Published in final edited form as:

Int J Parasitol. 2009 February ; 39(3): 267–279. doi:10.1016/j.ijpara.2008.06.015.

***Schistosoma mansoni* arginase shares functional similarities with human orthologs but depends upon disulphide bridges for enzymatic activity**

Jennifer M. Fitzpatrick^{a,2}, Jose M. Fuentes^{b,2}, Iain W. Chalmers^{a,1}, Thomas A. Wynn^c, Manuel Modolell^d, Karl F. Hoffmann^{a,1}, and Matthias Hesse^{c,e,*}

^aDepartment of Pathology, University of Cambridge, Tennis Court Road, CB2 1QP, UK

^bCIBER de Enfermedades Neurodegenerativas (CIBERNED), Departamento de Bioquímica y Biología Molecular y Genética, EU Enfermería y TO, Universidad de Extremadura, Cáceres, Spain

^cImmunopathogenesis Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases (NIAID), NIH, Bethesda, MD, USA

^dMax-Planck-Institut für Immunbiologie, Freiburg, Germany

^eDepartment of Microbiology and Immunology, Cornell University College of Veterinary Medicine, VMC C5 147, Ithaca, NY 14853, USA

Abstract

Schistosome helminths constitute a major health risk for the human population in many tropical areas. We demonstrate for the first time that several developmental stages of the human parasite *Schistosoma mansoni* express arginase, which is responsible for the hydrolysis of L-arginine to L-ornithine and urea. Arginase activity by alternatively activated macrophages is an essential component of the mammalian host response in schistosomiasis. However, it has not been previously shown that the parasite also expresses arginase when it is in contact with the mammalian host. After cloning and sequencing the cDNA encoding the parasite enzyme, we found that many structural features of human arginase are well conserved in the parasite ortholog. Subsequently, we discovered that *S. mansoni* arginase shares many similar molecular, biochemical and functional properties with both human arginase isoforms. Nevertheless, our data also reveal striking differences between human and schistosome arginase. Particularly, we found evidence that schistosome arginase activity depends upon disulphide bonds by cysteines, in contrast to human arginase. In conclusion, we report that *S. mansoni* arginase is well adapted to the physiological conditions that exist in the human host.

Keywords

Helminth; Metabolism; Enzyme; Evolution; Adaptation; Urea; Trematode; Drug target

1. Introduction

Parasitic worms comprise an important group of human pathogenic organisms affecting the lives of approximately 2 billion people mostly in developing countries in the tropics and sub-tropics (Savioli et al., 2002). One specific genus within Platyhelminths, the *Schistosoma*, causes chronic inflammation of the liver, intestine or bladder for about 200 million people in 74 countries. Current treatment programs depend upon chemotherapy mostly with praziquantel, or in some areas with oxamniquine or metrifonate, which effectively kills adult worms in established infections. Although the drugs are still very effective, there are unquestionable reasons for investigating and establishing new means of treatment. The current chemotherapy does not provide prophylactic protection and re-infections occur frequently after treatment. Concerns about the development of praziquantel-resistant schistosomes give new urgency for investigating potential new drug targets (Doenhoff et al., 2002; Cioli et al., 2004). The characterisation of pivotal metabolic pathways in schistosome species is essential for the identification of new targets.

The hydrolysis of L-arginine to L-ornithine and urea by arginase is a fundamental catabolic process as indicated by the ubiquity of this enzyme in the biosphere (Jenkinson et al., 1996). Arginase family members are represented in species from all three major lineages (archaea, eubacteria, eukaryotes) of life on earth (Ouzounis and Kyrpides, 1994). In higher vertebrates the best-described function of arginase is the detoxification of ammonia as an essential enzyme of the urea cycle in the liver (ureotelic organisms). Although ureotelic organisms express two or more isoforms of arginase, only one participates in the urea cycle. Additional functions for arginase in these organisms include the production of L-ornithine as a precursor for the generation of glutamate, L-proline and polyamines or the control of nitric oxide (NO) production by extra-hepatic arginase in various tissues. The latter affects different physiological functions such as macrophage activation (Modolell et al., 1995) and smooth muscle cell contraction (Christianson, 2005).

The function of arginase in microbes and invertebrates is mostly unknown. It is speculated that the urea cycle evolved from a biosynthetic pathway for L-arginine and appeared for the first time in amphibians as an adaptation to air-breathing in a terrestrial environment (Takiguchi et al., 1989). Many invertebrates are uricotelic organisms and eliminate excess nitrogen in the form of solid uric acid (Jenkinson et al., 1996). Nevertheless, a large number of uricotelic organisms possess arginase. It is speculated that their generation of L-ornithine by arginase feeds into the production of L-proline, glutamate and polyamines used for collagen synthesis, energy metabolism and cell proliferation (Jenkinson et al., 1996). Urea can also regulate osmosis (Yancey et al., 1982). Recently, an arginase family member was identified and characterised in the trematode parasite *Fasciola gigantica* (Mohamed et al., 2005), a close relative of schistosomes. Here, the enzyme seems to be essential for nitrogen metabolism.

