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Genomic organisation and transcription characterisation of the gene encoding *Leishmania* (*Leishmania*) *amazonensis* arginase and its protein structure prediction☆

Edson R. da Silva[a], Tiago M. Castilho[a], Fabiana C. Pioker[a], Carlos H. Tomich de Paula Silva[b], Lucile M. Floeter-Winter[a,*]

[a]*Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 1374 São Paulo, SP 05508-900, Brazil*
[b]*Centro Pesquisa, Inovação e Difusão (CEPID-FAPESP), Instituto de Física, Universidade de São Paulo, São Carlos, Brazil*

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Abstract

The genomic organisation of the gene encoding *Leishmania* (*Leishmania*) *amazonensis* arginase as well as its flanking regions were characterised. The size of the transcribed RNA was determined, allowing us to map the genomic sites signalling for RNA *trans*-splicing and putative polyadenylation regions. The general organisation was compared with genes encoding other proteins already described in organisms of the Trypanosomatid family. The complete nucleotide sequence of the arginase open reading frame was obtained and the three-dimensional structure of the enzyme was inferred by a computational analysis of the deduced amino acid sequence, based on the established crystal structure described for *Rattus norvergicus* arginase. The human liver arginase sequence was analysed in the same way and the comparison of the presumed structure of both the *Leishmania* and human enzymes identified some differences that may be exploited in chemotherapeutic studies.

Keywords: Arginase; Trypanosomatids; *trans*-Splicing site; Protein modelling; Chemotherapy; *Leishmania*

1. Introduction

Arginase (L-arginine amidinohydrolase, E.C. 3.5.3.1) is a binuclear manganese metalloenzyme (Reczkowski and Ash, 1992; Kanyo et al., 1996) that catalyses the hydrolysis of L-arginine to L-ornithine and urea. Two distinct isoforms of this enzyme have been described. The best known isoform, which is mainly expressed in the liver, is related to the ornithine–urea cycle or the Krebs–Henseleit cycle. For years, its physiological role has been considered to involve the excretion of urea in ureotelic animals. The other currently described isoform is found in the mitochondria of non-hepatic cells such as macrophages (Cook et al., 1994; Gotoh et al., 1996; Jenkinson et al., 1997; Morris et al., 1997; Chang et al., 1998). Although its physiological role is not yet well defined, its activity has been implicated in the regulation of nitric oxide (NO) synthesis, modulating the availability of L-arginine and consequently being linked to the cytotoxic processes in immunological mechanisms of protection against infectious diseases (Nathan, 1995).

The activity of arginase in parasitic protozoa of the Trypanosomatidae family was shown to be genus specific and has been used as an identification and classification tool (Yoshida and Camargo, 1978). *Leishmania* is one genus in that family in which arginase activity is detectable and is considered to play a role in ornithine production (Camargo et al., 1978). *Leishmania* species are the causative agents of a large spectrum of clinical diseases, generically known as leishmaniasis, which are a serious health problem in tropical and subtropical regions (Ashford, 2000). As they are obligate intracellular parasites of macrophages, studies on their mechanisms of survival ultimately invoke cytokine activation of these cells mediated by NO (Reiner and Locksley, 1995). The ability of NO to kill infectious agents has usually been demonstrated by adding nitric oxide synthase (NOS) inhibitors that abolish both NO and N^{ω}-hydroxy-L-arginine generation (Assreuy et al., 1994). A defence mechanism against *Leishmania* infection is mediated by N^{ω}-hydroxy-L-arginine, produced by NOS-II, through the inhibition of arginase from parasites (Iniesta et al., 2001).

☆ Nucleotide sequence data reported in this paper are available in GenBank™ under accession number: AAC95287.

* Corresponding author. Tel.: +55-11-3818-7329; fax: +55-11-3818-7329.

E-mail address: lmfwinte@icb.usp.br (L.M. Floeter-Winter).

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Besides these taxonomic and physiological interests, the molecular characterisation of the arginase-encoding gene is of interest for the study of its use as a chemotherapeutic target. So, based on conserved regions of the nucleotide sequence of some eukaryotic arginases already described (Sumrada and Cooper, 1984; Haraguchi et al., 1987; Ohtake et al., 1987; Xu et al., 1993), we designed two degenerated oligonucleotides and used them in a polymerase chain reaction (PCR) with *Leishmania* (*Leishmania*) *amazonensis* genomic DNA as template. The complete gene was then isolated from a *L.* (*L.*) *amazonensis* cosmid library (Uliana et al., 1999) using the PCR fragment as a probe. Chromosomal localisation and Southern blot analysis were used to determine the genomic organisation of the gene in *L.* (*L.*) *amazonensis* and in two other *Leishmania* species, *Leishmania* (*Leishmania*) *major* and *Leishmania* (*Leishmania*) *mexicana*. The characterisation of the transcribed product revealed that *L.* (*L.*) *amazonensis* utilises at least four *trans*-splicing signals in the maturation of arginase mRNA, independently of the life cycle stage. Finally, the complete determination of the nucleotide sequence of the *L.* (*L.*) *amazonensis* arginase coding region allowed the prediction of the enzyme structure and the comparison of functional sites with those described for human arginase.

2. Materials and methods

2.1. Organisms

Promastigotes of *L.* (*L.*) *amazonensis* MHOM/BR/1973/M2269, *L.* (*L.*) *mexicana* (MNYC/BZ/1962/M379) and *L.* (*L.*) *major* (MHOM/IR/1983/LT252), from the collection of the Department of Parasitology, University of São Paulo were grown at 25°C in M199 medium (Gibco-BRL). Amastigotes of *L.* (*L.*) *amazonensis* MHOM/BR/1973/M2269 were recovered from in vitro infection of J774A.1 macrophages cultured in RPMI medium (Gibco-BRL) at 35°C.

2.2. Purification and analysis of nucleic acids

DNA was purified and handled as previously described (Uliana et al., 1991). Total RNA was obtained using the TRIZOL (Gibco-BRL) reagent protocol and fractionated under glyoxal denatured conditions (Mcmaster and Charmichael, 1977; Sambrook et al., 1989). The poly A + fraction was fractionated in oligo dT column (Chirgwin et al., 1979).

