

Cloning of a trypanosomatid gene coding for an ornithine decarboxylase that is metabolically unstable even though it lacks the C-terminal degradation domain

(*Crithidia fasciculata*/protein turnover/polyamines/PEST region)

FREDRIK SVENSSON*, CAROLINA CERIANI†, EVA LÖVKVIST WALLSTRÖM*, INGER KOCKUM*, ISRAEL D. ALGRANATI†, OLLE HEBY‡, AND LO PERSSON*§

*Department of Physiology and Neuroscience, Lund University, S-223 62 Lund, Sweden; †Instituto de Investigaciones Bioquímicas “Fundación Campomar,” University of Buenos Aires and Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina, 1405 Buenos Aires, Argentina; and ‡Department of Cellular and Developmental Biology, Umeå University, S-901 87 Umeå, Sweden

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ABSTRACT Mammalian ornithine decarboxylase (ODC) is among the most labile of cellular proteins, with a half-life of usually less than an hour. Like other short-lived proteins ODC is degraded by the 26S proteasome. Its degradation is not triggered by ubiquitination, but is stimulated by the binding of an inducible protein, antizyme. Truncations and mutations in the C terminus of mammalian ODC have been shown to prevent the rapid turnover of the enzyme, demonstrating the presence of a degradation signal in this region. Moreover, ODCs from the trypanosomatid parasites *Trypanosoma brucei* and *Leishmania donovani*, which lack this C-terminal domain, are metabolically stable, and recombination of *T. brucei* ODC with the C terminus of mammalian ODC confers a short half-life to the fusion protein when expressed in mammalian cells. In the present study we have cloned and sequenced the ODC gene from the trypanosomatid *Crithidia fasciculata*. To our knowledge, this is the first protozoan shown to have an ODC with a rapid turnover. The sequence analysis revealed a high homology between *C. fasciculata* ODC and *L. donovani* ODC, despite the difference in stability. We demonstrate that *C. fasciculata* ODC has a very rapid turnover even when expressed in mammalian cells. Moreover, ODC from *C. fasciculata* is shown to lack the C-terminal degradation domain of mammalian ODC. Our findings indicate that *C. fasciculata* ODC contains unique signals, targeting the enzyme for rapid degradation not only in the parasite but also in mammalian cells.

Ornithine decarboxylase (ODC), which catalyzes the first step in the polyamine biosynthetic pathway, is an enzyme regulated by several unique mechanisms (1–3). The extensive regulation of ODC most likely reflects the importance of the polyamines in the cell. The polyamines are ubiquitous in nature and have been shown to be essential for a variety of cellular processes ranging from control of the membrane potential to cell growth (3–5). Thus ODC is a potential target for therapeutic agents against cancer and various parasitic diseases (3, 6). A remarkable finding is that humans afflicted with African sleeping sickness refractory to other antitrypanosomal drugs can be cured by treatment with an ODC inhibitor, 2-difluoromethylornithine (DFMO) (6, 7).

Mammalian ODC is regulated at a multitude of levels, including transcriptional, translational, and posttranslational

(1). The polyamines exert a strong and rapid feedback control of ODC synthesis and degradation, providing the cell with an efficient mechanism for the regulation of polyamine levels (1–3). An important characteristic of mammalian ODC is its very rapid turnover (2). In fact the biological half-life of ODC, which may be as short as a few minutes, is one of the shortest known for a mammalian enzyme. The turnover of ODC is partly regulated by polyamines (2). An excess of polyamines stimulates the degradation of ODC through the induction of a specific protein, antizyme, that binds strongly to ODC and targets it for degradation (2, 8, 9). The polyamines induce the synthesis of antizyme through a unique mechanism involving a frameshift during translation of the antizyme mRNA (10, 11). Also the degradation of ODC occurs through an extraordinary mechanism, involving an ATP-dependent but ubiquitin-independent process catalyzed by the 26S proteolytic complex called the proteasome (12, 13). This is the first example of a non-ubiquitinated protein being degraded by the 26S proteasome.

Molecular analysis of the ODC protein has revealed sequences important for the short half-life of the enzyme. Truncation of the C-terminal part of mouse ODC converts the enzyme into a stable protein (14). Furthermore, mammalian ODC, like many other proteins with short half-lives, contains so-called PEST regions (15) that are rich in proline (P), glutamic acid (E), aspartic acid (D), serine (S) and threonine (T). Mammalian ODC has two PEST regions, one of which is located within the C terminus of the protein (14). ODC from *Trypanosoma brucei*, the causative agent of African sleeping sickness, is a stable protein in the parasite (16) as well as when expressed in mammalian cells (17, 18). Compared with mammalian ODC, *T. brucei* ODC is truncated at the C terminus and therefore lacks one of the PEST sequences (17, 18). Recombining *T. brucei* ODC with the C terminus of mammalian ODC confers a short half-life to the fusion protein expressed in mammalian cells (17, 18). Also the ODC from *Leishmania donovani*, the causative agent of visceral leishmaniasis, lacks the sequence corresponding to the C terminus of mammalian ODC and is a stable protein in the parasite (19). Other parasites that express an ODC with a slow turnover rate are *Leishmania mexicana* (20) and the malaria parasite *Plasmodium falciparum* (21). However, the amino acid sequences of these ODCs are not yet known.

