RESEARCH COMMUNICATION

Experimental evidence for structure–activity features in common between mammalian histidine decarboxylase and ornithine decarboxylase

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Common protein motifs between histidine decarboxylase (HDC) and ornithine decarboxylase (ODC) were detected by computational analysis. Mutants were generated and expressed *in vitro*. In both enzymes, terminal PEST-region-containing fragments are not essential for decarboxylation (PEST regions are sequence

fragments enriched in proline, glutamic acid, serine and threonine residues in a hydrophilic fragment flanked by cationic amino acids). The substitution of a very well conserved histidine residue by alanine causes a severalfold increase of the apparent K_m values for the respective substrates.

INTRODUCTION

Histamine is a polyamine involved in a variety of physiological processes in animals that include allergy, gastric secretion, neurotransmission and cell growth. The histamine-producing enzyme in mammals, histidine decarboxylase (HDC, EC 4.1.1.22), was purified and its cDNA sequence reported in 1990 [1,2]. Although several HDC isozymes with different pI values and membrane affinity exist, it seems likely that these are derived from a single gene [1,3]. Mammalian HDC gene products can be subject to several post-transcriptional modifications including proteolytic processing and phosphorylation [2–6]. The primary translation product (656–662 amino acids as deduced from the cDNAs, or 73–75 kDa on the basis of SDS/PAGE electrophoresis) must be processed to render the mature subunit (53–55 kDa) of the homodimeric enzyme. The precise sequence of the mature subunit and the processing mechanism are still unknown. Sequenced peptides of native mouse mastocytoma HDC (mmHDC) correspond to fragments from amino acids 132–464 of the cDNA-deduced sequence [7]. The first 479 amino acids must include the necessary structures for the reconstitution of the active enzyme [8]. However, the presence of the N-terminus in the native enzyme has not been proven, and alternative processing mechanisms cannot be excluded.

Mammalian HDC, aromatic-L-amino-acid decarboxylase (DDC, EC 4.1.1.28) and glutamate decarboxylase (EC 4.1.1.15) are group II pyridoxal phosphate (PLP)-dependent L-amino-acid decarboxylases (L-aaDCs) that seem to have a different evolutionary origin compared with mammalian ornithine decarboxylase (ODC, EC 4.1.1.17) [9]. All are low-abundance and unstable PLP-dependent enzymes with important physiological roles. Knowledge of structure–function relationships among these enzymes has been limited by the difficulties in isolating sufficient amounts of proteins for molecular studies [6–8,10]. Of the mammalian L-aaDCs, ODC is the best characterized enzyme at the molecular level and for which abundant information on protein structure–activity and turnover exists [11–13]. Mam-

malian ODC is a homodimeric enzyme (461 amino acids, 51–53 kDa/monomer). Mutagenesis experiments have revealed residues that are essential for decarboxylation located in the first 423 amino acids of the primary sequence. The C-terminal amino acids 423–461 are important for protein stability and contain a PEST region (amino acids 423–449) in a domain that is not essential for ODC activity [13–15]. PEST regions are defined as sequence fragments enriched in proline, glutamic acid, serine and threonine residues in a hydrophilic fragment flanked by cationic amino acids. They can act as constitutive or conditional signals providing mechanisms for intracellular degradation of key metabolic proteins [16].

Although ODC showed no significant sequence similarity to group II L-aaDCs in mammals, we have detected common motifs among their cDNA-deduced sequences by computational analysis [17]. These include the presence of PEST regions. Mammalian HDCs and DDCs present a hydrophilic N-terminus containing a PEST region located in the region between amino acids 40 and 70. Mammalian HDCs have at least one other PEST region located after residue 503, just in the fragment that seems to be removed during monomer maturation.

In addition, mammalian HDC contains a histidine residue (His-274) in a hydrophobic surrounding [fragment 120–275 in rat HDC (rHDC)] that shows sequence similarity to the residues 193–198 of mammalian ODC and to other fragments of group II L-aaDCs [17]. In a previous screening of ODC mutants it was observed that substitution of His-197 by alanine led to a reduced activity when expressed *in vitro* [11,15]. Here we characterize the effect of the His/Ala substitution in both HDC and ODC.