Host L-arginine metabolism has a pivotal role in murine models of schistosomiasis. NO-production by classical activated macrophages can kill the parasite during the early invasive stage of the infection (James and Glaven, 1989; Oswald et al., 1994; Wynn et al., 1994; Ahmed et al., 1997; James et al., 1998) but causes enhanced liver damage in later disease stages (Herbert et al., 2004). Upregulated expression of arginase-1 by alternatively activated macrophages can limit this NO-mediated damage, but can also contribute to egg-induced fibrosis and granuloma formation during chronic schistosomiasis (Hesse et al., 2001; Pesce et al., 2006). Essential functions for pathogen-expressed-arginase in the survival and virulence of mammalian pathogens have been reported for *Helicobacter pylori* (Gobert et al., 2001; Zabaleta et al., 2004) and the kinetoplastid parasites *Leishmania mexicana* and *Leishmania major* (Kropf et al., 2003; Roberts et al., 2004). Hence, we speculated that schistosome species possess their own arginase. This hypothesis received support from previous data, showing that

host-derived L-arginine is relevant for the invading parasite and can be actively taken up by a cationic transporter system (Haas et al., 2002; Skelly et al., 1999).

Our results show that *Schistosoma mansoni* expresses its own arginase (SmARG) and here we detail its biochemical properties and expression pattern. Furthermore, we demonstrate that schistosome arginase is expressed by all life stages in intimate contact with the final mammalian but not with the intermediate molluscan host. The biochemical and structural data indicate that schistosome arginase is highly adapted to a mammalian environment. Although it has many similarities with mammalian arginase, we discovered important differences between these arginase family members that could allow the generation and application of specific inhibitors without affecting the host enzymes.

2. Material and methods

2.1. Parasite material

A Puerto Rican strain of *S. mansoni* was used in this study. Adult schistosomes were perfused from percutaneously infected Tuck Ordinary (TO) mice (Harlan, UK) challenged 2-7 weeks earlier with 125 cercariae (Smithers and Terry, 1965). Cercariae were shed from *Biomphalaria glabrata* intermediate snail hosts. Miracidia were hatched from *S. mansoni* eggs harvested from 7-week-infected mouse livers. Mechanically transformed schistosomula were prepared (James and Taylor, 1976) and cultured at 37 °C in DMEM (Sigma, UK) supplemented with 10% FCS, 2 mM L-glutamine and 100 µg/ml pen/strep for either 18-h or 3 days in an atmosphere of 5% CO₂. Six-day-old schistosomula were obtained from the lungs of infected mice and sporocysts, provided by Dr. Timothy Yoshino (University of Wisconsin, USA), were prepared as previously described (Yoshino and Laursen, 1995). Freshly shed cercariae were separated into heads and tails for arginase activity and Western blot analyses by vigorous vortexing and subsequent separation by density centrifugation with Percoll as described (Lewis, 2001). Linoleic acid stimulated cercaria (culture supernatants and bodies from 2 h cultures) and mechanically transformed schistosomula (culture supernatants and bodies from 3 h cultures) (Lewis, 2001) were also assayed for arginase activity and arginase presence by Western blot, respectively.

2.2. Mouse infections

Female 42-day-old C57BL/6Ai mice were obtained from Taconic Farms (Taconic, Germantown, NY) and housed under specific pathogen-free conditions in a National Institute of Health American Association for the Accreditation of Laboratory Animal Care-approved animal facility. For ear infections, mice were anaesthetised and each ear exposed to 500 cercariae for 20 min. Mice were subsequently euthanised at indicated time points, the ears removed and stored at -80 °C until further processing. The National Institute of Allergy and Infectious Diseases (NIAID) animal care and use committee approved all experimental procedures.

2.3. RT-PCR analysis

For the analysis of murine arginase-1 expression, individual ear samples were homogenised in STAT60 reagent (Tel-Test, Inc., Friendswood, TX) and total RNA isolated by chloroform/ethanol extraction. mRNA was isolated from 50 µg total RNA using magnetic beads technology according to the manufacturer's protocol (Miltenyi Biotec, Inc, Auburn, CA) and transcribed into cDNA. The cDNA (10 µl) was used as template for analysing the expression of murine arginase-1 by quantitative PCR as described using specific primers and the SYBR-Green method (Pesce et al., 2006). Data were normalised to the expression level of the housekeeping gene hypoxanthine-phosphoribosyltransferase (HPRT) in each sample and compared to the expression level for arginase-1 in non-infected control samples using the $\Delta\Delta C_t$ -method as

described by Applied Biosystems for the ABI Prism 7700/7900HT Sequence Detection Systems.