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Abbreviations: ODC, ornithine decarboxylase; DFMO, 2-difluoromethylornithine.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. Y08233).

§To whom reprint requests should be addressed at: Department of Physiology and Neuroscience, Lund University, Sölvegatan 19, S-223 62 Lund, Sweden. e-mail: lo.persson@mphy.lu.se.

Recently, it was demonstrated that the nonpathogenic trypanosomatid *Crithidia fasciculata* contains an ODC that has a fast turnover (22). The protozoan flagellates of the genus *Crithidia* are monogenetic trypanosomatid parasites that colonize the digestive tract of infected flies. Inhibition of protein synthesis in the parasite, using cycloheximide, resulted in a rapid loss of ODC activity with a half-life of about 30 min (22). In addition, the polyamine-mediated repression of ODC, usually found in organisms expressing a short-lived ODC, was absent in this parasite (22). To our knowledge, this is the first demonstration of a metabolically unstable ODC in a trypanosomatid. Because this experimental system may conceal information important for the understanding of the molecular mechanisms involved in the rapid turnover as well as the feedback control of ODC, we have isolated and sequenced the ODC gene from *C. fasciculata*. The deduced amino acid sequence of *C. fasciculata* ODC was almost 70% identical to that of *L. donovani* ODC (19). We demonstrate that the protein has a very fast turnover even when expressed in mammalian cells. However, the fact that *C. fasciculata* ODC lacks the part corresponding to the C terminus of mammalian ODC may be taken to denote that other signals mediate the fast turnover of the protein.

MATERIALS AND METHODS

Materials. L-[1-¹⁴C]ornithine (57 mCi/mmol; 1 Ci = 37 GBq) was obtained from New England Nuclear. [³⁵S]dCTP (1000 Ci/mmol) was purchased from Amersham. The eukaryotic expression vector (pSVL) containing the simian virus 40 late promoter and origin of replication was obtained from Pharmacia. The λGEM-11 genomic library from *C. fasciculata* was a kind gift from D. S. Ray (23). All restriction enzymes, DNA ligase, *Taq* DNA polymerase, and avian myeloblastosis virus reverse transcriptase were obtained from Boehringer Mannheim. Genomic clones of *T. brucei* ODC and *L. donovani* ODC were generously provided by C. C. Wang (16) and B. Ullman (19), respectively.

Cell Culture. A *C. fasciculata* laboratory strain (ATCC 11745) was cultured at 28°C in a defined medium (HOSMEM II) (24). The cultures were supplemented with 50 units of penicillin and 50 μg/ml of streptomycin. African green monkey COS-7 cells (ATCC CRL 1651) were grown at 37°C in DMEM containing 10% fetal calf serum, nonessential amino acids, and antibiotics (50 units of penicillin and 50 μg/ml of streptomycin) in the presence of 5% CO₂/95% air.

Isolation of Nucleic Acids. Genomic DNA (25) and total RNA (26) were isolated from *C. fasciculata* according to published methods.

Isolation of an ODC DNA Fragment from *C. fasciculata* Using PCR. A DNA probe for the isolation of the ODC gene from *C. fasciculata* was obtained by amplifying an ODC-specific DNA fragment using degenerate primers from conserved regions of ODC. The sense primer 5'-GGCGAATT-CRTNTWYGCNAAYCCNTGYAA-3' and the antisense primer 5'-GGCGAATTCACNCCRTCTTACRTAR-TA-3' corresponded to the conserved regions containing the amino acids (V/I)(Y/F)ANPCK and YYVNDG(V/L), respectively. The PCR was carried out in the presence of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μM of each dNTP, 100 pmol of each oligonucleotide primer, and 2.5 units of *Taq* DNA polymerase in a volume of 100 μl. The program used was as follows: 95°C for 15 s, 45°C for 30 s, and 75°C for 2 min (35 cycles) followed by a final extension step for 5 min at 75°C. Analysis of the PCR products on a 1% agarose gel gave a single band of about 700 bp which was in agreement with the expected size of the ODC fragment. The fragment was subcloned into pBluescript KS⁺ (Stratagene) and partially sequenced using the dideoxy chain termination method.

Isolation of the *C. fasciculata* ODC Gene. A λGEM-11 *C. fasciculata* genomic library (23) was screened for ODC positive

sequences using the cloned PCR product labeled with [³²P]dCTP. The plaque-forming units were blotted onto Hybond N filters (Amersham) and prehybridized at 42°C in 6× SSPE (20× SSPE = 3 M NaCl/0.2 M NaH₂PO₄/20 mM EDTA, pH 7.4), 0.5% SDS, 10% dextran, 50% formamide and 1 mg/ml DNA for 3–4 h. The ³²P-labeled PCR product was then added and the filters were hybridized overnight. The filters were washed for 2 × 5 min and 2 h at room temperature in 2× SSC (20× SSC = 3 M NaCl/0.3 M sodium citrate, pH 7.0) and 0.1% SDS followed by two high stringency washes for 30 min at 65°C in 0.1× SSC and 0.1% SDS. Positive plaques were further purified by new rounds of plating and screening. One of the clones was selected for further analysis.

