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 $\mu\text{mol CO}_2/\text{min/mg}$ 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 K_m value for L-ornithine was determined as 44 μM . The K_i 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, 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. By far the best known and most 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-[1-¹⁴C]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 $\times$ SSC, 1% (w/v) SDS, 50% formamide, 5 $\times$ 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 $\times$ 10⁵ c.p.m. per ml. Wash conditions were 1 $\times$ SSC/1% SDS

Abbreviations used: amp, ampicillin; α -DFMO, α -difluoromethylornithine; DTE, dithioerythritol; IPTG, isopropyl β -D-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 × 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 (λ gPODC) 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 *Bgl*II restriction site and the 3' antisense oligonucleotide possessed an *EcoRI* restriction site. The PCR product was digested, purified and cloned into the *Bgl*II–*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 D_{600} 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 × 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 × 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 100 000 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 × 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 L-ornithine (50 nCi). The reaction was initiated by addition of purified rPODC and incubated for 20 min at 25 °C.

The K_m 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

Figure 1 Genomic DNA Southern blot analysis

P. redivivus genomic DNA (8 µg per lane) was digested with *EcoRI* (lane E), *SaI* (lane S), *Bam*HI (lane B), *DraI* (lane D) and double-digested with *EcoRI* and *SaI* (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 (λ gPODC) 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, *Bam*HI; D, *DraI*; E, *EcoRI*; S, *SaI*. Introns are marked by arrowheads.