EXPERIMENTAL

Reverse-transcription PCR was carried out using mRNAs from 16-day old Wistar rat fetal liver and two primers: H1 (5'-TCAAGACATATGATGGAGCCCAGTG-3') and H2 (5'-

Abbreviations used: L-aaDC, L-amino-acid decarboxylase; rHDC, rat histidine decarboxylase; mmHDC, mouse mastocytoma histidine decarboxylase; rODC, rat ornithine decarboxylase; mODC, mouse ornithine decarboxylase; DDC, aromatic-L-amino-acid decarboxylase; PLP, pyridoxal phosphate; PEST regions, sequence fragments enriched in proline, glutamic acid, serine and threonine residues in a hydrophilic fragment flanked by cationic amino acids.

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TCCCTCAGGATCCTACTCCAGGGATAGCC-3'). H1 introduced a unique *Nde*I site in the N-terminus, and H2 introduced a stop codon at position 513 and a unique *Bam*HI site. Substitutions of the reported rHDC cDNA sequence [2] are shown in italics, and initiation and stop codons are underlined. The PCR product was ligated into a pGEM-T plasmid (Promega). A *Sac*II-*Bam*HI insert from the recombinant pGEM-T-rHDC plasmid was subcloned into a pBluescript SK-II plasmid (Stratagene), and both strands sequenced by the Sequenase system (Amersham International) using T7, SP6, PCR primers and some other internal primers (Pharmacia). The first ATG was 35 bp downstream from the T7 promoter transcription start site. A transition ATC/GTC (Ile-463 → Val substitution) and a silent transversion GTC/GTG (Val-453) were detected when compared with the reported rHDC sequence [2]. This sequence was named 1/512.rHDC and the recombinant plasmid p1/512.rHDC.

In order to obtain the N-terminally truncated version (69/512.rHDC), p1/512.rHDC was digested with *Kpn*I and *Bam*HI; the new insert was purified and ligated to *Sac*I/*Bam*HI-digested pBluescript SK-II. The linear construction was blunt-ended by Klenow treatment and circularized by T4 ligase. Orientation of the insert was assessed by restriction mapping and sequencing. The first ATG for expression in a T7 RNA polymerase/*in vitro* translation system was located 92 bp downstream of the T7 promoter (M-69 codon) in the full rHDC cDNA.

The His/Ala-274 substitution was generated by two independent PCR reactions [18], using p1/512.rHDC as template. One reaction used the T7 and M1 (5'-GGAGGGACTGTG-GCTCGCCGTCGATGCTGCTTATGC-3') primer combination, while M2 (5'-GCATAAGCAGCATCGACGGCGAGC-CACAGTCCCTCC-3') and H2 were used in the second reaction. M1 and M2 are complementary oligonucleotides corresponding to the sequence encoding residues 268–280 of rHDC with two substitutions introducing an Ala-274 codon (GCC) instead of a His codon (CAC). Both PCR products were purified, denatured and annealed together (40 °C for 30 min) before ten elongation–denaturation cycles using *Taq* polymerase and dNTPs. This was followed by 25 PCR cycles using T7 and H2 as primers. The product, termed H-274/A.rHDC, was subcloned into pBluescript SK-II and sequenced as described for p1/512.rHDC. JM-109 or

XL1-Blue cells were used as host cells. Computational analyses of the sequences were carried out using the University of Wisconsin Genetics Computer Group Programs.

The rat ODC (rODC) cDNA was obtained from pODC71 [19] and subcloned into the pGEM-3Zf+ vector between *Eco*RI and *Hind*III sites generated by PCR from base 27 upstream from the initiation codon up to base 91 downstream from the stop codon. The recombinant plasmid has the first ATG initiation codon 37 bases downstream from the T7 promoter transcription start. Wild-type mouse ODC (mODC) and the H/A-197 ODC mutant were described previously [15].

Purified plasmids were transcribed and translated using the TnT®-coupled reticulocyte-lysate system according to the manufacturer's (Promega) instructions [using, in a total volume of 25 µl, 0.5 µg of recombinant DNA, with additional 10 mg/ml poly(ethylene glycol)-300 and 10 µM pyridoxal phosphate (PLP)]. After a 120 min incubation, aliquots were removed for further experiments. Unlabelled methionine was added to the extract to be used for HDC activity determinations. HDC activity was assayed by measuring the production of ¹⁴CO₂ from L-[1-¹⁴C]histidine (ARC, St. Louis, MO, U.S.A.) in a mixture containing 0.2 mM dithiothreitol, 10 µM PLP, 10 mg/ml poly(ethylene glycol)-300, 100 mM potassium phosphate, pH 6.8, and 8 µl of the product of the TnT® reaction. The released ¹⁴CO₂ was measured as previously described for ODC activity determinations [20]. Activities are expressed as means ± S.E.M for at least three different experiments. Expression reactions carried out in parallel with 0.5 µg of pBluescript SK-II were used as blanks. When the radioactivity was not higher than 15% of that of the blanks, samples were considered as not having detectable activity. ODC activity was measured as described previously [15,20]. Apparent *K_m* values were calculated using a range of concentrations 0.02–2.02 mM (ODC) or 0.03–3.20 mM (HDC). *De novo* synthesis of polypeptides was monitored by SDS/PAGE (denaturing conditions) and fluorography of parallel expression reactions supplemented with [³⁵S]methionine (Amersham International) [15,20]. Signals were quantified using a FujiBas system (Fuji). No band of molecular mass greater than 42 kDa was detected when pBluescript SK-II was expressed in the presence of [³⁵S]methionine. ODC was expressed by using the same protocol but avoiding the use of poly(ethylene glycol)-300.