2.3. Oligonucleotides, PCR and reverse transcription/PCR

Two degenerated oligonucleotides were design based on preferential codon usage for *Leishmania* (Langford et al., 1992). Their positions are indicated in Fig. 1 and their sequences are: ArgF:5′ T(C/G/T)TGG(A/G)T(C/G/T)GA-(C/T)GC(C/G)CA(C/T)(A/G)C(C/G)GA(C/T)AT(C/T)AA-(C/T)AC(C/G) 3′ArgR: 5′ AG(A/G)CC(A/G)CC(C/G)(A/C)(C/G)C/G)AC(C/G)GG(A/C)GT(A/G)CC(C/G)GT(C/G-)GC(C/G)GG 3′. These primers were used for standard PCR (30 cycles; 94°C for 60 s, 54°C for 60 s, 72°C for 60 s) to amplify genomic DNA.

Two additional primers, also shown in Fig. 1, were designed to amplify the open reading frame (ORF) of the arginase. *Eco*RI restriction sites (underlined) were added to the sequence to help with cloning strategies. They are – Arg5L: 5′GGAATTCATGGAGCACGTGCAGCAGTAC-AA 3′ and Arg3L: 5′GGAATTCCGTCCCAGACACA-CGCTTGAGG3′. PCR conditions for these primers were 30 cycles; 94°C for 60 s, 60°C for 60 s, 72°C for 60 s.

For the reverse transcriptase (RT)-PCR assay 5 μg of total RNA, obtained as described in Section 2.2, were incubated with 200 U of SuperScript II RT (Gibco-BRL) and oligonucleotide Arg3L as primer. The RT reaction was performed at 42°C for 50 min. The enzyme was heat inactivated and 10% of the reaction product was used in the following 35 cycles of PCR reaction, 94°C for 60 s, 60°C for 60 s, 72°C for 120 s, using the primers:

MEDL: 5′CGCTATATAAGTATCAGTTTC3′ and ARG-SC3: 5′GTTCGGCTTCAGCACCTGCGGTAC3′.

2.4. Probe labelling and hybridisation conditions

DNA fragments used as probes were uniformly labelled by random primed synthesis (Feinberg and Vogelstein, 1983, 1984), with MegaPrime (Amersham), in the presence of [α-^{32}P] dATP and [α-^{32}P] dCTP (sp. act. 3000 Ci/mmol, Amersham).

Hybridisation procedures were performed using the 'Rapid Hyb Buffer' as described by the manufacturer (Amersham Pharmacia). Different washing conditions were used to achieve desired stringencies, as indicated in the figure legends.

2.5. Cloning and sequencing strategies

Desired fragments, purified from agarose gels by electroelution, or PCR products were ligated to appropriate sites in pUC 19, pBS (Stratagene), pMOS (Amersham) or pGEM-T Easy Vector (Promega). Nucleotides sequences were determined automatically as described in Sanger et al. (1977) using an ALF Express System (Amersham Pharmacia) or an ABI Prism™ 310 Genetic Analyzer (Perkin–Elmer).

2.6. Pulse field gel electrophoresis

Chromosomes of *L.* (*L.*) *amazonensis*, *L.* (*L.*) *major* and *L.* (*L.*) *mexicana* were prepared by modifications of described methods (Schwartz and Cantor, 1984; Anand, 1986). Agarose blocks containing 6×10^6 promastigotes of each species were submitted to running conditions at 8 V/cm, 10°C, with pulses of 180 s for 18 h, 240 s for 18 h and 300 s for 18 h in 0.5× TBE buffer (45.5 mM Tris, 45.5 mM boric acid, EDTA 1 mM, pH 8.0).

The gel was stained with ethidium bromide and prepared for transfer to a Hybond N$^+$ nylon membrane (Amersham)

