**Panagrellus redivivus** ornithine decarboxylase: structure of the gene, expression in *Escherichia coli* and characterization of the recombinant protein

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A Southern blot analysis of the *Panagrellus redivivus* ornithine decarboxylase (ODC) gene suggests that it is a single-copy gene that resides on a genomic 3.2 kb EcoRI fragment. Phage clones possessing ODC gene sequences were isolated from a genomic EMBL-4 library and purified. The phage DNA inserts were analysed and a 3.2 kb EcoRI fragment containing the entire ODC gene was isolated. The nucleotide sequence analysis of this fragment reveals that the gene is interrupted by two introns of 47 and 49 bp. In the 5' non-translated region of the gene, putative AP1, VPE2 and c-Myc binding sites were identified. The ODC cDNA was expressed in a bacterial system as a His-fusion protein and the enzyme was purified by Ni²⁺-chelating affinity chromatography. The subunit molecular mass, as deduced from the cDNA and shown by SDS/PAGE, is 47.1 kDa. On the basis of gel filtration analyses it is shown that the active enzyme is a dimer. The specific enzyme activity was determined to be 4.2 µmol CO₂/min/mg protein. The enzyme is dependent on pyridoxal 5'-phosphate as a cofactor, and the presence of dithioerythritol or other thiol-reducing agents is essential for maximal activity. The *Kₘ* value for L-ornithine was determined as 44 µM. The *Kₘ* values for putrescine, α-difluoromethylornithine, α-hydrazino-ornithine and α-methylornithine were calculated as 51, 34, 0.34 and 42 µM respectively.

**INTRODUCTION**

The polyamines spermidine and spermine and their precursor putrescine have been reported to be essential for cell proliferation and differentiation [1–3]. Although a variety of effects of the polyamines have been reported to be essential for cell proliferation and differentiation [1–3], their exact biological function is still unknown. Ornithine decarboxylase (ODC; EC 4.1.1.17) is the first and apparently rate-limiting enzyme in polyamine biosynthesis, catalysing the decarboxylation of ornithine to putrescine. Inhibition of ODC leads to the growth arrest of animal cells. In the most well-known and widely studied inhibitor for ODC is α-difluoromethylornithine (α-DFMO) [4,5], an enzyme-activated irreversible inhibitor that acts by alkylating the active site of the enzyme [6]. α-DFMO is used for the treatment of African trypanosomiasis and has considerable potential as a chemotherapeutic or chemopreventative agent for other diseases. The ODC is one of the most highly regulated enzymes known [7,8] and has an extremely short half-life. Regulation of ODC activity can be found at the transcriptional and translational levels, as well as at the level of mRNA and protein stability [9–13]. In mammalian ODC genes several recognition sites for transcription factors have been found, e.g. TATA boxes, c-AMP responsive elements, AP-2 binding sites and GC boxes [14,15]. It has also been shown that the human ODC gene is regulated by the c-Myc–Max protein complex [16].

Until now very little information has been available about the regulation of polyamine concentrations in parasitic nematodes. To establish a model organism for nematodes, we investigated the polyamine biosynthesis of the free-living nematode *Panagrellus redivivus*, which can be maintained under axenic culture conditions. The ODC cDNA of *P. redivivus* has been isolated previously [17]. Here we report the structure of the ODC gene and the biochemical properties of the recombinant ODC expressed in *Escherichia coli*.

**EXPERIMENTAL**

**Biological material**

The wild-type strain of *Panagrellus redivivus* was maintained as previously described [17].

**Chemicals**

Restriction enzymes were purchased from Boehringer Mannheim. The expression vector pTrcHisB was from Invitrogen (San Diego, CA, U.S.A.), the vector pBSKS from Stratagene (Heidelberg, Germany), and His-bind resin was purchased from Novagen (Madison, WI, U.S.A.). L-Ornithine, putrescine, α-methylornithine and α-hydrazino-ornithine were from Sigma (Deisenhofen, Germany), α-DFMO was a gift from Marion-Merrell-Dow (Cincinnati, OH, U.S.A.), L-[1-¹³C]ornithine (57 mCi/mmol) was obtained from Amersham-Buchler (Braunschweig, Germany), and Protosol was from New England Nuclear (Bad Homburg, Germany).