All parasite material was homogenised in TRIZOL reagent (Invitrogen, UK) and total RNA was isolated as recommended by the manufacturer. Potential genomic DNA contamination was prevented by treatment of total RNA with DNase I (Ambion Inc.) prior to reverse transcription, which was carried out as previously described (Wynn et al., 1993). The reverse transcription (RT)-PCR primers for SmARG (5'-GGA TGA AAT GGT CAC GGA GT-3' and 5'-GTC TAA ATC CCG CAA ACC AA-3') and α -tubulin (5'-GGC GGT GGT ACT GGT TCT GGG-3' and 5'-CAT TTA GCG CAC CAT CGA AGC-3') were synthesised at Sigma-Genosys (Cambridge, UK). Reverse transcriptase (-) negative controls were included to detect contaminating genomic DNA. Thirty-one cycles of PCR were used to amplify the SmARG fragment (389 bp), whereas 26 cycles were used to amplify the α -tubulin fragment (325 bp). The PCR conditions and cycle numbers were chosen for a linear relationship between input RNA and final PCR product. All amplicons were electrophoresed on a 2% agarose gel and stained with ethidium bromide. Images were captured by a digital camera and analysed by gel documentation software (Kodak 1D 2.0 electrophoresis documentation and analysis system 120, Eastman Kodak Co., New Haven, CT, USA and NIH Image (version 1.62)). Amplification of α -tubulin served as an internal control for the amount of RNA and cDNA from each sample.

2.4. SmARG cDNA cloning

A *S. mansoni* sequence (T7 end of BAC clone 7D11, CHORI-103 library) sharing high similarity to *Schistosoma japonicum* arginase (Accession No. AAQ16108) was identified from entries maintained at The Institute for Genomic Research (TIGR, <http://www.tigr.org/tdb/e2k1/sma1/>). PCR primers (5'-ACT TGG GCA TGC TGA AGC TA-3' and 5'-GTC GTC CAA CCA CGG TAT TT-3') were designed to amplify a 160 bp fragment from BAC clone 7D11 and this product was used as a template for oligonucleotide design to clone the full-length arginase cDNA. Using gene-specific oligonucleotide primers, the 5' and 3' cDNA sequences encoding the N- and C-terminal domains of SmARG were isolated by 5' Rapid Amplification of cDNA Ends (RACE) and 3' RACE strategies (GeneRacer Kit, Invitrogen, UK) using cercaria RNA. Sequencing of the cDNAs encoding SmARG was performed at the Department of Genetics, University of Cambridge in both orientations using Big Dye v3.1 fluorescent chemistry and an Applied Biosystems 3100 Genetic Analyser.

2.5. Multiple sequence alignment, phylogeny and homology modelling

Multiple sequence alignment of SmARG was performed using the ClustalW program maintained at the EMBL-EBI website (<http://www.ebi.ac.uk/clustalw/index.html>) (Chenna et al., 2003). For phylogenetic reconstructions, arginase protein sequences were aligned using the MUSCLE program maintained at the EMBL-EBI website (<http://www.ebi.ac.uk/muscle/index.html>) (Edgar, 2004) and 162 conserved amino acids residues (selected using gBlocks; (Castresana, 2000) version 0.91b) of each chosen sequence were then used in the analysis. Data were analysed via Bayesian inference using MrBayes (ver. 3.1.1) (Huelsenbeck and Ronquist, 2001) and the Whelan and Goldman (WAG) protein substitution model (Whelan and Goldman, 2001) as previously described (Fitzpatrick et al., 2007). The consensus phylogram, rooted on the two bacterial arginases from *Staphylococcus aureus* and *Haloarcula marismortui*, was subsequently visualised using TreeView (Page, 1996). Human arginase 1 crystal structure (Di Costanzo et al., 2005) was used as a template to build models for SmARG. An alignment of the protein sequences was initially generated by ClustalW and refined manually. Models were created using MODELLER software (Martí-Renom et al., 2000) and the validity of the chosen models were tested using WHATIF (Rodriguez et al., 1998) and ProSA-web (Wiederstein and Sippl, 2007). Models were visualised using MacPyMOL software (DeLano Scientific LLC).

2.6. Cloning, expression and purification of recombinant SmARG

This work was performed as paid service by the core protein expression facility at Cornell University (Ithaca, NY). Briefly, based on the cDNA sequence for SmARG, specific primers were generated (forward: 5'-CGA ATT CTG AAA TCA GTC-3'; reverse: 5'-ATC TCG AGT TAT TGT TTT GTC-3') and the open reading frame (ORF) was amplified by PCR from a template plasmid using a proofreading polymerase. The initiating Met codon was not included and the first Leu (Leu2) codon (TTG) was changed (CTG). The product was inserted into the EcoRI-XhoI sites of the pGEX 4T-2 3C His expression vector. The recombinant protein was expressed in *Escherichia coli* strain DH5 α (Invitrogen, Carlsbad, CA). The bacterial lysate was purified over a GST FF column (GE Healthcare, Piscataway, NJ) in PBS, 1% NP40 and 1 mM DTT and eluted in the same buffer + 10 mM glutathione. The purified protein was then digested with PreScission protease (GE Healthcare), precipitated with 40% ammonium sulphate and dialysed against PBS + 1 mM DTT before running it back through the GST column to remove any residual GST.