Subcloning and Sequencing of the *C. fasciculata* ODC Gene. The isolated bacteriophage DNA was digested with *Sfi*I or *Sac*I followed by fractionation on a 0.8% agarose gel. The *Sfi*I digest showed a 12-kb insert and the *Sac*I gave three specific bands of about 4.5, 4.3, and 2.3 kb. The *Sac*I fragments were blotted onto a Hybond N membrane and hybridized to the cloned PCR product as described. The 4.5-kb fragment was shown to hybridize to the ³²P-labeled PCR product. The isolated fragment was then subcloned into pBluescript KS⁺ and both strands were sequenced using the dideoxy chain termination method with the T7 sequencing kit from Pharmacia. Additional sequencing was done with an Applied Biosystems model 373A sequencer using the *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). This fragment was shown to contain the complete ORF of *C. fasciculata* ODC as well as long stretches of the 3' and 5' untranslated regions, including the 5' splice acceptor site.

Southern and Northern Blot Analyses. Genomic DNA (10 μg) from *C. fasciculata* was digested with various restriction enzymes. The resulting DNA fragments were separated on a 0.8% agarose gel, transferred to Hybond N (Amersham) membranes, and hybridized to the ³²P-labeled PCR product as described. For identification of the *C. fasciculata* ODC transcript 20 μg of total RNA was separated on a 1% agarose gel containing 2.2 M formaldehyde and blotted onto a Hybond N membrane. The membrane was then hybridized to the ³²P-labeled PCR product.

Mapping of the 5' Splice Acceptor Site of the ODC Transcript. The 5' end of the ODC mRNA was determined by amplification and sequencing of the 5' terminus of the mature ODC transcript. The reverse transcription was performed at 42°C for 60 min in the presence of 300 ng random hexanucleotides, 125 nM of each dNTP, 20 units human placenta ribonuclease inhibitor, 50 μg total RNA from *C. fasciculata*, and 50 units of avian myeloblastosis virus reverse transcriptase in a total volume of 20 μl. To amplify the 5' end of the *C. fasciculata* ODC cDNA, 2.0 μl of the reverse transcriptase reaction was added to a PCR containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μM of each dNTP, 20 pmol of each oligonucleotide primer, and 2.6 units of *Taq* DNA polymerase/*pwo* DNA polymerase (expand high fidelity PCR system, Boehringer Mannheim). The sense primer, 5'-CCA-TCTAGAGCTATATAAGTATCAGTTTCTGTA-3', contained 27 nt of the 39-nt mini-exon that is trans-spliced onto the 5' end of the transcript (27, 28). The antisense primer, 5'-CGCACCATCGGCAACTCGTGCC-3', contained a nucleotide sequence from the coding region of the *C. fasciculata* ODC gene. The PCR program included a primary denaturation for 5 min at 95°C and 30 cycles at 96°C, 45°C, and 72°C for 15, 30, and 60 s, respectively, followed by a final extension at 72°C for 5 min. The PCR product was then further amplified by nested PCR using two new antisense oligonucleotides, 5'-TCTGGATGTACACGTTGGAGAGG-3' and 5'-GGCAGAGAAGCTCATCCCC-3'. The PCR resulted in a product of about 900 bp, which was subcloned into the pCR II vector using a TA cloning kit (Invitrogen) and then sequenced as described above.

Expression of ODCs from *C. fasciculata*, *T. brucei*, and *L. donovani* in COS Cells. As has been demonstrated for many other transcripts from trypanosomatids, the translational initiation sequences of ODCs from *C. fasciculata* (present study), *T. brucei* (29) and *L. donovani* (19) do not comply with the Kozak consensus sequence (30, 31). Thus, to be able to express these ODCs in a mammalian cell we modified the context of the initiation codon to CCACCATG by PCR. The modified ODC DNAs were then subcloned into the mammalian expression vector pSVL.

For transfection, COS cells were harvested during exponential growth and resuspended in 0.8 ml fresh growth medium at a density of 10×10^6 cells/ml. The transfection was performed by electroporation. The cells were mixed with 15 μ g of DNA and then pulsed with 0.3 kV at 250 μ F. Following a 5-min recovery period at room temperature the cells were seeded in fresh medium at a density of 28,000 cells/cm². The cells were analyzed 2 days after transfection. The turnover of ODC was determined by following the decay of ODC activity after cycloheximide treatment (50 μ g/ml).