**DNA purification, restriction analyses, Southern blot, isolation and analyses of genomic phage clones**

Genomic DNA was isolated from a mixed-stage population of worms, essentially by the method of Jowett [18], cleaved with restriction enzymes (Boehringer Mannheim) and size-fractionated in a 0.9 % agarose gel. The DNA was blotted on to nylon membranes (GeneScreen Plus; New England Nuclear, Bad Homburg, Germany) and probed with a 0.9 kb genomic *P. redivivus* PCR fragment generated as previously described [17]. Standard hybridization conditions for GeneScreen Plus membranes [5 × SSC, 1 % (w/v) SDS, 50 % formamide, 5 × Denhardt’s solution, 100 µg of tRNA] were performed overnight at 42 °C in the presence of at least 100 ng of ³²P-labelled DNA at 5 × 10⁸ c.p.m. per ml. Wash conditions were 1 × SSC/1 % SDS.

Abbreviations used: amp, ampicillin; α-DFMO, α-difluoromethylornithine; DTE, dithioerythritol; IPTG, isopropyl β-β-thiogalactoside; LB medium, Luria–Bertani medium; ODC, ornithine decarboxylase; PLP, pyridoxal 5-phosphate; rPODC, recombinant ODC; SL, spliced leader.

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at 65 °C (two washes of 30 min each) and 0.2 x SSC/1% SDS at 65 °C (30 min).

Screening of the genomic EMBL-4 phage library [19] under standard high-stringency conditions and subsequent preparation of phage DNA was performed as described by Sambrook et al. [20]. The phage DNA was digested with restriction enzymes, separated in 1 % (w/v) agarose gels, blotted on to nylon membranes and hybridized with the genomic PCR fragment. Hybridization conditions were as described above. A 3.2 kb EcoRI fragment from one of the genomic phage clones (AgPODC) was subcloned in pBSKS and subjected to sequence analysis. The DNA sequence determinations were performed by the dideoxy chain termination method. Secondary structure analyses were performed with HIBIO DNASIS from Hitachi.

Production and characterization of recombinant P. redivivus ODC protein

Sequence-specific primers based on the cDNA sequence of P. redivivus ODC (P-ODC 18: [17]) were used to amplify the complete coding region of the ODC cDNA. The 5' oligonucleotide possessed a BglII restriction site and the 3' antisense oligonucleotide possessed an EcoRI restriction site. The PCR product was digested, purified and cloned into the BglII–EcoRI cleaved expression vector pTrcHisB, which is a pUC derivative. The DNA insert is positioned downstream and in frame with a sequence that encodes an N-terminal fusion peptide [(His)6]. The vector pTrcHisB::rPODC was used to transform E. coli strain BL21. Cultures of the E. coli strain BL21 harbouring the expression plasmid were grown in Luria-Bertani (LB) medium supplemented with ampicillin (amp) (LB/amp; 50 µg/µl) overnight, diluted 1:100 in fresh LB/amp medium and grown until the D600 reached approx. 0.5. To induce the lac promoter, the culture was supplemented with 0.8 mM isopropyl-β-D-thiogalactoside (IPTG) and incubated for a further 90 min. Cells were harvested, washed in 1 x His-tag binding buffer (5 mM imidazole, 50 mM NaCl, 20 mM Tris/HCl, pH 7.9) and stored at -70 °C until used. For the purification, soluble extracts were prepared after resuspending the thawed cell pellets in 1 x His-tag binding buffer. These cell suspensions were sonicated 10 times for 15 s at 70 W in an ice bath. The recombinant ODC (rPODC) was purified from the 10000 g (4 °C for 1 h) supernatant of the lysed cells by using the pET His-tag Ni2+–chelating affinity chromatography system (Novagen) with imidazole competition for the elution of the protein. Subsequently the sample was applied to Sephadex G25 previously equilibrated with 40 mM Tris/HCl, pH 7.5, containing 1 mM dithioerythritol (DTE) and 1 mM EDTA. A portion of the protein was applied to a HiLoad Superdex S 200 FPLC column (1.6 cm x 60 cm; Pharmacia) with a flow rate of 1 ml/min to determine the molecular mass of the active protein. The subunit size of rPODC was determined by SDS/PAGE [10 % (w/v) gel] [21]. The gel was stained with Coomassie Blue. Protein concentrations were estimated by the Bradford method [22].