2.7. Enzymatic activity, kinetic, pH-dependency and inhibitor analysis

Arginase activity was measured as described by Munder et al. (1998) with some modifications. Briefly, arginine hydrolysis was carried out in a final volume of 200 μ l, containing 0.5 M L-arginine pH 9.7 and 1 mM MnCl₂ at 37 °C for 10 min. After the addition of 800 μ l of an acid mixture containing H₂SO₄ (96%), H₃PO₄ (85%) and bi-distilled H₂O (1:3:7 v/v) and 100 μ l of 3% isonitrosopropiophenone (ISPF, dissolved in absolute ethanol) samples were heated at 100 °C for 30 min. The colorimetric reaction was measured at 540 nm. The calibration curve was prepared with increasing amounts of urea between 1.5 and 120 μ g. One unit (U) of enzymatic activity is defined as the amount of enzyme that catalyses the formation of 1 μ mol urea/min. Kinetic parameters were determined as previously described (Fuentes et al., 1991, 1994a). The effect of pH on arginase activity was tested by incubating recombinant enzyme aliquots for 30 min and in the presence of 1 mM MnCl₂ at different pH values. Acetic/acetate buffer (50 mM) was used in the pH range between 4 and 5, 50 mM Tris/malate between pH 5 and 8 and 50 mM glycine/NaOH between pH 8 and 11. The pH of maximum arginase activity was determined with 0.5 M L-arginine buffered at different pH values between 6 to 12 and in the presence of 1 mM MnCl₂. In all assays, the pH was checked in the reaction medium. Recombinant SmARG activity in the presence of different amino acids and borate (all 10 mM) was investigated at pH 10, 37 °C and with 1 mM MnCl₂ and 0.5 M L-arginine in the reaction mix. Furthermore, recombinant SmARG was incubated with 0.1 mM N^ω-hydroxy-L-arginine (NOHA) or 0.1 mM N^ω-hydroxy-nor-L-arginine (norNOHA) at pH 10, 37 °C for 60 min. Then L-arginine was added at increasing concentrations to treated or non-treated enzyme (control) and urea production determined after 10 min incubation at 37 °C.

2.8. Tissue preparation and protein determination

Deep-frozen ear tissue was crushed with a pestle in an Eppendorf tube (1.5 ml), resuspended in 0.5 ml 50 mM Tris-HCl buffer, pH 7.5, and homogenised with an Ultra-Turrax (IKA, Mühlheim, Germany). After addition of 0.5 ml 0.1% Triton X-100, the tubes were shaken for 10 min at 25 °C and centrifuged at 15,000g. The protein concentration was measured according to Bradford, using Coomassie brilliant blue as described by Bio-Rad.

2.9. Determination of molecular mass, bivalent cation dependency and role of disulphide bonds

The molecular mass of the arginase subunit was determined by SDS-PAGE under denaturing conditions using a 12.5% resolving gel. After Coomassie blue staining, protein bands were compared to Prestained Precision Plus Protein Standard (Bio-Rad).

The molecular mass of native enzyme was calculated by gel filtration in a Sephacryl S-300 superfine column (2.6 × 85 cm) using as standards Ferritin (440 kDa), aldolase (158 kDa), ovalbumin (43 kDa) and ribonuclease (13.7 kDa)

The role of bivalent cations for arginase activity was determined after dialysis for 24 h as previously described (Fuentes et al., 1994b). The enzyme was subsequently incubated with selected cations (10 mM) in 25 mM Tris-HCl pH 7.5 for 2 h at 37 °C. Following incubation, L-arginine was added (0.5 M) and urea production was measured after 10 min. The optimal cation concentration for enzymatic activity was determined by incubating dialysed enzyme with different concentrations of MnCl₂ (from 0.01 to 10 mM). Dialysed enzyme was also used for thermal stability studies.

The effect of sulphhydryl group modifications on arginase activity was studied using DTT, reduced glutathione, 2-mercaptoethanol and iodoacetamide, all dissolved in 25 mM Tris-HCl pH 7.5. Undialysed recombinant enzyme was incubated for 30 min at 37 °C with each of the reagents (10 mM final concentration) before 0.5 M L-arginine was added and the urea concentration analysed after 10 min incubation at 37 °C. Likewise, aliquots of dialysed enzyme were incubated for 30 min at 37 °C with each of the reagents. Then each aliquot was divided into two fractions and enzyme activity was determined directly or after incubation with 10 mM MnCl₂ for 2 h at 37 °C.