Figure 1 Common protein motifs detected among mammalian ODC and group II L-aaDCs producing 1,4-diamine

The different cDNA-deduced sequences are represented, showing the location of PEST regions and the histidine-containing hydrophobic motif found in every reported PLP-dependent L-aaDC. Simple traits show terminal fragments known to be poorly conserved or non-essential for maintaining decarboxylase activity. Some relevant amino acid residues are numbered.

Table 1 Apparent K_m values for the amino acid substrate of rHDC and mODC mutants expressed *in vitro*

Extract	K_m (mM)
rHDC	
1/512	0.11
69/512	0.28
H/A-274	0.75
mODC	
Wild-type	0.03
H/A-197	0.55

RESULTS AND DISCUSSION

In contrast with prokaryotic pyruvate-dependent HDCs [21], structure–function studies on PLP-dependent HDCs are scarce, specially in eukaryotic organisms. The putative PLP-binding site of mammalian HDC (Lys-308 in rHDC) was located by computer-assisted sequence comparison with other related PLP-dependent L-aaDCs (see program MOTIFS, GCG, Wisconsin and [2,7]). In rat and humans, HDC and DDC show 52–54% identity in the first 480 amino acids of their deduced sequence. Studies carried out by Yamamoto et al. [7,8] suggest that, in mmHDC, the C-terminus (residues 479–661) is not essential for HDC activity, having no counterpart in DDC (Figure 1).

We have constructed recombinant plasmids to express *in vitro*-truncated versions of the rat HDC. The *in vitro* expression system has been used extensively for structure–function studies of mammalian ODC [11,13,15]. When expressing the 1/512.rHDC insert (Figure 2, lane 2), the major ^{35}S -labelled bands were of 55 and 53 kDa. A 48 kDa band accounted for 35% of the total signal. Other less intense bands of 44–46 kDa were also observed. Because sizes are in agreement with initiation in consecutive ATG codons (Met residues 17, 69, 80, 97 etc.), these band could be the result of internal initiation of translation. Lower minor bands are also observed after *in vitro* expression of ODC cDNAs (Figure 2, lane 1, and [15]).

HDC activity was found after *in vitro* expression of p1/512.rHDC. Both rODC and rHDC polypeptides were synthesized *de novo* at very similar rates (Figure 2, lanes 1 and 2). Both decarboxylating activities were measured at 0.6 mM ornithine or 0.8 mM histidine, concentrations that have been reported as saturating for the enzymes purified from native sources [1,22]. At these concentrations, HDC activity (1500 ± 30 pmol/h per 0.5 μg of DNA) was 52% of the rODC activity. A 40-fold difference is observed between both specific

activities of homogeneous purified extracts from mouse tissues, being higher for ODC [1,22]. Both enzymes are low-abundance proteins *in vivo* [5]. ODC represents 10^{-3} – 10^{-5} % of the total protein in mammalian cells [20,23]. Parallel time courses of both ODC and HDC activities in the same mammalian cells show that *in vivo* ODC activity is also several-fold higher than HDC activity [24,25]. To our knowledge, this is the first report of the reconstitution of a group II L-aaDC activity by an *in vitro* expression system. It will provide a useful tool for rapid activity screening of HDC mutants and also for structure–function studies of other group II PLP-dependent L-aaDCs.