The ornithine decarboxylase activity assay

The ODC activity was measured as previously described [23]. The reaction mixture contained, in a final volume of 250 µl, 40 mM Tris/HCl, pH 7.5, 100 µM pyridoxal 5-phosphate, 1 mM DTE, 1 mM EDTA and 100 µM α-or-nithine (50 nCi). The reaction was initiated by addition of purified rPODC and incubated for 20 min at 25 °C.

The Km for ornithine was determined by varying the concentration of non-radioactive ornithine in the standard assay procedure. α-DFMO kinetic analysis was performed as follows. Enzyme samples were preincubated with different concentrations of α-DFMO in 40 mM Tris/HCl, pH 7.5, 1 mM DTE and 1 mM EDTA for various lengths of time at 25 °C. At each sampling time an aliquot was removed. Samples were incubated at 25 °C for 15 min. Analyses of putrescine, α-hydrazino-ornithine and α-methylornithine kinetics were performed with different inhibitor concentrations at various ornithine concentrations. Samples were incubated at 25 °C for 20 min. The values represent the mean of two (putrescine, α-methylornithine and α-hydrazino-ornithine) or five (ornithine) independent experiments, each performed in duplicate. HPLC analyses were performed as previously described [24] to demonstrate the formation of putrescine from ornithine. The kinetic analyses for substrate and inhibitors were calculated with the program GraphPad INPLOT.

RESULTS

Genomic restriction mapping

The P. redivivus ODC gene and its flanking sequences were analysed by genomic Southern blotting. The previously described P. redivivus genomic DNA (8 µg per lane) was digested with EcoRI (lane E), SacI (lane S), BamHI (lane B), DraI (lane D) and double-digested with EcoRI and SacI (lane S/E). The DNA fragments were separated on a 0.9% agarose gel and transferred to a GeneScreen Plus membrane. The filter was hybridized with the radiolabelled P. redivivus genomic PCR product previously described [17], washed and exposed overnight to X-ray film. The sizes of the DNA markers (1 kb Ladder, Life Technologies) are indicated on the left.

Figure 2 Partial restriction map of the genomic phage clone insert

A partial restriction map of the EMBL-4 phage clone (AgPODC) containing the entire coding region of the P. redivivus ODC gene is shown diagrammatically. The 0.9 kb genomic PCR product is shown below. Restriction endonuclease sites: B, BamHI; D, DraI; E, EcoRI; S, SacI. Introns are marked by arrowheads.
genomic PCR product [17], shown in Figure 2 as a black bar, was used to probe a genomic DNA Southern blot (Figure 1). The PCR product hybridized strongly with a 3.2 kb EcoRI fragment, two SalI fragments of 0.9 and 6 kb, one BamHI fragment of 5 kb, one DraI fragment of 5 kb and two SalI–EcoRI fragments of 0.9 and 1.6 kb.

**Isolation and characterization of genomic phage clones**

The PCR product was also used to isolate and partly map genomic phage clones from a *P. redivivus* genomic library [19].

One of these phage DNA inserts was shown to contain the entire ODC gene on a 3.2 kb EcoRI fragment. The partial map of this clone (λgPODC) is shown in Figure 2. The restriction enzyme pattern of the isolated clone is consistent with that determined by the genomic Southern blot analysis. The 3.2 kb EcoRI fragment was subcloned into pBS and subjected to sequence analysis. The nucleotide sequence of the entire coding region, as well as 683 bp upstream from the ATG and 175 bp downstream from the TGA stop codon, were determined (Figure 3). The sequence analyses revealed the presence of two small introns. In the 5′ non-translated region, one putative AP1 (TGASTMA, bp 366–372), was identified, as described in the text. The SL1 splice acceptor site is double underlined and the poly(A) signal sequence is in bold letters.
Production and purification of rPODC

The complete *P. redivivus* ODC cDNA was expressed by using pTrcHisB in *E. coli*. As much as 500 µg of purified rPODC could be obtained from 1 litre of bacterial culture. The recombinant protein represents approx. 4% of the total soluble protein of the bacteria. The yield of the purification was approx. 20%. Figure 4 (lane 3) shows the purified enzyme, which was produced in a soluble form residing in the cytosol of the bacteria. The enzyme could be stored at 4 °C in 40 mM Tris/HCl, pH 7.5, containing 1 mM DTE and 1 mM EDTA, for several days without significant loss of activity.