2.10. Western blot analysis

Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane as previously described (Fuentes et al., 2000). Primary antibody against Arginase 1 (rabbit polyclonal antibody raised against rat Arginase 1, cross-reacting with several Arginase 1 from other species but not with Arginase 2 (Diez et al., 1994)), was used at a 1/10,000 dilution for specific staining of arginase. Peroxidase-conjugated secondary antibodies (Bio-Rad) (1:5000) and chemiluminescence (ECLTM-plus reagent) were used according to the manufactures recommendation (GE Healthcare) for detection.

3. Results

3.1. Increased schistosome-mediated arginase activity in the skin of infected mice

Recent studies have demonstrated that skin-invading schistosomulae can actively manipulate the response of dermal dendritic cells (Angeli et al., 2001b). Therefore, we speculated that invading larvae might avoid a host-protective classical macrophage response by inducing alternative activation of skin-residing macrophages. To test our hypothesis we infected C57BL/6 mice by exposing their ears to 500 cercariae of *S. mansoni* for 30 min. At indicated time points after infection the mice were euthanised and the ears removed. The ear tissue was processed accordingly and analysed for arginase activity or for arginase-1 mRNA expression using specific primers for murine arginase-1.

We detected significantly enhanced arginase activity immediately after exposure to cercariae and at 12 h after infection (Fig. 1A). At 24 h the activity was highly variable and somewhat reduced and then increased again at 48 h p.i. The higher variability at the later time points could reflect the migratory behaviour of schistosomulae in the skin, which start to leave the tissue at about 24 h p.i. (He et al., 2005). In contrast, there was no increase in murine arginase-1 mRNA expression in infected ears at any time point compared to uninfected ears (Fig. 1B). Since murine arginase-2 is constitutively expressed, these results reveal that elevated arginase activity after infection with *S. mansoni* cercariae does not stem from alternative activation of skin-residing host macrophages or dendritic cells.

Recently, arginase has been isolated and characterised from the trematode parasite *F. gigantica* (Mohamed et al., 2005). There was also some evidence reported for arginase activity in *S. mansoni* some 40 years ago (Senft, 1966) and the NCBI protein database contains a sequence for an arginase-ortholog in *S. japonicum* (AAQ16108). Therefore, we investigated whether parasite arginase could be responsible for the elevated arginase activity in infected skin. When we tested soluble protein extract from cercariae and adult worms, two life stages in direct contact with the mammalian host, we found high arginase activity in the two stages (Fig. 1C). Hence, increased arginase activity in skin during schistosome invasion is likely derived from parasite arginase and not by alternatively activated murine macrophages and/or dendritic cells.

3.2. *Schistosoma mansoni* arginase displays many sequence and structural similarities to human and mouse arginase

To initiate structural and enzymatic characterisation of SmARG, we first identified a *S. mansoni* DNA sequence in the TIGR genomic database displaying high similarities to the sequence of arginase from *S. japonicum* and designed specific primers to amplify a 160 bp fragment. Using the sequence derived from this amplicon, we cloned and sequenced the full-length cDNA for SmARG, which encoded a 364 amino acid protein (NCBI Accession No. AAP94031) (Fig. 2). When aligned to the protein sequence of orthologous *S. japonicum*-arginase (SjARG) and the two human arginase isoforms (HsARG-1, HsARG-2), we discovered many similarities. Particular amino acids with essential functions for the enzymatic activity of mammalian arginase are well conserved in both schistosome species. We detected the six histidine and aspartic acid residues (H101, H126, D124, D128, D232 and D234), which form the catalytic centre of HsARG-1 by binding two manganese ions (Christianson, 2005), in HsARG-2 as well as in both schistosome arginases. Furthermore, histidine 141 (HsARG-1), which has a proposed function as proton shuttle (Di Costanzo et al., 2005), is also preserved in the other three enzymes (His160 (HsARG-2), His171 (SmARG) and His172 (SjARG), respectively). In addition to the similarities for the catalytic centre of arginase, the sequence data reveal that four amino acid residues (R255, E256, E262 and R308), which are important for the subunit interaction in homotrimeric HsARG-1 (Lavulo et al., 2001; Sabio et al., 2001), are also well conserved in all aligned sequences.

Based on the known crystal structure for HsARG-1 (Di Costanzo et al., 2005) we generated a homology model for SmARG (Fig. 3). Similar to our sequence data, our model predicts many structural similarities between the human and the trematode arginase. Most importantly, the model suggests that the six histidine and aspartic acid residues, involved in the binding of the manganese ions in both human arginases, will form an almost identical active site within the SmARG protein. Therefore we expect that SmArg displays many similar functional properties when compared to human arginases. However, further comparison of the two structural models revealed a remarkable difference. In the SmARG model C291 and C332 are found in close proximity suggesting that in the native protein, a potential disulphide bond may form between these two residues. Importantly, the C291 residue is only conserved in both schistosome arginases but not in the two human arginases. However, C332 is substituted by a serine in SjARG and in HsARG-2, while conserved in HsARG-1. Therefore, the ability to form a disulphide bond at this particular location seems to be a specific feature of SmARG.