ODC Assay. The COS cells were sonicated in ice-cold 0.1 M Tris-HCl (pH 7.5) containing 0.1 mM EDTA and 2.5 mM dithiothreitol, and the debris was sedimented by centrifugation at 30,000 $\times g$ for 20 min. The expression of *C. fasciculata* ODC in the COS cells, which was much less than that of *T. brucei* ODC or *L. donovani* ODC, was determined after precipitation of the endogenous COS ODC using an excess of a specific antibody against mammalian ODC (32). The ODC activity was determined by measuring the release of ¹⁴CO₂ from L-[1-¹⁴C]ornithine in the presence of saturating levels of pyridoxal 5'-phosphate (0.1 mM) and L-ornithine (0.5 mM). Protein concentrations were determined by the method of Bradford (33).

Computer Analysis. The sequence analyses were performed with the GAP/BESTFIT comparison program and the multiple sequence alignment program PILEUP of the Wisconsin package, Genetics Computer Group.

RESULTS

Isolation of the ODC Gene from a *C. fasciculata* Genomic Library. A suitable probe for screening of the *C. fasciculata* λ GEM-11 genomic library was obtained by amplifying an ODC-specific DNA fragment from *C. fasciculata* by PCR using degenerate oligonucleotides based on conserved amino acid sequences in ODC molecules from various eukaryotes. We obtained a 689-bp PCR product that was subcloned and partly sequenced. The sequence revealed a high homology to the ODC gene from *L. donovani* (results not shown), strongly indicating that the PCR product contained a sequence from the *C. fasciculata* ODC gene. This PCR product was then used to screen the genomic library. Out of 50,000 screened plaques about 10 were positive. One of the positive plaques, containing a clone with a \approx 12-kb insert, was used for further analysis. A 4.5-kb restriction fragment of the clone was selected for subcloning and sequencing.

Sequencing of the ODC Gene from *C. fasciculata*. The nucleotide sequence of the *C. fasciculata* ODC clone was found to contain a single long ORF of 2163 bp encoding a protein of 721 aa with a calculated molecular mass of 78,913 Da (Fig. 1). The deduced amino acid sequence showed a high homology with ODCs from other eukaryotes (Fig. 2). In addition to the ORF, \approx 1100 and \approx 350 nt of the 5' and 3' flanking regions, respectively, were sequenced from the clone. Analysis of the codon usage revealed that the ORF of *C. fasciculata* ODC, like those of other genes from *C. fasciculata* and *L. donovani*,



FIG. 1. Nucleotide and amino acid sequences of the *C. fasciculata* ODC gene and its predicted protein product. The 2163 nt ORF is numbered starting with the A in the first ATG. The 721 aa are numbered with the initiating methionine as number 1. The sites of the two degenerate PCR primers used for obtaining the ODC-specific probe are underlined. Nucleotides 5' to the splice acceptor site are printed in lowercase letters.