Characterization of the active rPODC

HPLC analysis of the reaction product formed in the ODC activity assay confirmed the formation of putrescine (results not shown). The molecular mass of the rPODC monomer as determined by SDS/PAGE is approx. 47 kDa. This result is consistent with the value calculated from the amino acid sequence of the cDNA. FPLC gel filtration of the active recombinant protein on a Superdex S-200 column resulted in one peak of activity corresponding to a protein of approx. 90 kDa (results not shown), indicating that this enzyme is active as a dimer.

The specific activity of *P. redivivus* recombinant ODC was found to be $4.2 \pm 0.18 \mu mol \text{CO}_2/\text{min per mg of protein}$ ($n = 2$). It was shown that the enzyme activity is dependent on the concentration of PLP. Maximal ODC activity was measured in the presence of 100 µM PLP. The apparent $K_m$ for the substrate ornithine was determined as $44 \pm 3 \mu M$. Putrescine, the product of the enzyme reaction, with an apparent $K_i$ of 51 µM, was shown to be a competitive inhibitor (Figure 5). α-Hydrazinoornithine and α-methylornithine were also competitive inhibitors with $K_i$ values of 0.34 and 42 µM respectively. $K_f$ for the irreversible inhibitor α-DFMO was 34 µM (Figure 6) and $\tau_{1/2}$ (enzyme half-life under saturating conditions) was 1.58 min.

DISCUSSION

In the present study we investigated the genomic structure of the *P. redivivus* ornithine decarboxylase. Southern blot analyses suggested that the ODC gene of *P. redivivus* is a single-copy gene. The nucleotide sequence of the 5′ flanking region of the ODC gene was shown to contain a consensus splice acceptor site upstream from the coding region, located exactly where the previously described cDNA ends. Upstream from this splice acceptor site no splice donor site could be found. *Caenorhabditis elegans* transcripts that contain a consensus splice acceptor site...
upstream from the coding region and no corresponding 5′ splice donor site were described as targets for trans-splicing [25]. A 5′ rapid amplification of cDNA ends demonstrated that a spliced leader (SL1) is trans-spliced to the 5′ end of the ODC transcript and revealed that the previously described cDNA [17] was complete, other than the spliced leader sequence. Trans-splicing of nematode mRNAs was first discovered by Krause and Hirsh [26] and is a very common way of generating 5′ mRNA ends in different genera of nematodes [27]. The presence of a SL1 precursor RNA in P. redivivus had already been shown by Bektesh et al. [27]. Macrae et al. [28] also reported trans-splicing of SL1 to the C. elegans ODC message.

The 5′ untranslated region of the P. redivivus ODC mRNA comprises 212 bp; it is thus unusually long compared with most other ODC messages [14,28,29]. This region can form stable secondary structures \( \Delta G = -370 \text{kJ/mol} \) \(-88 \text{kcal/mol}\), which has also been predicted for the mammalian ODC mRNAs. These structures may play a role in regulating ODC expression. Sequence analyses of the 5′-flanking region of the P. redivivus ODC gene revealed several putative binding motifs, two for c-Myc, one for AP1 and one VPE2 element. The c-Myc protein binds to random DNA sequences and to at least two specific elements [30]. It binds as a homodimer or as a heterodimer together with Max [31] and functions in transcriptional regulation as well as in DNA replication [32]. Homodimers of Jun or heterodimers between members of the Jun and Fos or Jun and activating transcription factor (ATF) family can bind to the AP1 motif [33–35]. Binding sites for these factors were also found in the promoter regions of ODC genes from other organisms [14,15,36]. In consequence we propose that the ODC of P. redivivus is regulated at the transcriptional level, in a manner similar to that of the mammalian ODCs. Additionally we found a VPE2 element that has been shown to play a role in the regulation of the expression of the vitellogenin [37] and metallothionein [38] genes of C. elegans. In these cases the element seems to play a role in regulating tissue-specific gene expression.