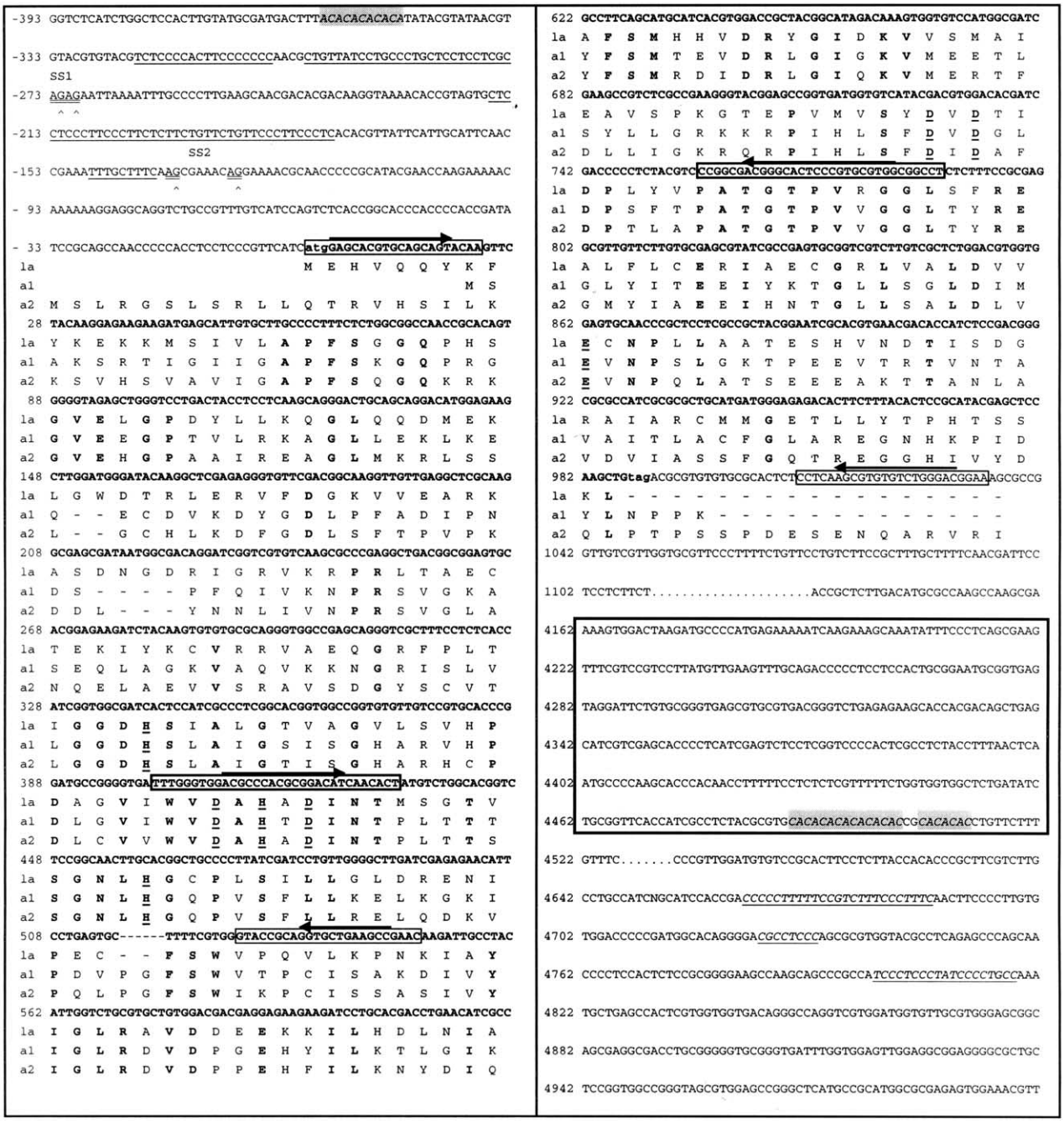

Fig. 1. Nucleotide sequence of *L.* (*L.*) *amazonensis* arginase and comparison of amino acid of *Leishmania* and human arginases. The complete sequence (arginase ORF and neighbouring regions), consisting in 6392 nucleotides, is deposited in GenBank (gb|AAC95287.1| AF038409). Specific regions are indicated in the Figure: the ORF is represented in bold (start codon and stop codon are in lower case, +1 is the A of the start codon). The two major *trans*-splicing signalling regions (SS1 and SS2) are presented double underlined at the dinucleotides 'AG' following the underlined polypyrimidine tracts. The repetitive dinucleotide 'CA' motifs are in italics and grey highlighted. A large box indicates putative polyadenylation region. The oligonucleotides used in PCR or RT-PCR assays are given in boxes: ArgF (nucleotides 401–432), ArgR (788–757), Arg-SC3 (549–526), Arg5L (1–23) and Arg 3L (1034–1010), and the arrows indicate their orientation. Aligned with the nucleotide sequence are the deduced amino acids from *L.* (*L.*) *amazonensis* (la), described in this paper as well as the known amino acids from human arginases, type I (a1) and type II (a2). The amino acids responsible for coordination of the Mn_A^{2+}, His 139, Asp 243 and Asp 245 and Mn_B^{2+}, His 114, Asp 137 and Asp 141 as well as the amino acids responsible for L-arginine interaction, Glu 288 and His 154 are underlined. Identical amino acids amongst the three sequences are in bold.

following a modified protocol from Amersham. Briefly, the gel was irradiated with 240,000 μJ and then treated for DNA depurination in 200 ml of HCl, 0.2 M, with a change after 15 min. DNA was denatured by immersing the gel in 200 ml of 0.5 M NaOH/1.5 M NaCl for 30 min, and the treatment was repeated once. The gel was then neutralised in 200 ml of 0.5 M tris/1.5 M NaCl (pH 8.0) for 30 min and finally left to stand for 30 min in 20× standard saline citrate

(SSC) (3 M NaCl and 0.3 M Na_3.citrate, pH 7.6) before transfer to the nylon membrane.

2.7. Computational analysis of nucleotides and amino acids sequences

GenBank sequence request or submission (Benson et al., 2000) were performed using Entrez, BLAST and Bankit programs (Altschul et al., 1990, 1997; Gish and States, 1993. Clustal-W (Thompson et al., 1994), Esee Eye Ball (Cabot, 1987) and DNA (Schwindinger and Warner, 1984) programs were used for amino acid prediction and sequence analysis. The size of the DNA or RNA molecules fractionated by electrophoresis was calculated using the Kodak Digital Science program.

2.8. Molecular modelling

The frameworks for the models of human and *Leishmania* enzymes were based on the crystal structure for the rat liver arginase (Brookhaven code 1 rla), which shares 86 and 41% sequence identity with human and *L.* (*L.*) *amazonensis* sequences, respectively. Homology modelling was performed by submitting amino acid sequences to URL (http://www.expasy.ch/swissmod/) using the PROMOD program (Peitsch, 1995, 1996; Guex and Peitsch, 1997) and by applying the local MODELLER 6.0 program (Sali and Blundell, 1993) to both human (liver arginase) and *Leishmania* sequences based on their alignments with the rat sequence. The alignments used in the MODELLER 6.0 program were performed with the Clustal-W program (Thompson et al., 1994). The resulting models were evaluated for their residue interactions and atomic contacts and subsequently improved using the DGROTA option of the WHATIF program (Vriend, 1990), for side chain substitutions, and by using the loop searching algorithm of Jones and Thirup (1986), as indicated in the graphic program O (Jones et al., 1991). The stereochemistry of the structures was evaluated with the PROCHECK program (Laskowski et al., 1993). Residue environment analysis was performed with the VERIFY_3D program, which uses an implementation of the algorithm of Luthy et al. (1992), and atomic contacts were assessed with the QUALTY option of the WHATIF program.

3. Results

3.1. Cloning and determination of Leishmania arginase nucleotide sequence

The amino acid sequences of arginase from *Homo sapiens* (Haraguchi et al., 1987), *Rattus norvergicus* (Ohtake et al., 1987), *Xenopus laevis* (Xu et al., 1993) and *Saccharomyces cerevisiae* (Sumrada and Cooper, 1984) were retrieved from the GenBank data base and aligned using Esee Eye Ball or Clustal-W programs. The degenerate oligonucleotides ArgF and ArgR (see Section 2.3) were then designed from the conserved regions highlighted in Fig. 1. The PCR fragment of 388 bp obtained with *L.* (*L.*) *amazonensis* DNA as template was cloned into the pMOS-blue vector (Amersham). Nucleotide determination of the cloned fragment confirmed its similarity with the arginase sequence. This fragment was further used as probe in the screening of a cosmid genomic library of *L.* (*L.*) *amazonensis* (Uliana et al., 1999). The cosmid DNA was then characterised and a restriction map was drawn. Fig. 2A shows a schematic representation of the genomic locus, the restriction sites of the initial cosmid clone that allowed sequencing strategies and the probe used in the genomic organisation study.

The complete nucleotide coding region and deduced amino acid sequence of the arginase as well as the nucleotide sequence of neighbouring upstream and downstream regions is shown in Fig. 1. From the initial ATG to the stop codon TAG, the ORF is 990 nucleotides long. The analysis of codon usage indicated the same relations as previously described (Langford et al., 1992). The other signalling sites such as the *trans*-splicing acceptor site and polyadenylation site region will be discussed in Section 3.3.