<i>C. fasciculata</i>	MSNHVSLSN ASYHNHANYT ELVPLEPTIAQ TPTHFAHDE NHVTITENGV TMTTTSAAA MMTATAPLPV SPVHAFDEAA QRRLLARKTA LLEQYRMYPO SADRLLAQRR HHEASHLRDA -120
<i>L. donovani</i>	MGDHDVALCH VSRYNHANYT AFVPLETVSD DTGCDLSLHD SASE RIRMAPPASA SK AGAA EERLHPYERR LLDQYQIHLQ PANRNFLSRA DSAAGREETA -100
<i>T. brucei</i>
Mouse
PEST I	
<i>C. fasciculata</i>	EDSDGAALSP SILSPDTCNK TTAAYAQTVA MSPNEKGGAD RHTVPAASQD LVDLFFLEGS DAVNGMSFSA YPIYGERTAA ERRAAVRAVF EKYGVDMQLP ASPAVLAAAF AHRQRRFRV -240
<i>L. donovani</i>	QSDGHDVALCH VSRYNHANYT AFVPLETVSD DTGCDLSLHD SASE RIRMAPPASA SK AGAA EERLHPYERR LLDQYQIHLQ PANRNFLSRA DSAAGREETA -200
<i>T. brucei</i>
Mouse
PLP	
<i>C. fasciculata</i>	LP...RDSVE TREYQWRRLS NVYIQKGVK...ATMAAANP TGSN...K PAYEAEDPFY MIDLGRVVEQ MARWRHELPM VRPFAVKCN PNLMAIEVLG ALGAGFDCA KDIHLVLDN -350
<i>L. donovani</i>	IAPINKSALE TREYQWRRLS NLYTQKGVKD AASAADAAT TATNGAVPAA PAYEPDPPY IIDLGRVVEQ MARWRHELPM VRPFAVKCN PNPFAVLEVLG ALGAGFDCA KEEIHMVLGR -320
<i>T. brucei</i>M DIVNDDLSC RFL.EGFNTR DALCKKIS.M NTCDEGDPFF VADLDGIVRK HETWKKLPR VTFPFAVKCN DDWRVLGTLA ALGTGFDCA NTEIQRVGR. -98
MouseM SSFTKDEFDC HILDEGFTAK DILDQKINEV SSSDDKDAFY VADLDGILKK HLRWLKALPR VTFPFAVKCN DSRAIVSTLA AIGTGFDCAS KTEIQLVQG. -100
PEST II	
<i>C. fasciculata</i>	HLVSDPDDIV FANPCKQLGD LREARACGVT YVTVDNVPEM EKIGRMLPSA RAIIRIKTND AAKCAFSTK FGAGMDDVES LLQAARQHN EVYGVSFVHG SGNGDQSAVY SALRDSYRVE -470
<i>L. donovani</i>	QLVASPDDII FANPCKQLGD LREARACGVT YVTVDNPEM EKISRMLPSA HAIIRIKTND SKAQCSFSTK FGAPLEDVHG LLEAARQHN TVCGVSFVHG SGNGDQSAVY SAVRDAYQVE -440
<i>T. brucei</i>	.IGVPEKII YANPCKQVSH IRYARSDGVD VMTFDCEGVD EKVAKTHPKA KMLVRISTDD SLARCLRSVK FGAKVEDCRF ILEQAKKLI DVTCVSFVHG SGSDTSFVFA QAISDSRVE -217
Mouse	.LGVPAERVI YANPCKQVSO IKYASNSGVQ MMTFDESEIL MKVARAHPKA KLVLRITADD SKAVCLRSVK FGATLTKSRL LLERAKELNI DVIQVSFVHG SGCTDPTFV QAVSDARCVF -219
DFMO	
<i>C. fasciculata</i>	QLAAGYGFNC TVLDIGGGYP GTEPIPGSGE TSFETIARAM RPVLEELFGG G.DVTIIEEP GRYFTAATHA LAMNVFATRT LRLSDVEKEN CQAFQNVNVM DEPEEYQYVY NDGLYHSFNC -589
<i>L. donovani</i>	QQAVQYGFNC TILDIGGGYP GTEVEGSGN TSFEIARTI RPVLAEELFGG G.DVTIIEEP GRYFTAASHA LLMNVFASRT LRLSDVEKVR .QAFQSVNVM DEPEEYQYVY NDGLYHSFNC -558
<i>T. brucei</i>	DMGTGLGNM HILDIGGGYP GTR.....DAF LKFEIAGVI NNALEKHPF DKLITVAEP GRIVYASAFI LAMVIAKVV TFGVQTDVGA HAE..... SNAQSFMYV NDGVYGSFNC -326
Mouse	DMATEVGFNM HILDIGGGYP GSE.....DTK LKFEIITSVI NPALDKYFYS DSGVRIIAPF GRIVYASAFI LAVNIIAKTI VWKQEGSD EDE..... SNEQTFFMYV NDGVYGSFNC -328
PEST I	
<i>C. fasciculata</i>	IVFDHAHPTL LLLNDGDGAD DTAEGVANAA VDDVDSEEG EPTVDGAPTN DSELFVANDR RRLNTRRPLR ITTIFGPTCD SMDCILKQOP FPPEMLGDWL LAPDMGSYTT AAGCFNNGFA -709
<i>L. donovani</i>	ILFDHAHPTL LLLNDGDGAD GVESGTEAAA ...VCSEEG ETSLSGLAN DLEFMSANDR RRSFARRPLR ITTIFGPTCD SMDCILKQOP FPPEMLGDWL LVPDMGSYTT AAAGFNGFA -675
<i>T. brucei</i>	ILYDHAVVRP LQPRE.....PIEN EKLYPSS.....VWGPTCD GLDQIVERY LPEMQVBEWL LFEEDMGAYTV VGSSEFNGFO -399
Mouse	ILYDHAHVKA LIQKR.....PKPD EKYYSS.....IWGPTCD GLDRIVERCN LPEMHVGDWM LFENMGAYTV AAASFNGFO -401
DFMO	
<i>C. fasciculata</i>	TRRREWSSV AL*..... -721
<i>L. donovani</i>	TRRLEWSSV DLCARPRVY TREGNTRCV SE*..... -707
<i>T. brucei</i>	SPTIYVVSF LPHVVRBELK SQKS*..... -423
Mouse	RPNIYVMSR PMWQLMKIQI SHGFPEVEVE QDDGTLPMSQ AQESGMDRHP AACASARNTV * -461

FIG. 2. Comparison of the amino acid sequences of ODC proteins from *C. fasciculata*, *T. brucei*, *L. donovani*, and mouse. The deduced amino acid sequences were compared using the multiple sequence alignment program PILEUP. Amino acids that are identical in all of the species are printed in red. Amino acids that are not identical in all four species but identical in *C. fasciculata* and *L. donovani* are printed in blue. The binding sites for the cofactor pyridoxal 5'-phosphate and the irreversible inhibitor DFMO (34) are indicated. The PEST regions in *C. fasciculata* ODC are indicated by green lines.

contains an over-representation of G or C at the third codon position (35). About 85% of the codons used contain a G or C residue as the third nucleotide in the *C. fasciculata* ODC gene.