Adaptation to living conditions in their hosts often causes functional and structural similarities between parasite molecules and their respective host counterparts. We investigated the relation of SmARG with selected arginases from other species in a rooted phylogenetic tree (Fig. 4). Not surprisingly, we found a close relation between the two known arginases in schistosome species. Interestingly, schistosome arginase clusters with arginase from Arthropoda and Vertebrata species but not with exemplary arginase from the phylum Nematoda in our tree.

Arthropoda and Nematoda belong to the same superphylum of Ecdysozoa, while Vertebrata are members of the superphylum Deuterostomia and schistosomes, as platyhelminthes, are part of the superphylum Lophotrochozoa. The relatively close distance between schistosome and mammalian arginase supports our claim that schistosome arginase has adapted well to the physiological environment in their mammalian host. Similar to our data, other phylogenetic analysis show that *Leishmania* arginase clusters with arginase from fungal species (Sekowska et al., 2000) and *Caenorhabditis elegans* arginase clusters closely to bacterial arginases (Perozich et al., 1998), lending strong support to our inferred phylogenetic tree.

3.3. Enzymatic and biochemical characterisation of SmARG indicates a close functional relation to human arginase

Next, we determined essential biochemical and functional features of SmARG. We cloned the full-length ORF into an expression vector and expressed recombinant SmARG in *E. coli*, so that large quantities of the protein could be easily prepared. After purification we used the recombinant enzyme to determine the molecular weight, Michaelis-Menten constant and other important parameters (Table 1). As predicted from the sequence data, when compared with the two human isoforms, SmARG shows many similarities, particularly to HsARG1. We also found that SmARG has the highest reaction rate of all three enzymes. However, the mitochondrial HsARG2 has a higher substrate affinity as indicated by the lower K_m value, while HsARG1 has lower substrate affinity than SmARG. The high reaction rate combined with an intermediate substrate binding affinity suggests that SmARG has the highest specific biological activity of all three tested arginases.

The pH optimum for all enzymes is in the same basic range, while the thermal stability of HsARG1 is somewhat higher than for SmARG indicating an enhanced conformational stability for the human enzyme. The optimal Mn^{2+} concentration for all three enzymes is the same, as could be expected from the similarity in their DNA sequence (Fig. 2) and the predicted structural similarities for the Mn^{2+} -binding site (Fig. 3).

We also determined the molecular mass for a SmARG subunit to be 40 kDa, which made it slightly larger than HsARG1 but smaller than HsARG2. When we analysed the size of native SmARG by gel filtration chromatography on a Sephacryl S 300 column, calibrated with several protein markers, we obtained a molecular weight of 240 kDa. As such, we conclude that native SmARG forms a hexamer.

3.4. Expression of arginase is upregulated in all life stages which interact with the definitive mammalian host

Recent technological advances allow unravelling of the transcriptome of schistosome parasites faster and in far more detail than ever before (Hokke et al., 2007). The emerging picture shows that the expression of parasite genes can differ by gender (Fitzpatrick et al., 2005) or life stage (Jolly et al., 2007) and thereby reflects specific functions. Therefore, we investigated the mRNA expression profile of arginase in different life stages of *S. mansoni* and also in both genders of adult worms.

We isolated total RNA from a variety of different life stages and also from separated male and female adult worms. Next we determined the relative expression of arginase mRNA by semi-quantitative RT-PCR using specifically designed primers. Our expression profile shows that arginase expression is highly upregulated in infective cercariae and during the first days of infection of the mammalian host (Fig. 5). Arginase expression subsides at 2 weeks p.i. before it is upregulated again in the maturing worm. We found only marginal differences between female and male worms. These results suggest that arginase is not specifically involved in egg production or fertilisation. Strikingly, we did not detect any arginase expression in sporocysts

and only very low expression in miracidia. Microarray data confirm our semi-quantitative PCR results from the different life stages and, in addition, show only low SmARG-expression in schistosome eggs (data not shown). Apparently, arginase metabolism is not necessary during the invasion of snails, the subsequent transition into sporocysts and the asexual proliferation of the sporocysts. Our data indicate that hydrolysis of L-arginine by arginase is only relevant when the parasite is in contact with its mammalian host and is in complete agreement with arginase activity levels found in the skin of cercariae-infected mice (Fig. 1A) and in isolated cercariae and adult worms (Fig. 1C).

3.5. Arginase is located in the head but not in the tail of cercariae and it is not secreted upon transition into schistosomula

We reported arginase activity (Fig. 1C) and mRNA expression (Fig. 5) in infective cercariae. A recent analysis showed that cercariae mostly up-regulate the expression of genes involved in energy production (Jolly et al., 2007). Arginase can contribute to the energy metabolism of invertebrates by feeding into proline and glutamate production (Jenkinson et al., 1996). Hence, similar to other enzymes for the energy metabolism of cercariae (Skelly et al., 1993), we hypothesised that arginase would be found preferentially in the tail of infective larvae if it was required for the delivery of energy for tail movements in water.