Based on the nucleotide sequence for *L.* (*L.*) *amazonensis*, two oligonucleotides, delimiting the ORF (see Section 2.3), were synthesised and used to PCR *L.* (*L.*) *mexicana* genomic DNA. The amplified fragment was cloned and the determination of nucleotide sequence revealed a composition almost identical to that of *L.* (*L.*) *amazonensis*. Twelve differences were detected at the nucleotide level (T63C, T69C, T510C, G258A, G933A, G987A, A663G, C750T, C922T, A917T, G921C) resulting in only two amino acid changes (D306V, nucleotide 917; R308C, nucleotide 922), corresponding to a nucleotide similarity of 98.8% between the arginase sequence of the two organisms.

3.2. Genomic organisation of arginase gene in Leishmania

Genomic DNA from *L.* (*L.*) *amazonensis* was single and double digested with the restriction enzymes *Bg*/II, *Kpn*I, *Pst*I and *Sph*I. These enzymes were chosen since their localisation in the arginase ORF was known by sequence analysis and therefore could be used as references in mapping the sites localised upstream and downstream to the ORF (Fig. 2A). Digestion with these enzymes also produced restricted fragments employed as anchor probes to determine the orientation of outside sites.

Fig. 2B shows the Southern blot of *L.* (*L.*) *amazonensis* fractionated DNA probed to fragment *Pst*I/*Sac*I (Fig. 2A). The general restriction pattern indicated a single, or few copy genes presenting a homogeneous genomic organisation. Besides the dominant bands detected, that certainly correspond to arginase-coding sequences, the probe also detected a faint band in some of the digests, especially with *Pst*I, even in high stringency washing conditions. This fragment probably represents a sequence sharing

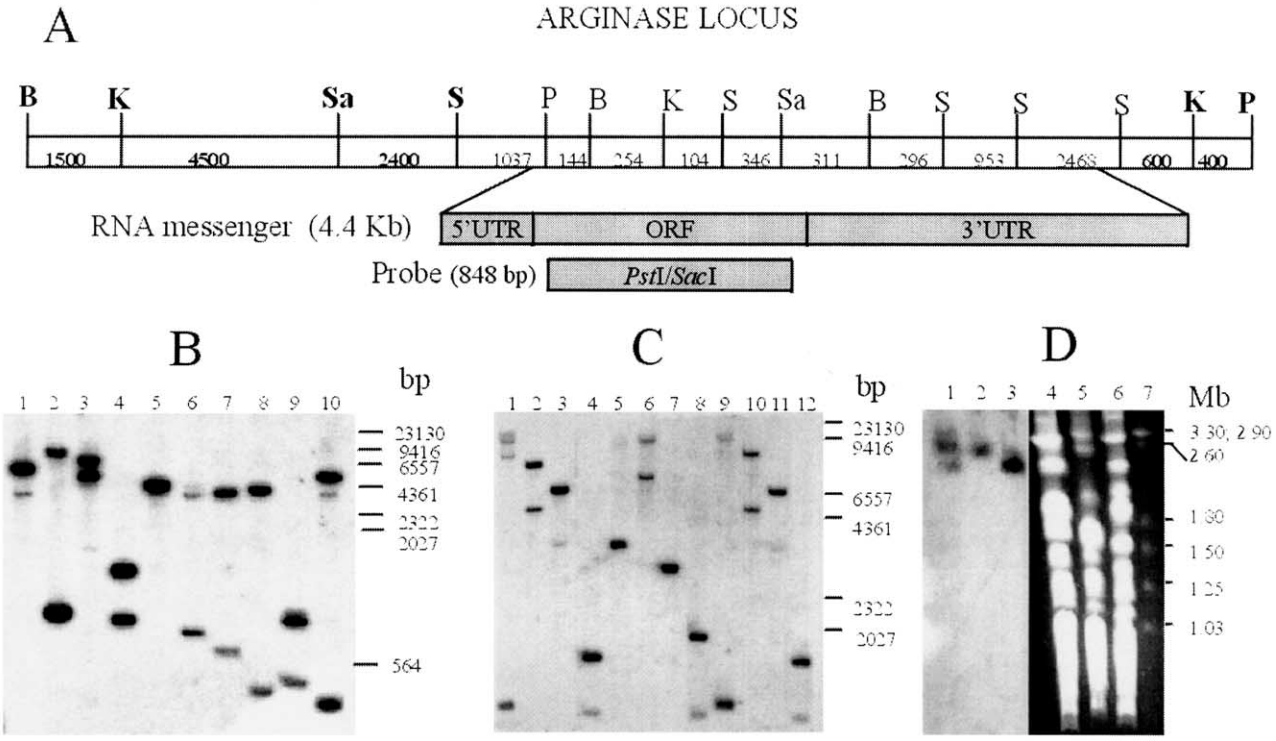

Fig. 2. Genomic organisation of *L.* (*L.*) *amazonensis* arginase-coding region. (A) Genomic restriction map: *Bgl*I (B), *Sac*I (Sa), *Kpn*I (K), *Sph*I (S) and *Pst*I (P) indicate the position of restriction site at the sequence, deduced by Southern blot analysis (bold) or by sequence analysis (regular). The number in the boxes indicates the fragment size in bp. The fragment used as probe as well as boxes indicate the transcribed region below the map. (B) Southern blot analysis carried out with samples of 10 μg of genomic DNA from *L.* (*L.*) *amazonensis* were digested with the following restriction enzymes (1) *Pst*I, (2) *Bgl*II, (3) *Kpn*I, (4) *Sph*I, (5) *Sac*I, (6) *Pst*I/*Sac*I, (7) *Bgl*II/*Sac*I, (8) *Kpn*I/*Sac*I, (9) *Sph*I/*Pst*I, (10) *Kpn*I/*Pst*I. The fragments were fractionated on 1.2% agarose gels and probed with fragment *Pst*I/*Sac*I (Fig. 2A) using the following washing conditions: 2× standard saline citrate, 1% sodium dodecyl sulphate, 60°C. The lines indicate the migration of λ-*Hin*dIII markers. Exposure time was 66 h. (C) Comparative arginase genomic organisation was carried out by Southern blot containing samples of 10 μg of genomic DNA from *L.* (*L.*) *amazonensis* (lanes 1–4), *L.* (*L.*) *major* (lanes 5–8) and *L.* (*L.*) *mexicana* (lanes 9–12), digested with *Bgl*I (1, 5 and 9), *Kpn*I (2, 6 and 10), *Pst*I (3, 7 and 11) and *Sph*I (4, 8 and 12). The products were fractionated on 0.8% agarose gels and the Southern blot was washed as described in (B). The lines indicate the migration of λ-*Hin*dIII markers. Exposure time was 66 h. (D) Agarose blocks containing approximately 6×10^6 *L.* (*L.*) *amazonensis* (lane 1), *L.* (*L.*) *major* (2) and *L.* (*L.*) *mexicana* (3) promastigotes were submitted to pulse field gel electrophoresis, as described in Section 2.6. The fractionated chromosomes were blotted and washed as described for (B/C). The membrane was exposed for 72 h. Lane 7 shows the migration of molecular marker from *Hansenula wingei* (MegaBase™ IV DNA standard-Gibco-BRL).