The predicted amino acid sequence of *C. fasciculata* ODC is longer than that of ODC from other species, including *L. donovani*. ODCs from *C. fasciculata* and *L. donovani* (19) consist of 721 and 707 aa, respectively, whereas the enzyme from mammals (36), yeast (37), *T. brucei* (16, 29), *Neurospora crassa* (38), *Xenopus laevis* (39), and the nematode *Panarellus redivivus* (40) contains between 423 and 484 aa. Comparison of the amino acid sequence for ODC from *C. fasciculata* with those of other eukaryotic species reveals several conserved regions (Fig. 2). The sequence PHFAVKCN, which resembles a consensus sequence of PXXAVK(N), probably contains the lysine (K) to which the pyridoxal 5'-phosphate cofactor binds (34). The sequence GPTCD is conserved in all eukaryotic ODCs sequenced so far and contains the cysteine (C) that is likely to be the major binding site of the irreversible inhibitor DFMO (34). The deduced amino acid sequence of *C. fasciculata* ODC contains a ≈ 250 aa N-terminal extension, which is absent in the mouse ODC. The amino acid sequence of *C. fasciculata* ODC shows a marked homology with that of *L. donovani* ODC (Fig. 2). Optimal alignment gives a 69% identity between the two sequences. The homologies between the amino acid sequence of *C. fasciculata* ODC and those of mouse, yeast, and *T. brucei* ODC are much lower, with identity scores of 37–40%.

The C terminus of *C. fasciculata* ODC is shorter than those of ODCs from *L. donovani* and *T. brucei* (Fig. 2). Compared with mouse ODC, the *C. fasciculata* ODC lacks 48 of the C-terminal amino acids whereas the ODCs from *L. donovani* (19) and *T. brucei* (16) lack 28 and 36 aa, respectively. This region of the mammalian ODC has been demonstrated to be essential for the extremely fast turnover of the protein (14), which may explain the finding that ODCs from *L. donovani* (19) and *T. brucei* (16) are proteins with a very slow turnover.

Analysis of the ODC Gene Locus in *C. fasciculata*. Several of the protozoan parasites have some of their genes organized in tandemly repeated arrays (41, 42). To achieve information on the organization of the ODC gene locus in *C. fasciculata* we

analyzed genomic DNA using Southern blot analysis. The DNA was digested with restriction enzymes that either did not cut at all (*EcoRI*), or cut only once (*PstI* and *SacI*), within the ODC gene. After blotting, the restriction fragments were hybridized to the labeled 689-bp PCR product that was used for the isolation of the ODC gene. As shown in Fig. 3, *PstI*, which cuts within the sequence corresponding to the probe, gave rise to three fragments; one ≈ 4 kb and the others ≈ 2 kb each. The 4-kb fragment probably resulted from incomplete cutting of the DNA, because the amount of this fragment varied greatly between experiments. *SacI* (which does not cut within the region covered by the probe) and *EcoRI* both gave rise to a single band of two different sizes (≈ 6 and ≈ 15 kb, respectively). Taken together these results suggest that the ODC gene is not arranged in tandemly repeated units but may be present as a single copy in the genome of *C. fasciculata*. However, a titration of the gene copy number is needed for ultimate confirmation.

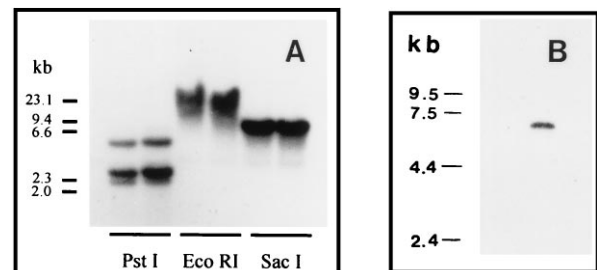


FIG. 3. Southern (A) and Northern (B) blot analyses of DNA and RNA from *C. fasciculata*. (A) Genomic DNA from *C. fasciculata* was digested with *PstI*, *EcoRI*, and *SacI* and fractionated on a 0.8% agarose gel. The digest was blotted to a Hybond N membrane and hybridized to a 32 P-labeled *C. fasciculata* ODC-specific PCR probe. The size markers were *HindIII*-digested λ DNA. (B) Total RNA from *C. fasciculata* was fractionated on a 1% agarose gel containing 2.2 M formaldehyde, blotted to a Hybond N membrane, and then hybridized to a 32 P-labeled *C. fasciculata* ODC-specific PCR probe. Mammalian ribosomal RNA and a commercial RNA ladder (GIBCO) were used as size markers.

Analysis of the ODC mRNA in *C. fasciculata*. Northern blot analysis of total RNA from *C. fasciculata* revealed a single transcript of ≈ 6.5 kb (Fig. 3). The maturation of mRNAs in *C. fasciculata*, like in other trypanosomatids, involves a trans-splicing event in which a 39-nt mini-exon is added to the 5' end of the transcript (27, 28). To determine the site for this splicing event we amplified and sequenced a region of *C. fasciculata* ODC cDNA corresponding to the 5' terminus of the mature ODC transcript. One of the primers used for amplification contained part of the 39-nt mini-exon and the other one contained a sequence from the ORF. The PCR gave rise to one major amplification product of about 900 bp in size. Sequencing revealed that the site for the trans-splicing of the mini-exon to the ODC transcript was located at nt -301 from the predicted translation initiation site, thus giving rise to a 5' untranslated region of 340 nt.