gg aag tca att ttc <u>acg tga</u> tgc ttt caa aat acg cac aat ttt tga	47	C CTC ATC CTC CGG ATC AGT GTC AGC GAC CCG ACG GCG CAA TGC CAA	1245
atc tta aac gct ttc att gcc act ttg acc ttt tag tcc cat taa gca	95	S L I L R I S V S D P T A Q C Q	171
aag atg agc atc acc tcc tgt cta caa cct <u>act gat aat</u> gcg tta ctt	143	CTC GGA ATC AAG TTT GGT TGC GAC CCT GAA ACC GTC GCC CCA AAA CTG	1293
tta gct ttt atg aca tgc gtc aaa aaa gaa gga act tcc cga tat tag	191	L G I K F G C D P E T V A P K L	187
ttg atg agc agg ttg ttc acg act acg aat gct gaa gaa atg cga ttt	239	CTT CGA AAA GCG GCA GAT ATG GGA ATG AAT GTC ATC GGA ATC TCT TTC	1341
tga tga cct caa agg gga caa aaa agg agt ttc act gca <u>gtc tct tat</u>	287	L R K A A D M G M N V I G I S F	203
caa aga gta ttc cgc gtc cgc ctt ttc tag aat aag ggt aca ctg gtt	335	CAT GTC GGA AGC GGC TGC AAT GAA CCA GCT ACC TTC CGC ACG GCT TTG	1389
atg tgc acg gtg atg ata gag cat tca ttt <u>tga cta acc</u> gta atg gta	383	H V G S G C N E P A T F R T A L	219
caa gtt ggg ttg ggg aag ctt gaa gtc gtc gga atg cgc cgc tta atg	431	GAG TAT GCT CGT GGT CTG TTC GAT CTA GGC ATC AGC CTC GGC CTC TCC	1437
ggg ttg gac ttt gta ctg tag cta att tga atg ctt <u>tgc aga</u> aga ctt	479	E Y A R G L F D L G I S L G L S	235
gac att ctt ctc ctt tca taa ttt cta ccc gcg caa ggt cac tgt cca	527	ATG ACA CTG CTT GAC ATT GGT GGC GGG TTC CCC GGC GTC GAC ACA GCC	1485
ctt gct gct tgc ggt att atc ctc cgc tct ggt cga cag aaa ctt ctt	575	M T L L D I G G G F P G V D T A	251
gtc gcc caa aat ccc gtc cct gcg gaa atc cac att cgc cgt cgg cac	623	CAT ATT AGC CTT GAC GCC TGT GCT GCT GTT ATC AAT CCA GCT CTC GAG	1533
gga aag gcc gtg ctt gaa caa cga taa cgc tcc gaa gcc tcc ttg gtg	671	H I S L D A C A A V I N P A L E	267
acg ttt gaa aat ATG ACC CTC ACC GGC TGT GTT GAC TAC TAC GAG ATC	719	GAA CTC TTC CCG CTT GAT AGT TGC CCG GAT GTT GAA GTC ATC GCC GAG	1581
M T L T G C V D Y Y E I	12	E L F P L D S C P D V E V I A E	283
ATC GCT GGC ACG AAG GTT GCC GTC TGT CGC AAT GCC ATC GAC AAC AAA	767	CCT GGC AGG TAC TTT GCT TGT GCT GCT GTG TCA GTC ACT ACA AAT GTC	1629
I A G T K V A V C R N A I D N K	28	P G R Y F A C A A V S V T T N V	299
ACC GTC GCA ACG GCT ATT GCT GCA ACC AGG ACT GTA AAT GGC AAC GAT	815	ATT GCC AGT GTC AAA GTC CCG GCT AGC CGA ATC ACT GAA AAA Ggt aca	1677
T V A T A I A A T R T V N G N D	44	I A S V K V P A S R I T E K	313
GAC CCT TTT GTC GTG ATG AAT GTG AGC ACC ATC ATG GCT AAG GTC ATT	863	atg cca aat tca tat tca aat ttc aaa acc aaa gta ttt cag CC GAC	1724
D P F V V M N V S T I M A K V I	60	A D	315
CAA TGG CAA CGA ACG ATG CCA CGA GTG GCT CCA TGC TAT GCC GTG AAG	911	GAT GTC AAC CGC GAC GGA TAC ATG TAC TAC ATG AAC GAC GGC GTC TAT	1772
Q W Q R T M P R V A P C Y A V K	76	D V N R D G Y M Y Y M N D G V Y	331
TGT AAT GAC GAT AAA GTG TTG TTG AGA ACC TTG GCG GAT CTG GGC ATG	959	GGC TCT TTC AAC TGC AAG TTG TTC GAT CAC TAT CAA CCT CGT GGA ATG	1820
C N D D K V L L R T L A D L G M	92	G S F N C K L F D H Y Q P R G M	347
GGC TTC GAC TGC GCC AGC AAG GCC GAA ATC GAG AAA GTC ATT GGG CTG	1007	CCA TTG GCT GAG CAC GAT GCC GAT GAA CCA CGC TTC CCG GTT TGC GTT	1868
G F D C A S K A E I E K V I G L	108	P L A E H D A D E P R F P V C V	363
GTT GGA CCG GAA AAG ATT GTC TAC GCG AAT CCG TGC AAG ACC CGC GGA	1055	TGG GGA CCA ACT TGT GAC GGT TTG GAC CAA GTA GAG GAA TCT TCC GTC	1916
V G P E K I V Y A N P C K T R G	124	W G P T C D G L D Q V E E S S V	379
TTC ATT GCC CAT GCT GAG GCC GCT GGA GTT AAG CGA ATG ACG TTC GAT	1103	ATG CCG AGA CTC TAT GAG GGT GAC TGG TTG TAC TAT CCT GAC ATG GGC	1964
F I A H A E A A G V K R M T F D	140	M P R L Y E G D W L Y Y P D M G	395
AGC GTT GAG GAG TTG ACC AAG ATT AAG CAA AAC CAT GCC GAT CCG AGg	1151	GCC TAC ACA TCG GTT GCC GCC AGC ACC TTC AAC GGT TTT GAT AAG CCG	2012
S V E E L T K I K Q N H A D P	155	A Y T S V A A S T F N G F D K P	411
ttt gtt tga cac act tca tga ttg cct gct tca gaa tca ata tta cag	1199	AAA ACT TAC TAC TTC ATC GAT GAG GCA ACG CTT GGA AGC ATT GTT CGC	2060
		K T Y Y F I D E A T L G S I V R	427
		AAA GCA GAT TCT GCT CCT CGC GGT TGA cga ctg cct gat gcg tgc cca	2108
		K A D S A P R G *	435
		aca cca agt ata atc gat gaa cga aaa tct gtt gaa att gaa aat aaa	2156
		atg ata tgt taa aaa acg ttt tat gtg aaa tga cca aat gca gtt tca	2204
		aat tgg tga ctg gca act att taa atg gtg tca gat atg aaa cac tgc	2252
		aaa aag taa t	