some similarity with arginase, whose identity is unknown or which may be a gene family related to arginase (Perozich et al., 1998). Indeed, a GenBank search for sequence similarity pointed to several other known sequences, however, only the isolation and characterisation of the nucleotide sequence of this fragment could elucidate its identity. It is interesting to note that a *L. major* sequence deposited in GenBank (accession number AL121851/CAB58296) shows 41% identity of nucleotides and possibly is the coding sequence for agmatinase, an enzyme that belongs to the polyamine pathway and converts agmatine (produced from arginine by arginine decarboxilase action) to putrescine. This enzyme could be a member of the arginase family.

Genomic DNA of *L.* (*L.*) *major* and *L.* (*L.*) *mexicana* was also restricted with the same enzymes and probed to the same fragment (Fig. 2C). The comparison of the hybridisation patterns amongst the three *Leishmania* that infect mammals showed a conserved genomic organisation of the arginase gene between *L.* (*L.*) *amazonensis* and *L.* (*L.*) *mexicana*. The faint band pattern was also observed in *L.* (*L.*) *mexicana* DNA, but not in *L.* (*L.*) *major* DNA that showed distinct restriction patterns.

The use of the same probe on pulse field fractionated chromosomes indicated sequence complementation with only one band of 2.2 Mb for *L.* (*L.*) *major* and 2.0 Mb for *L.* (*L.*) *mexicana*, and with two bands of 2.4 and 1.8 Mb for *L.* (*L.*) *amazonensis* (Fig. 2D). This last observation could be due to the sequence detection in homologues at different stages of condensation or due to the presence of the gene sequence in two distinct chromosomes. This heterogeneous distribution pattern observed for *L.* (*L.*) *amazonensis* chromosomes may also be due to the presence of similar DNA sequences, as detected in the Southern blot.

3.3. Arginase transcription pattern

Total and poly A+ enriched RNA obtained from promastigotes of the three *Leishmania* species were fractionated and the Northern blot was probed with the *Pst*I/*Sac*I fragment (Fig. 2A). A prevalent RNA molecule of 4.4 kb