Turnover of *C. fasciculata* ODC Expressed in a Mammalian Expression System. *C. fasciculata* is the only protozoan known to have an ODC that is turning over rapidly (22). However, as revealed in the present study, *C. fasciculata* ODC lacks the region corresponding to the C terminus of mammalian ODC, which has been shown to be essential for the fast turnover of the enzyme (9, 14, 17, 18). This finding indicates that the "C-terminal rule" of ODC, in which the rapid turnover of the enzyme is dependent on the presence of a specific C-terminal region, is not generally applicable. It is conceivable, though, that the rule is only valid in mammalian cells and thus, when expressed in such a system, *C. fasciculata* ODC would be metabolically stable. To determine the turnover of *C. fasciculata* ODC in a mammalian cell we subcloned the ODC gene from *C. fasciculata* into the mammalian expression vector pSVL and then expressed it transiently in COS cells. To discriminate between endogenous COS ODC and expressed *C. fasciculata* ODC, we used a specific antibody against mammalian ODC (32). This antibody does not react with *C. fasciculata* ODC but only precipitates the COS ODC. As shown in Fig. 4, transfection of COS cells with the expression vector containing the ODC gene from *C. fasciculata* gave rise to ODC activity in the COS cells that was not precipitated by the ODC antibody, demonstrating the accuracy of the clone and the efficiency of the system. The turnover of *C. fasciculata* ODC in the COS cells was determined by measuring the decay of nonprecipitable ODC activity after inhibition of protein synthesis by cycloheximide. As seen in Fig. 5, *C. fasciculata* ODC turns over rapidly, with a half-life of about 1 h. In comparison, ODCs from *T. brucei* (Fig.

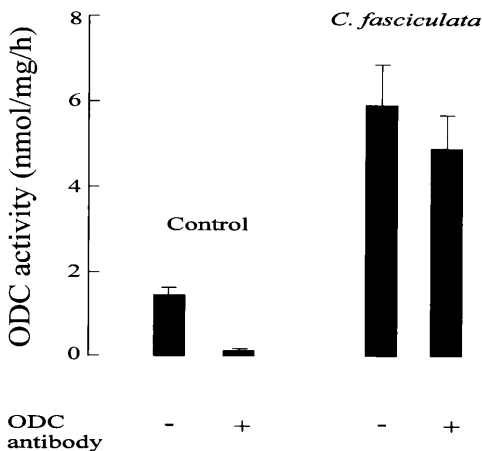


FIG. 4. Expression of *C. fasciculata* ODC in mammalian cells. COS cells were transfected with pSVL (control) or pSVL containing the ODC gene from *C. fasciculata*. Two days after transfection the cells were harvested and analyzed for ODC activity. In some samples, ODC activity derived from endogenous COS ODC was immunoprecipitated using an antibody specific against mammalian ODC (mean \pm SEM, $n = 6$).

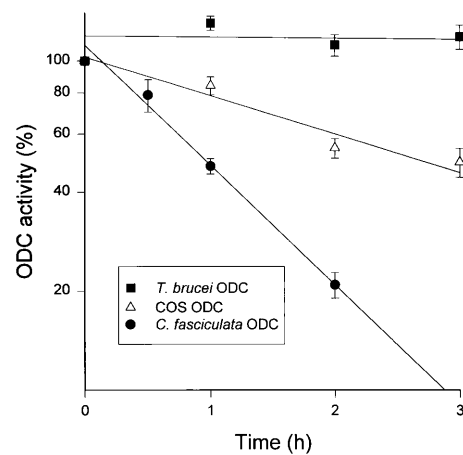


FIG. 5. Turnover of *C. fasciculata* ODC and *T. brucei* ODC expressed in mammalian cells. COS cells were transfected with pSVL or pSVL containing the ODC gene from *C. fasciculata* or *T. brucei*. The turnover of ODC in the cells was determined by following the decay of ODC activity after addition of cycloheximide. The *C. fasciculata*-specific ODC activity was determined after immunoprecipitation of the endogenous COS ODC using an antibody specific against mammalian ODC. The fraction of endogenous ODC in the COS cells expressing the *T. brucei* ODC was less than 1% (mean \pm SEM, $n = 3-5$).

5) and *L. donovani* (not shown) were found to be stable proteins when expressed in the COS cells. The half-life of the endogenous COS ODC was estimated to be about 3 h.

DISCUSSION

ODC is among the most labile enzymes in the mammalian cell with a half-life often shorter than 1 h (2). The molecular mechanisms as well as the necessary sequences for this rapid degradation are presently being unraveled. It has previously been shown that removal of or mutations in the C terminus of mammalian ODC can transform the protein into a stable one (14, 18, 43). Moreover, when this region of mammalian ODC is fused to stable proteins it confers a rapid turnover rate to these proteins (17, 18, 44). That the C terminus of mammalian ODC is indeed essential for the rapid turnover of the enzyme is also supported by the findings that ODCs from *T. brucei* (16) and *L. donovani* (19), which are stable proteins, lack the corresponding region. Based on these results it has been assumed that the C terminus is necessary for a rapid turnover of the protein in mammalian cells (9, 18). However, we now show that the *C. fasciculata* ODC lacks this region but nevertheless turns over rapidly in the parasite (22) as well as in mammalian cells. Thus, it appears that the function of the C-terminal domain of mammalian ODC in the rapid turnover of the enzyme is taken over by some other part(s) of the *C. fasciculata* ODC.

