-H241N and GadB-H241L. Total proteins extracted from uninduced and induced JM109(pREP4) cultures containing plasmids pQ*gadB*, pQ*gadB*H241A, pQ*gadB*H241N and pQ*gadB*H241L, respectively, were compared. The rates of synthesis of mutant forms were comparable to those obtained from wild-type GadB (Fig. 2). However, none of the three GadB mutant forms, unlike the wild-type enzyme, was found in the soluble fraction of cells (cf. lane 3 of His241 mutants vs. lane 3 of wild-type GadB). Attempts to increase the solubility by growing the bacteria at different temperatures (37°C to room temperature) and in different growth media were unsuccessful. The insolubility of the mutant forms cannot be just assigned to the large overexpression of GadB. Indeed, wild-type GadB, when overexpressed, is recovered in large amounts in the soluble fraction, even though some of it is also detected in the insoluble fraction (Fig. 2, rightmost panel). In the case of wild-type GadB the presence of some of the overexpressed protein in the insoluble fraction is reasonably explained because the cell has to cope with the abnormally high overexpression levels of this protein, which is thus sequestered into inclusion bodies as a response to a stressful condition. Different is the case of the three GadB His241 mutants, which are exclusively detected in the insoluble fraction (Fig. 2, lanes 4), likely because of the deleterious effect of replacing a residue essential for correct protein folding. By means of immunoblot analysis it was possible to detect very low levels of expression of the mutants in the soluble cell supernatant ($\leq$0.3 mg/l bacterial culture; data not shown). Following the standard purification protocol, an attempt to purify the GadB-H241N mutant from an 8-l culture was made, but highly impure enzyme was obtained (data not shown). Moreover, instability of the partially purified protein material made any further progress in the purification unsuccessful.

The site-directed mutagenesis data can be interpreted in the light of the GadB crystal structure [10]. Position 241 in the GadB sequence appears to be structurally critical. It does not tolerate a small residue like Ala, which leaves a buried cavity and is not able to interact with Ser269 (and Ser271). A branched hydrophobic residue like Leu, which would not leave a significant cavity, is also not accepted at position 241, stressing the importance of hydrogen bonding interactions. However, neither is the H241N mutant viable. Modelling of the H241N mutation shows that Asn would be able to perfectly reproduce the hydrogen bonding interaction between the proximal side chain nitrogen of His241 and the side chain of Ser269 (data not shown). On the other hand, Asn241 cannot reproduce the second hydrogen bond of H241, that of the distal imidazole nitrogen with the side chain of Ser271. Analysis of multiple sequence alignments, however, does not support a critical role for the latter hydrogen bonding interaction, since Ser271 is not conserved within group II decarboxylases: it is most frequently replaced by residues able to accept or donate hydrogen bonds (like Asn, as in DOPA decarboxylase, Thr or Cys), but also by Gly, as in some prokaryotic histidine decarboxylases (from *Morganella morganii*, *Klebsiella planticola* and *Enterobacter aerogenes*) [2]. Notably, in DOPA decarboxylase (PDB entry 1JS3) the aforementioned hydrogen bond does not involve Asn298 (corresponding to GadB Ser271), but a 'bridging' water molecule, which in turn interacts with the side chain of Asn298.

The structural role of His241 appears to be that of stabilising the interaction between two strands of the central sheet of the large domain and, most importantly, of creating optimal packing in the hydrophobic region between the β-sheet itself and a long helix (125–149) of GadB (Fig. 1). Since His241 is completely conserved in group II decarboxylases, we propose

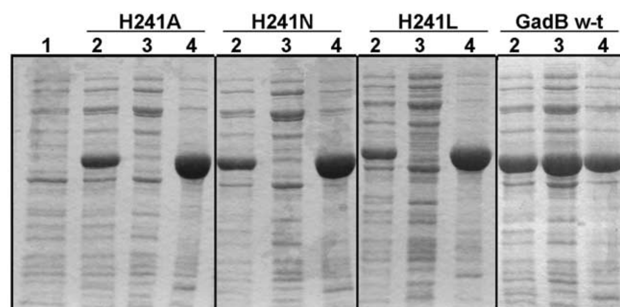

Fig. 2. 10% SDS–PAGE analysis of expression of GadB-H241A, H241N and H241L mutants and of the wild-type enzyme (left to right): lane 1, total cell extract before induction; lanes 2, total cell extract after a 3-h induction with IPTG; lanes 3, proteins from soluble material after cell lysis; lanes 4, proteins from the insoluble material after cell lysis.

that it is a critical residue for the correct folding and stability of this important group of enzymes.

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