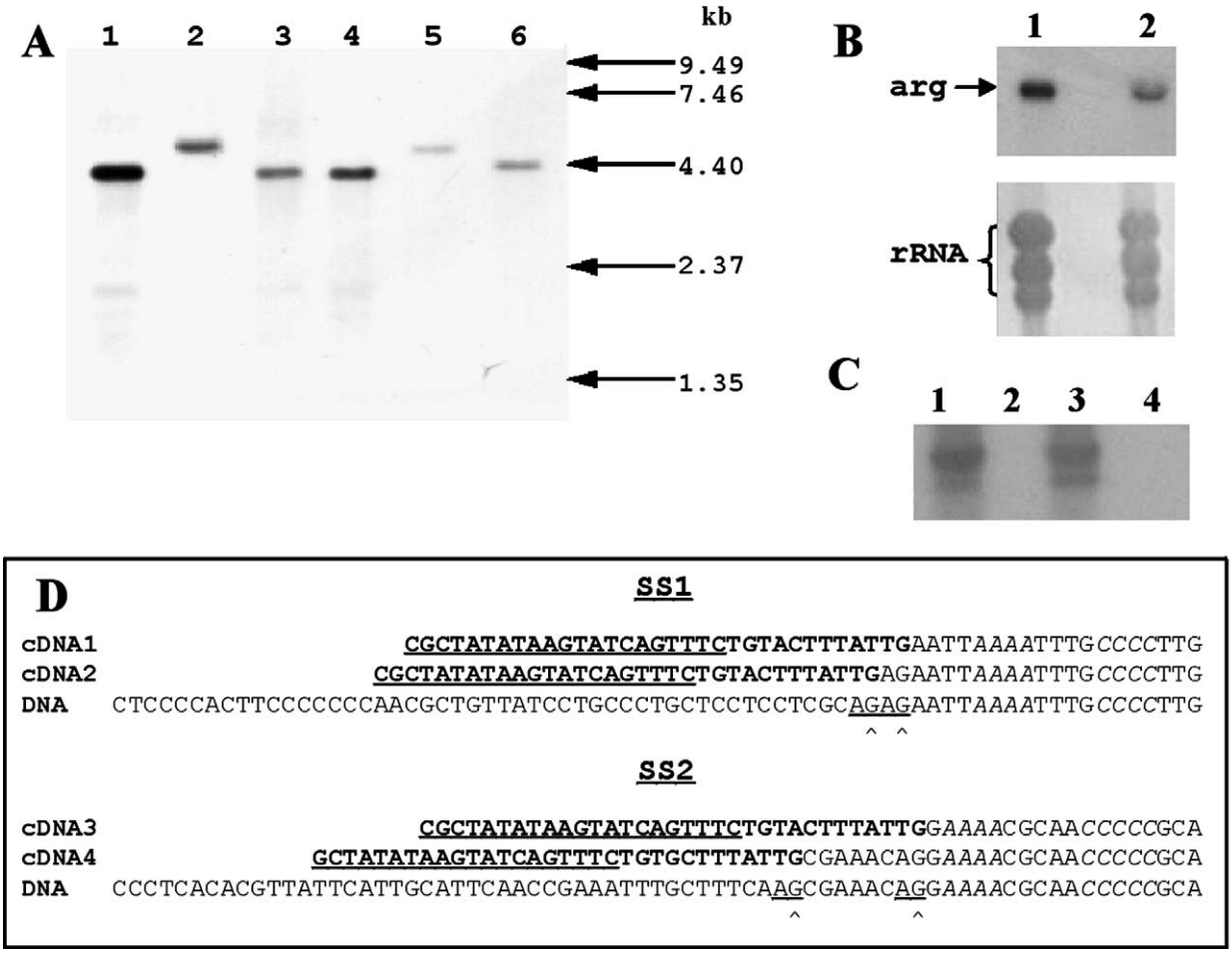

Fig. 3. Northern blot of *Leishmania* total and poly A + enriched RNA and the *trans*-splicing sites. (A) Total (10 μg; lanes 1–3) and poly A + RNA (purified from 10 μg of total RNA; lanes 4–6) from *L.* (*L.*) *amazonensis* (1; 4), *L.* (*L.*) *major* (2; 5) and *L.* (*L.*) *mexicana* (3; 6) promastigotes, respectively, fractionated on 1% agarose gel and blotted onto a nylon membrane, were probed to the *Pst*I/*Sac*I fragment (Fig. 2A) with rapid-hyb buffer (Amersham) and then washed twice in 2× standard saline citrate for 10 min at room temperature, and twice in 2× standard saline citrate, 1% sodium dodecyl sulphate, for 20 min at 65°C. Exposure time: 7 days. Molecular markers: RNA Ladder (Gibco-BRL). (B) Total RNA (10 μg) from promastigotes (1) and amastigotes (2) of *L.* (*L.*) *amazonensis* fractionated and probed as described in A. rRNA insert represents the loading control stained with methylene blue. (C) Agarose gel fractionation of RT-PCR products with (1, 3) or without (2, 4) reverse transcription of RNA purified from amastigotes (1, 2) or promastigotes (3,4) of *L.* (*L.*) *amazonensis*. (D) The *trans*-splicing sites of the arginase gene were mapped by sequencing RT-PCR products. The joined mini-exon RNA (bold) and the 5′ untranslated region of arginase are depicted as cDNA (1–4) and aligned with the genomic sequence to indicate the *trans*-splicing acceptor site for each major *trans*-splicing signalling region (SS1 and SS2, Fig. 1). The MEDL primer that corresponds to part of the splice-leader sequence is underlined in all cDNA sequences; the AG dinucleotides are underlined in the DNA sequence; and the 'AAAAN$_{4-6}$CCCC' motif is indicated in italics.

was detected, as seen in Fig. 3A, for *L.* (*L.*) *amazonensis* and *L.* (*L.*) *mexicana*. In *L.* (*L.*) *major* RNA, a larger molecule of 5.0 kb was detected. The presence of a single band in both total and poly A + enriched RNA preparations indicates that it is not a precursor molecule but a mature mRNA (Fig. 3A). Besides molecule length difference, the lower intensity of hybridisation observed for *L.* (*L.*) *major* RNA allowed to infer that sequence similarity is higher between *L.* (*L.*) *amazonensis* and *L.* (*L.*) *mexicana* than between *L.* (*L.*) *amazonensis* and *L.* (*L.*) *major*. Using higher stringency conditions of washing hybridisation (0.1× SSC, 1% sodium dodecyl sulphate at 65°C) the *L.* (*L.*) *major* was not detected (not shown). In fact, the use of previously described ORF primers in a PCR using genomic *L.* (*L.*) *major* DNA as template was not successful. The faint bands detected in Fig. 3A probably correspond to an unspecific hybridisation of the probe with the large amount of rRNA present in the samples since they co-migrate with that molecules and were eliminated using the higher stringency conditions (not shown).

Northern blotting of RNA purified from the amastigote stage of *L.* (*L.*) *amazonensis*, recovered from in vitro infection of J774A.1 macrophages, also resulted in the same mRNA molecule detected in promastigotes (Fig. 3B). The comparable intensity of the hybridisation signal indicates that arginase expression is not stage regulated. *Leishmania*

(*L.*) *amazonensis* was also cultivated in the presence of 2.3 or 5.0 mM of L-arginine, conditions that induce arginase expression (Yoshida and Camargo, 1978). Northern blots of RNA from the log phase of those cultures, probed as described earlier, produced the same mRNA bands; however, the arginase mRNA concentration did not change in the induced cells (data not shown).

To detect possible *trans*-splicing acceptor sites present upstream of the ORF, we used RT-PCR strategy as an approach to correctly map the 5′ end of arginase mRNA. A cDNA template was obtained by reverse transcription primed by Arg3L, located at the 3′ end of the ORF (see Fig. 1). To ensure anchoring to the transcribed ORF, a subsequent PCR was driven with the pair of primers ARG-SC3 nested in relation to the cDNA template (Fig. 1) and a mini-exon sequence based primer (MEDL). Agarose gel fractionation of PCR products revealed at least two major bands that did not appear in the negative control not submitted to reverse transcription (Fig. 3C). The determination of nucleotide sequence of PCR products, shown in Fig. 3D, indicated the existence of at least four positions signalling *trans*-splicing and the consequent addition of mini-exon RNA. By analysing these sites in Fig. 1, pyrimidine tracts followed by the dinucleotide AG were mapped into two major regions, SS1 (−272 and −270) and SS2 (−137 and −129). The same RT-PCR strategy was applied to amastigote RNA and the amplified products produced the same pattern as observed for promastigote RNA (Fig. 3C). This observation indicates that, besides the absence of a stage regulated transcription, the use of *trans*-splicing sites is also non-stage specific.

Considering the Northern blot result together with RT-PCR results, the polyadenylation site was estimated to be about 3150 nucleotides downstream from the stop codon. In fact, in that position, a putative polyadenylation site, presenting motifs expected for poly A + addition in *Leishmania* (Ullu et al., 1996), was observed (box in Fig. 1). The nucleotide sequence flanking that site presented pyrimidine tracts and AGs (Fig. 1), possibly indicating *trans*-splicing signalling for the neighbour gene.