Although information on the size of the ODC expressed in *C. fasciculata* is lacking, the ORF shown in Fig. 1 is most probably the one used *in vivo*. The following observations support this notion: (i) the predicted initiation codon is the first ATG 3' to the splice acceptor site, (ii) all reading frames 5' to this ATG contain multiple stop codons; (iii) there is a codon bias for G or C as the third nucleotide also in the codons encoding the N-terminal part of the enzyme, and (iv) the deduced amino acid sequence corresponding to the sequence between the first and the second ATG of the *C. fasciculata* ODC gene is highly homologous to the N terminus of the *L. donovani* ODC.

C. fasciculata ODC shows a relatively high homology to the core region of mammalian ODC (Fig. 3). The main differences are found in the N-terminal and in the C-terminal regions. The N terminus of *C. fasciculata* ODC is extended by 256 aa as

compared with that of mouse ODC. *L. donovani* ODC also has a long (219 aa) N-terminal extension, whereas the *T. brucei* ODC does not. The C terminus of mammalian ODC contains one of the two PEST regions of the enzyme (17). The PEST sequences are regions identified in proteins with a rapid turnover (15). Although *C. fasciculata* ODC lacks the C-terminal PEST region of mammalian ODC, it contains two regions which fulfil the requirements of PEST regions; the regions between aa 118–140 and 596–649. Especially the latter one appears to be a very strong PEST sequence. Interestingly, these parts of the *C. fasciculata* ODC are missing in the mammalian ODC. *L. donovani* ODC, which is a stable enzyme (19), lacks the region corresponding to the first PEST sequence of *C. fasciculata* ODC, and has a much weaker PEST sequence [according to the algorithm described by Rogers *et al.* (15)] in the region corresponding to the second PEST sequence of *C. fasciculata* ODC.

Mammalian ODC is degraded by the 26S proteasome (13, 45) in an energy-dependent but ubiquitin-independent process (12). Thus far, ODC and c-Jun (46) are the only proteins known to be degraded by this protease system without being ubiquitinated. Instead, degradation of mammalian ODC is stimulated by a specific protein, named antizyme, that binds very strongly to the enzyme and also inhibits its activity (2, 8, 9, 47). It has been suggested that antizyme acts in place of ubiquitin in ODC degradation (9). The recent finding that fusion of the N terminus of antizyme to other proteins induces their rapid degradation is consistent with this hypothesis (48, 49). The antizyme binding site within mouse ODC has been mapped to the region between aa 117 and 140 (47), which corresponds to aa 368–391 of *C. fasciculata* ODC. *T. brucei* ODC, which does not bind antizyme (47), shows a low degree of homology with mouse ODC in this region. Because the homology between *C. fasciculata* ODC and mouse ODC in this part of the enzyme is even lower, it can be assumed that antizyme does not bind to *C. fasciculata* ODC. Although it is not known at what stage of evolution that antizyme appeared, it is conceivable that the trypanosomatids, like *C. fasciculata*, *T. brucei*, and *L. donovani*, do not express this protein. As for *C. fasciculata*, the rapid turnover of its ODC may instead be induced by a different mechanism. Nevertheless, it is clear from the present study that this mechanism is active in the mammalian cell and capable of inducing a rapid degradation of *C. fasciculata* ODC.

In spite of the fact that there is a high homology between the *C. fasciculata* ODC and the *L. donovani* ODC (Fig. 2), there is a marked difference in stability between the two proteins. *C. fasciculata* ODC has a very rapid turnover, whereas *L. donovani* ODC is a stable protein (19). Due to the high homology between the two proteins it should be possible to make various hybrids, which should provide information on what parts of the protein are essential for this “C-terminal-independent” rapid turnover of *C. fasciculata* ODC.

Recent experiments carried out with *Herpetomonas samuelpessoai* have shown that this trypanosomatid parasite, like *C. fasciculata*, contains a metabolically unstable ODC (C.C., unpublished results). Both *C. fasciculata* and *H. samuelpessoai* are monogenetic trypanosomatids parasitizing on a single insect host, whereas *T. brucei*, *L. donovani*, *L. mexicana*, and *P. falciparum* are digenetic parasites with more complex life cycles involving insects and mammals as alternating hosts. The fact that monogenetic parasites, in contrast to digenetic parasites, appear to express metabolically unstable ODCs may be related to environmental differences affecting the parasite's need to rapidly control its synthesis of polyamines or their metabolites. Such polyamine metabolites include the unique conjugate of glutathione and spermidine, called trypanothione, which plays an essential role in protecting the parasite against various stressful conditions (50).

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