We separated head and tail mechanically and isolated the two body parts by density centrifugation. Subsequently, we lysed the tissues and determined arginase activity in the two lysates. Our results show only marginal activity in the tail while we found strong activity in the head (Fig. 6A). In addition, we investigated the presence of arginase in the two lysates by Western blot. Similar to our activity data, most arginase was detected in the head and not in the tail (Fig. 6B). We also found no evidence that cercariae incorporate C¹⁴-L-arginine or release urea (data not shown). Thus, we conclude that arginase is not likely involved in the energy metabolism of free-living cercariae. The data also support our conclusion that elevated arginase activity in infected mouse tissue stems from invading parasites (Fig. 1).

When schistosomes invade their mammalian host they become vulnerable to the toxic effects of NO produced by classically activated macrophages (Wynn et al., 1994; James et al., 1998; Hoffmann et al., 1999). However, there are indications that the invading parasite employs sophisticated strategies to manipulate the activation and effectiveness of local innate immune cells (Angeli et al., 2001a,b). *Helicobacter pylori* releases bacterial arginase, which depletes the microenvironment of L-arginine, subsequently preventing bactericidal NO-production by host macrophages and inhibiting T-cell activity (Gobert et al., 2001; Zabaleta et al., 2004). We speculated that schistosomula might release arginase after entry into the skin, probably together with the protein cocktail stored in their acetabular glands. We tested this hypothesis by transforming cercariae mechanically into schistosomula or by agitating them chemically with linoleic acid, a major stimulatory lipid found in human skin. Both treatments cause the secretion of the acetabular gland contents (Stirewalt, 1974). After separating the parasites from the supernatant by centrifugation, we analysed the lysed parasite material and the supernatant for arginase activity and arginase expression by Western blot. We detected only very low arginase activity in the supernatant (Fig. 6C). Similarly, arginase was not found in the supernatant by Western blot (Fig. 6D). In contrast, arginase was expressed and active in the parasite lysate. These results show that arginase is not secreted in significant amounts by the skin-invading parasite. The last blot shows a difference in molecular weight between recombinant SmARG (40 kDa) and the native protein (32 kDa). We speculate that proteolytic modifications of the nascent protein might be responsible for these differences. Differences in molecular weight, which might stem from proteolysis, have also been indicated for mammalian arginase (Munder et al., 2005).

3.6. Activity of SmARG depends upon Mn^{2+} and can be inhibited by reducing agents and by the NOS-intermediate metabolite N^{ω} -hydroxy-L-arginine

We also analysed how certain amino acids and inhibitors impact the activity of SmARG. Branched-chain amino acids such as L-lysine inhibit both human arginases (Carvajal and Cederbaum, 1986; Fuentes et al., 1994a; Colleluori et al., 2001) and *F. gigantica* arginase (Mohamed et al., 2005). Surprisingly, we found that L-lysine and diaminopimelic acid, a precursor for L-lysine inhibiting liver arginase from guinea pig (Soler et al., 1981), failed to block SmARG activity. L-Ornithine was only moderately inhibitory (Fig. 7A). In contrast, L-cysteine and L-histidine strongly blocked SmARG-activity. These data indicate that both amino acids are essential for the function of SmARG.

Borate is a moderate non-competitive inhibitor of HsARG-1 ($K_i = 0.27$ mM) (Carvajal et al., 1999) and HsARG-2 ($K_i = 0.32$ mM) (Colleluori et al., 2001). Similarly, we found that borate moderately blocks SmARG-activity (Fig. 7A). Apparently, the interaction of tetrahedral borate anion with the binuclear manganese cluster of human arginase prevents the hydrolysis of L-arginine (Baggio et al., 1997; Carvajal et al., 1999). The structural similarities at the catalytic site between human arginases and SmARG (Fig. 3) explain why borate causes similar effects for all three enzymes.

The importance of manganese for the enzymatic activity of SmARG was further demonstrated when we replaced it with other divalent metal cations. Removal of Mn^{2+} by dialysis significantly reduced the enzymatic activity to 60% (Fig. 7B). Activity could be completely restored by adding Mn^{2+} but did not recover significantly in the presence of Co^{2+} , Mg^{2+} and Ca^{2+} . In addition, these cations did not compete effectively with Mn^{2+} in non-dialysed arginase. We conclude that SmARG has a low binding affinity for these cations. In contrast, Co^{2+} increases the enzymatic activity of HsARG-2 (Colleluori et al., 2001) and partially restores the activity of HsARG-1 (Carvajal et al., 1995). Furthermore, Zn^{2+} and Cu^{2+} are strong inhibitors of human and schistosome arginase. Arginase from *F. gigantica* is also sensitive to replacement of Mn^{2+} by other bivalent metal cations. Here cation replacement with Mg^{2+} , Ca^{2+} and Co^{2+} reduced the enzymatic activity when compared with Mn^{2+} (Mohamed et al., 2005).