Figure 3 Nucleotide sequence of the *P. redivivus* ODC gene

Numbers indicate the position in the nucleotide and amino acid sequences. Lower-case letters represent introns and flanking sequences; exons are given in capital letters. Underlined sequences in the 5' region represent potential regulatory elements identified, as described in the text. The SL1 splice acceptor site is double underlined and the poly(A) signal sequence is in bold letters.

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 *SaII* fragments of 0.9 and 6 kb, one *BamHI* fragment of 5 kb, one *DraI* fragment of 5 kb and two *SaII*-*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* EMBL-4 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 (λ gPODC) 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),

Figure 4 Production and purification of *P. redivivus* rPODC

Coomassie Blue-stained SDS/PAGE (10% gel) of the 100 000 g supernatants of lysed cells. Lane 1, *E. coli* BL21 containing pTrcHisB without insert induced with IPTG; lane 2, BL21 containing pTrcHisB::rPODC induced with IPTG; lane 3, purified rPODC.

Figure 5 Inhibition of rPODC by putrescine

Plot of $1/v$ against $1/[S]$ in the presence of fixed concentrations of putrescine: $\square$, 0 μM ; $\blacksquare$, 40 μM ; $\triangle$, 100 μM ; $\blacktriangle$, 200 μM .

one putative VPE2 (CTGATAA, bp 127–133) and two putative c-Myc (TCTCTTA, CACGTG, bp 14–19, bp 280–286) binding sites were identified. No consensus TATA box sequences were present in this region. The first nucleotide of the previously described cDNA was demonstrated to be the last nucleotide of a consensus splice acceptor site (UUUCAG). A 5' rapid amplification of cDNA ends revealed that a spliced leader (SL1) is *trans*-spliced to the 5' end of the ODC mRNA of *P. redivivus* (results not shown).

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 $^{\circ}\text{C}$ 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

Figure 6 Inhibition of rPODC by α -DFMO

Top panel: Time- and concentration-dependent inhibition of rPODC by α -DFMO: $\bullet$, 5 μM ; $\circ$, 10 μM ; $\blacksquare$, 18 μM ; $\square$, 36 μM α -DFMO. Bottom panel: K_i for α -DFMO. The times required for half inactivation ($t_{1/2}$) are plotted against the reciprocal of the inhibitor concentration.

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 ± 0.18 $\mu\text{mol CO}_2/\text{min}$ 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 ± 3 μM . 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 -Hydrazino-ornithine and α -methylornithine were also competitive inhibitors with K_i values of 0.34 and 42 μM respectively. K_i for the irreversible inhibitor α -DFMO was 34 μM (Figure 6) and τ_{50} (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 [$\Delta G = -370$ kJ/mol (-88 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

[39,44–46]. 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]. α -DFMO was found to have an apparent K_i of 34 μ M and a τ_{50} of 1.58 min. ODC isolated from rat liver has a K_i for α -DFMO of 39 μ M and τ_{50} is 3.1 min. This enzyme was also demonstrated to have a K_i for α -methylornithine of 40 μ M [48] and for α -hydrazino-ornithine of 0.5 μ M [49]. The nematode enzyme has K_i values for α -methylornithine and α -hydrazino-ornithine of 42 and 0.34 μ 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 μ mol CO₂/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 μ mol CO₂/min per mg [51] respectively, and the ODCs of vertebrates, which were shown to be in the range 19–75 μ mol CO₂/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_i values, however, are very different from each other. *P. redivivus* ODC has a K_i of 51 μ M whereas the mammalian enzyme has a K_i of 0.6 mM [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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