3.4. Comparison of Leishmania and human arginase primary and 3D structures

Using the established crystal structure data for the rat enzyme (Kanyo et al., 1996), we performed a simulation of the 3D structure of both human and *Leishmania* arginases. The main aspects of this simulation are represented in Fig. 4. The final models obtained with the PROMOD program were of better quality than those obtained with MODELLER 6.0. They presented good stereochemistry, as evaluated by the PROCHECK program, with final *G*-factors of 0.25 for the human model and −0.21 for the *Leishmania* model, values better than those expected for a

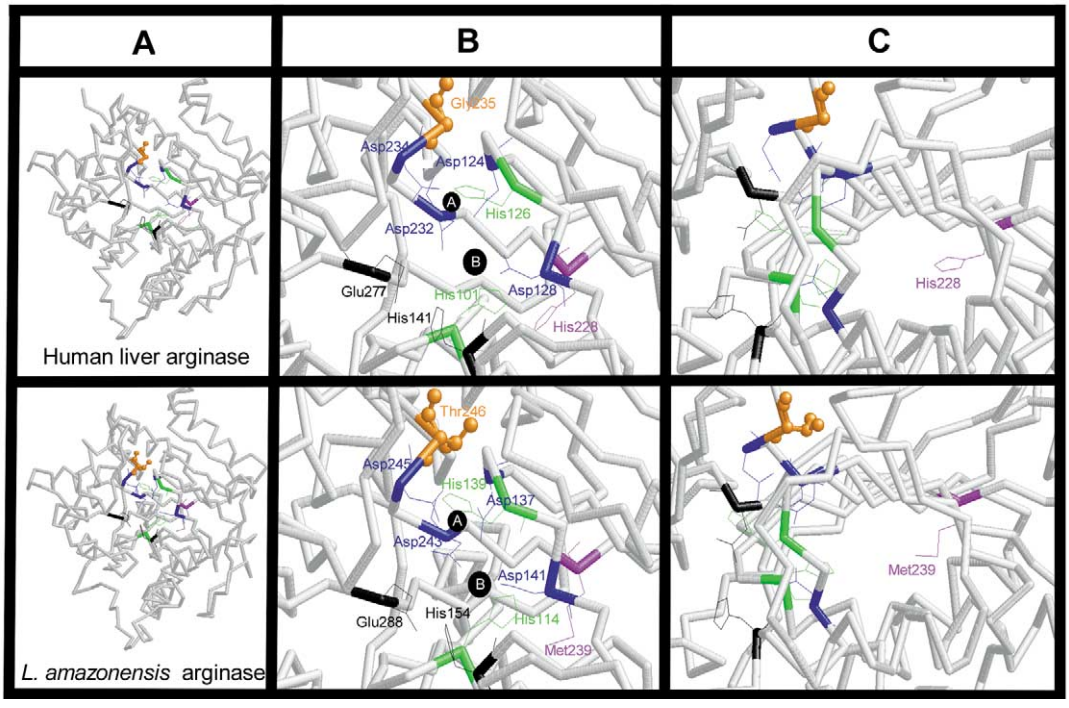

Fig. 4. *Leishmania* (*L.*) *amazonensis* and human liver type arginase 3D models. (A) The complete monomer model of both human and *L.* (*L.*) *amazonensis* enzymes. Amino acids that participate in the active site are indicated by colour. (B) Detail of the binuclear Mn^{2+} cluster (spheres A and B) and of the amino acids that directly interact with substrate in the active site. The coordinating of each Mn^{2+} atom (black sphere A and B) is made by two Asp residues (blue) and one His (green) residue. The His and Glu (black) bound direct to the substrate. (C) Difference in the space 'channel-like' structure caused by the presence of Met 239 (magenta) instead of His 228 (magenta), observed in human.

crystal structure at 2.1 Å; ideally, *G*-factors should be above −0.5 (Jones et al., 1991). Residue environment analysis (VERIFY_3D) resulted in a final index of 142.0 (human model) and 138.31 (*Leishmania* model), slightly worse than the expected mean value of 142.9 and 149.82, respectively, but fitting in the range for good quality structures (above 45% of the expected value). Atomic contact quality, as measured by the QUALTY module of the WHATIF program, gave final scores of −0.816 (human model) and −1.137 (*Leishmania* model), lower values than the threshold number (−1.2). Only scores showing higher values than that index indicate that the structure should be treated with caution. The value of −1.137 for the *Leishmania* model is an acceptable result, particularly when one considers the reduced sequence identity of the starting structure (rat liver arginase) with the *Leishmania* enzyme. The same criteria were used to evaluate the quality of the trimer model for *Leishmania* arginase, which presents G-factor of −0.20 and QUALTY index of −0.977.

At the primary sequence level, the alignment of the deduced amino acid sequences of *Leishmania* and human (liver and macrophage) arginases, presented in Fig. 1, disclosed a high degree of similarity, mainly at the catalytic centre. The amino acids that coordinate Mn^{2+} and the L-arginine binding in the active site of arginase in the rat (Kanyo et al., 1996) were localised in *L.* (*L.*) *amazonensis*. Figs. 1 and 4 identify His 139, Asp 243 and 245 that coordinate the Mn_A^{2+}; His 114, Asp 137 and Asp 141 that coordinate Mn_B^{2+} and His 154 and Glu 288 that are directly involved in L-arginine binding.

The monomer model of the two organisms and details regarding the active site showed they were highly conserved (Fig. 4A). However, differences could be seen between two non-conserved equivalent amino acids His 228 and Met 239, respectively, in human and *Leishmania* arginases forming a differential space channel-like structure (Fig. 4C). A difference in charge in a cleft could also be detected. The Gly 235 of the human arginase is uncharged, whereas in the equivalent position, *Leishmania* arginase presents a polar uncharged Thr 246 (Fig. 4B). These differences in the neighbourhood of the active site are not conserved and could be explored in the design of specific *Leishmania* arginase inhibitors.

4. Discussion

Southern and chromosomal blotting analysis showed a closer genomic organisation of arginase-coding sequence in *Leishmania* (*L.*) *amazonensis* and *L.* (*L.*) *mexicana* than in *L.* (*L.*) *major*. However, in the three species it is a low copy number gene restricted to a few chromosome loci. Indeed, the ORF of *L.* (*L.*) *amazonensis* and *L.* (*L.*) *mexicana* are almost identical (98.8% similarity). When compared with the arginase sequence described for other organisms, the ORF presents variable degrees of similarity. The deduced amino acid sequences presented a high level of conserved residues, mainly at the active site of the enzyme. The comparison of primary amino acid sequences of arginase from *L.* (*L.*) *amazonensis* with the two types of human arginase, type I (liver) and type II, showed 43 and 39% identity, respectively, or 58 and 54% similarity, if we consider the conservation of the amino acid character. The similarity of *Leishmania* arginase to human liver arginase (type I) greater than to that of the macrophage (type II) is interesting because of the functional and inducible properties of these enzymes. Murine resistance or susceptibility to the *Leishmania* infection is regulated by immune response Th1 or Th2 type inducing NOS II or arginase I, respectively (Nathan, 1995).