The sequence analyses of the coding region of the P. redivivus ODC gene confirmed the results obtained from the analysis of the previously described genomic PCR fragment [17]. The P. redivivus ODC gene has two small introns. The C. elegans ODC [28] was also shown to have two introns. Thus the two nematodes represent an evolutionary stage between the intronless ODC genes of Trypanosoma brucei [39], Leishmania donovani [40] and Saccharomyces cerevisiae [41] and the mammalian ODC genes with 11 introns. Other intermediate numbers of introns can be found in Neurospora crassa, which has only one intron [42], and Drosophila melanogaster, which has five [43].

The sizes of the introns in the C. elegans ODC gene, 57 and 47 bp [28], are very similar to those in the P. redivivus ODC gene (49 and 47 bp). However, only the position of the first intron is conserved. The D. melanogaster and human ODC genes also have introns in this position. The location of the second intron in the C. elegans ODC gene is the same as in the human and D. melanogaster ODC genes. P. redivivus shares the location of its second intron with a different intron in the human ODC gene. It can therefore be speculated that there are favoured positions for introns in the ODC gene that were conserved during evolution.

The results presented in this paper show that catalytically active P. redivivus ODC can be overexpressed in E. coli. The molecular mass of the monomer was determined by SDS/PAGE as approx. 47 kDa. This corresponds to the results obtained by the sequence analyses of the cDNA and shows that no post-translational processing of the protein is necessary to obtain catalytically active ODC. The molecular mass of the P. redivivus ODC monomer is very similar to those of other eukaryotes [39–46]. Gel filtration analyses revealed that the active protein is about 90 kDa and is thus a homodimer. This is consistent with the quaternary structure of other ODCs [44,45], with the exception of the T. vaginalis ODC, which is active as a tetramer [46].

The \( K_m \) determined for P. redivivus recombinant ODC is similar to those reported for mammalian ODCs [45,47]. \( \alpha \)-DFMO was found to have an apparent \( K_m \) of 34 \( \mu \text{M} \) and a \( \tau_{90} \) of 1.58 min. ODC isolated from rat liver has a \( K_m \) for \( \alpha \)-DFMO of 39 \( \mu \text{M} \) and \( \tau_{90} \) is 3.1 min. This enzyme was also demonstrated to have a \( K_m \) for \( \alpha \)-methylornithine of 40 \( \mu \text{M} \) [48] and for \( \alpha \)-hydrazino-ornithine of 0.5 \( \mu \text{M} \) [49]. The nematode enzyme has \( K_m \) values for \( \alpha \)-methylornithine and \( \alpha \)-hydrazino-ornithine of 42 and 0.34 \( \mu \text{M} \) respectively. Thus the vertebrate and nematode enzymes seem to be very similar in their kinetic properties. Differences are observed in the specific activity and the inhibition of the ODC activity by putrescine. The ODC of P. redivivus has a specific activity of 4.2 \( \mu \text{mol CO}_2/\text{min per mg of protein} \). This lies between the specific activities described for the ODCs from S. cerevisiae and T. pyriformis, which are 0.5 [50] and 0.3 \( \mu \text{mol CO}_2/\text{min per mg} \) [51] respectively, and the ODCs of vertebrates, which were shown to be in the range 19–75 \( \mu \text{mol CO}_2/\text{min per mg} \) [47,51]. Putrescine was shown to be a competitive inhibitor of the nematode enzyme, which was also true of the mammalian enzyme [45]. The \( K_m \) values, however, are very different from each other. P. redivivus ODC has a \( K_m \) of 51 \( \mu \text{M} \) whereas the mammalian enzyme has a \( K_m \) of 0.6 \( \mu \text{M} \) [52].

As previously reported [17], the P. redivivus ODC does not have any C-terminal PEST sequences, which have been discussed as being important in rapid protein degradation in vivo [53]. The lack of PEST sequences has also been described for the T. brucei ODC [39]. In contrast, vertebrate ODCs possess PEST sequences [53,54]. Because P. redivivus lacks this regulatory sequence it seems possible that enzyme activity is regulated in vivo by feedback inhibition of putrescine rather than by degradation due to the recognition of the PEST sequences.

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