The 69/512.rHDC insert encodes a 48 kDa polypeptide lacking both N-terminal and C-terminal PEST-region-containing fragments. This polypeptide was expressed *in vitro* (Figure 2, lane 4). The activity detected from 69/512.rHDC extracts was very similar (99 ± 14 %) to that detected using p1/512.rHDC, when normalized with respect to *de novo*-synthesized polypeptides. Apparent K_m values for histidine were also determined for both 1/512 and 69/512 extracts (Table 1). Affinity for histidine was higher in the 1/512.rHDC extract, but the apparent K_m of the 69/512.rHDC was very similar to that reported by Ohmori et al. [1] for the native enzyme purified from a mouse mastocytoma cell line (0.25 mM). Thus the PEST region-containing N-terminus of mammalian HDC is not essential for histidine decarboxylation, but its presence could affect the final conformation of the mammalian enzyme.

This result, together with data reported for other L-aaDCs, suggest that the terminal PEST-region-containing fragments do not seem to be essential for mammalian L-aaDC enzyme activities producing 1,4-diamines (Figure 1). In fact, mammalian ODC lacking the terminal fragment containing the C-terminal PEST-region shows full ODC activity [15]. In rat DDC, the first 57 amino acid residues that include a PEST sequence show no sequence similarity to those of the *Drosophila* enzymes, in spite of the high degree of sequence similarity between the rest of the sequence (58%) [28]. Neither group II Gram-negative bacteria HDC nor plant HDC-like protein (40–42 kDa/monomer) contain PEST regions in their N-termini [9,26,27]. The PEST-region-containing C-terminus of mammalian HDC is not only non-essential for activity but should be removed during HDC subunit maturation [16,17]. Thus the appearance of terminal PEST sequences in different group L-aaDCs (ODC, HDC and DDC) during evolution could provide a new post-translational regulatory mechanism for these enzymes.

Common motifs detected among eukaryotic L-aaDCs include a histidine residue in a hydrophobic environment [17]. No activity could be detected at non-saturating ornithine concentrations (17 μM) after *in vitro* expression of a H/A-197 mODC mutant [11,15]. Initially we measured the HDC activity of p1/512- and pH/A-274.rHDC products at 40 μM histidine. At 40 μM histidine, the velocity of histidine decarboxylation by p1/512.rHDC products was 50 ± 18 pmol/h per 0.5 μg of DNA, but it was non-detectable in the H/A-274.rHDC extracts, as is the case with H/A-197.mODC at low ornithine concentrations. When the substrate concentration was increased by 20-fold to 0.8 mM histidine, the velocity of histidine decarboxylation by H/A-274.rHDC extracts (715 ± 346 pmol/h per 0.5 μg of DNA) was still almost half of the activity detected from equal quantities of polypeptides expressed from p1/512-rHDC. Both p1/512.rHDC and pH/A-274.rHDC were expressed *in vitro* at very similar rates (Figure 2, lanes 2 and 3). The His/Ala substitution does not alter the predicted secondary structure of the fragment. Table 1 shows the apparent K_m values for histidine of both 1/512.rHDC and H/A-274.rHDC extracts together with those obtained under similar experimental conditions with wild-type

Figure 2 *In vitro* expression of rODC and rHDC mutant polypeptides

The Figure shows SDS/PAGE–fluorography of the ^{35}S -labelled polypeptides present in equal volumes of TnT® reactions carried out with p.rODC (lanes 1), p1/512.rHDC (lane 2), pH/A-274.rHDC (lane 3) and p69/512.rHDC (lane 4).

mODC and the H/A-197 mODC mutant. For both enzymes, the single His/Ala substitution caused a significant reduction in affinity for the amino acid substrate, apparent K_m values being higher than 0.5 mM, which are unusual concentrations of ornithine and histamine in mammalian fluids and tissues [29]. Concentrations higher than 4 mM would be necessary to measure both activities at zero reaction order.

These results suggest that this well-conserved histidine residue in a hydrophobic 'pocket' in ODC and HDC must be important in allowing access of the amino acid substrate to the catalytic site. A similar motif can be observed in every PLP-dependent L-aaDC described so far [9,17]. In addition, the highest degree of identity (57%) between fragments of mammalian and bacterial PLP-dependent HDCs is found in the sequences containing this motif (amino acids 268–281 in rHDC, and amino acids 192–205 in *Enterobacter aerogenes* HDC) [2,26]. Curiously, this histidine-containing motif is located in the central portion of the primary sequence reported as being well conserved or essential for decarboxylation in ODC, HDC and DDC (Figure 1).

In the present study we have tested a hypothesis based on structural analogies previously detected among group II and IV decarboxylases by computational analyses [17]. The present results provide experimental evidence with which to consider the possibility of evolutionary-convergence phenomena among L-aaDCs and give new insights for future structure–function studies.

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