The transcription product of the gene in *Leishmania* is a molecule about four times larger than the ORF. The mRNA maturation follows the pattern already described for trypanosomatids. Polycistronic transcription usually produces a pre-mRNA in which ORF are separated by an intergenic spacer. The mature mRNA is generated by two RNA processing reactions, the regular *trans*-splicing or addition of the 5′ mini-exon and the polyadenylation at the 3′ end (Ullu et al., 1996). The putative signals for both events were found in the boundaries of the arginase coding mRNA.

The data presented here show that the region required for *trans*-splicing, consisting of the AG dinucleotide following the polypyrimide tract, appeared more than once. In fact, the presence of more than one *trans*-splicing site for a single copy gene has been described before (Stiles et al., 1999; Nepomuceno-Silva et al., 2001), although its meaning is uncertain. In the evolutionary process, polycistronic transcription could lead to an accumulation of processing sites in intergenic regions to ensure that one of them would be correctly used. All mapped sites are located downstream of polypyrimidine tracts (as expected) and interestingly, upstream to an $AAAA_{N4\text{-}6}CCCC$ motif. Exonic sequences have been associated with *trans*-splicing (López-Estraño et al., 1998), but other experiments should be performed to evaluate the possible functional role of this motif.

The presence of a common repetitive dinucleotide CA motif may indicate the poly A + addition site at the 3′ end of the mature RNA. It is interesting to note that the same motif is also found at the 5′ region and is already related to regions of RNA processing (Landfear et al., 1986; Kapler et al., 1990; Kawazu et al., 1997), upstream to the mapped *trans*-splicing signalling regions (SS1 and SS2). This observation could indicate the polyadenylation site of the 5′ neighbour gene. Moreover, in agreement with the polycistronic transcription behaviour, the 3′ polyadenylation site was closely linked to another motif that could be signalling for *trans*-splicing. It has been demonstrated that the accurate choice of the poly A + site and the polyadenylation itself are coupled to downstream *trans*-splicing and the polypyrimidine tract could act by regulating both mechanisms (LeBowitz et al., 1993; Matthews et al., 1994).

The functional meaning of the almost 3.4 kb non-trans-

lated regions of arginase is not known, but neither a stimulation of mRNA expression nor a stage-specific transcription regulation could be detected. This suggests that the functional role of the enzyme may not be involved in stage-specific regulation of its mRNA maturation. Similarly, characterisation of the mRNA product of the arginase gene did not provide any clue about any regulation involving a particular functional role.

The initial characterisation of the arginase gene described in the present work is essential for the elucidation of the functional role of the enzyme. Iniesta et al. (2001) showed that N^{ω}-hydroxy-L-arginine produced by NOS-II of activated macrophage, blocks the growth of *Leishmania* inhibiting arginase. The characterisation of the complete arginase gene allowed the construction of arginase knock out *Leishmania* mutants or mutants showing an overexpression. The possible phenotypic changes caused by the absence or overexpression of the arginase gene will yield significant information about the functional role of the enzyme.

The alignment of the deduced amino acid sequences of *Leishmania* and human (liver and macrophage) arginases is presented in Fig. 1. The high degree of similarity, mainly at the catalytic centre can be clearly seen. The amino acids that coordinate Mn^{2+} and the L-arginine binding in the active site of arginase in the rat (Kanyo et al., 1996) were localised in *L.* (*L.*) *amazonensis*. Figs. 1 and 4 show His 139, Asp 243 and 245 that coordinate the Mn_A^{2+}; His 114, Asp 137 and Asp 141 that coordinate Mn_B^{2+} and His 154 and Glu 288 that are directly involved in L-arginine binding. These amino acids are conserved in all arginases that have been compared to date (Perozich et al., 1998), but the variation of amino acid composition shows that the *Leishmania* enzyme is closer to the human liver arginase (type I) than to the human macrophage enzyme (type II).

Using the crystal structure data established for the rat enzyme (Kanyo et al., 1996), we performed a simulation of 3D structure of both human and *Leishmania* arginases.

The increase in the atomic contact score for the model of *Leishmania* arginase, particularly for the trimer in comparison with the monomer model, suggests the in vivo existence of a trimeric structure for the *Leishmania* arginase. In addition, Arg 308 residue nucleates a network of salt bridges such as those observed in rat liver arginase (Lavulo et al., 2001). This residue forms an intramonomer salt bridge with Glu 262 and an intermonomer salt bridge with Asp 204 on the adjacent subunit. Both Glu 262 and Asp 204 residues are conserved, as is the case for rat, human, mouse and *Xenopus* arginases. These observations suggest a similar role in stabilising the oligomeric states of these enzymes. In *Leishmania* arginase, the Glu 262 and Asp 204 (positions 273 and 215) are conserved, but Arg 308 is absent. On the other hand, in many arginases from bacterial sources, enzymes which are hexameric (a dimer of trimers) rather than trimeric, these residues are not conserved (Perozich et al., 1998).

The various and small manual adjustments performed in the main chain at the S-shaped tail did not compromise this suggestion because the tail is not considered critical for the maintenance of the structural integrity of the arginase or for optimal activity, but Arg 308 plays a important role in stabilising the overall oligomer (Lavulo et al., 2001).

All of the evaluation methods used here suggest that the structure of both human and *Leishmania* arginase present no serious stereochemical problems or physico-chemical anomalies, supporting the reliability of the proposed models for a simple structural analysis of their active sites. The main aspects of this simulation are presented in Fig. 4. The monomer model for the data of the two organisms and details of the active site were highly conserved (Fig. 4A). However, differences could be seen between two non-conserved equivalent amino acids, His 228 and Met 239, respectively, in human and *Leishmania* arginases, forming a differential space channel-like structure (Fig. 4C). A difference in charge in a cleft could also be detected. The Gly 235, in human arginase is uncharged, whereas in the equivalent position *Leishmania* arginase presents a polar uncharged Thr 246 (Fig. 4B). These differences in the neighbourhood of the active site are not conserved and can be explored in the design of specific *Leishmania* arginase inhibitors.

The expression of the *L.* (*L.*) *amazonensis* arginase in a bacterial host is also being developed. The production of large quantities of purified enzyme will be used in crystallographic experiments. The analysis of the crystal will provide data for precise studies of its structure and the subsequent elaboration of specific inhibitors.

In the arginase superfamily proteins, the active site, including coordination of divalent metal Mn^{2+}, is conserved in all organisms (Perozich et al., 1998). Three-dimensional models for *Leishmania* and human arginases were constructed using the Promod Modelling Server. The comparison of these models will be explored, particularly to search for spatially significant differences in the neighbourhood of the active site, and to plan specific inhibitors of *Leishmania* arginase.

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