Mammalian arginase and most other arginases are not or only moderately sensitive to reducing agents (Greenberg et al., 1956; Mora et al., 1966; Soru, 1983), indicating that disulphide bonds are not important for their enzymatic activity. An interesting exception is arginase from *H. pylori*, which is strongly inhibited by DTT and 2-mercaptoethanol (2-ME) (McGee et al., 2004). When we tested the impact of reducing agents on the enzymatic activity of SmARG, we found that DTT, 2-ME and reduced glutathione diminished or abrogated its activity (Fig. 7C). The removal and/or replacement of Mn^{2+} had almost no detectable effect in the presence of 2-ME and reduced glutathione. Only for DTT did we find that dialysis of Mn^{2+} further reduced the activity of the enzyme in the presence of the agent. Addition of Mn^{2+} could partially restore the activity to the level of non-dialysed enzyme. Conversely, Iodoacetamide, an alkylating sulfhydryl agent, has no effect on SmARG-activity. These data indicate that, in contrast to its human orthologs, disulphide bonds by cysteines are essential for the enzymatic activity of SmARG.

Mammalian arginase is strongly inhibited by NOHA, a stable intermediate in the biosynthesis of nitric oxide and L-citrulline by mammalian NOS (Boucher et al., 1994; Daghigh et al., 1994; Colleluori and Ash, 2001). Substantial amounts of NOHA are released from the active site of NOS and can bind to intracellular arginase (Chenais et al., 1993; Klatt et al., 1993). Particularly in activated macrophages this competitive inhibition is essential for regulating the L-arginine metabolism between the two enzymes. Importantly, NOHA can also be released into the extracellular space, as shown for classically activated rat macrophages and rat endothelial cells (Hecker et al., 1995; Buga et al., 1996), where it can be taken up by other cells using the

y⁺ cationic transporter system. Since *S. mansoni* expresses an effective y⁺ cationic transporter (Skelly et al., 1999), we speculate that release of NOHA by classically activated host macrophages could regulate the activity of SmARG. Indeed, we discovered that SmARG is highly sensitive to NOHA (Fig. 7D). Activity of SmARG was strongly inhibited by NOHA and only high concentrations of L-arginine were able to generate some enzymatic activity.

Surprisingly, the NOHA analogue norNOHA showed only weak inhibition of SmARG. In contrast, norNOHA is a 40- to 100-fold more potent inhibitor of several mammalian arginases than NOHA (Tenu et al., 1999; Moali et al., 2000; Colletuori and Ash, 2001). NOHA differs from its analogue norNOHA by having one more CH₂-group. While the shorter analogue fits better into the substrate-binding site of mammalian arginase, certain structural differences of SmARG apparently prevent the binding of norNOHA. This appears to be an interesting similarity to mammalian NOS, which binds and uses NOHA as a substrate but does not interact with norNOHA (Tenu et al., 1999; Moali et al., 2000; Cox et al., 2001).

4. Discussion

Invading schistosomula are vulnerable to NO produced by classically activated murine macrophages (James and Glaven, 1989; Oswald et al., 1994; Wynn et al., 1994). Sufficient availability of L-arginine is required for the stability of the NOS-2 complex (El-Gayar et al., 2003) and therefore efficiently controls the production of NO by murine macrophages (Modolell et al., 1995; Rutschman et al., 2001). Some pathogens are able to limit L-arginine-availability for NO-production by inducing host arginase activity (Stempin et al., 2002; Kropf et al., 2005) or by releasing their own arginase (Gobert et al., 2001). Invading schistosome larvae have a variety of mechanisms at their disposal to manipulate the local immune response (Jenkins et al., 2005). Hence, it is conceivable that they also attempt to control toxic NO production by resident innate immune cells in the skin.

We found no evidence that invasive schistosomula induce host arginase expression in the skin upon primary contact. Nevertheless, our data demonstrate that arginase activity increases in the skin during infection and this finding is directly attributable to the parasite-produced ortholog. Schistosomula manipulate the local immune response in the skin by releasing excretory/secretory (ES)-products from their acetabular glands (Herve et al., 2003). While a recent proteomic analysis of ES-products from mechanically activated cercariae showed evidence for some arginase release (Knudsen et al., 2005), we did not detect the secretion of significant amounts of arginase by mechanically or chemically activated cercariae. Although small amounts of SmARG might be released from invading parasites, it is probably not sufficient to affect the L-arginine metabolism of local macrophages. Alternatively, schistosomula could deplete their microenvironment of L-arginine by a rapid intake of the amino acid via their y⁺ cationic transporter system and a subsequent hydrolysis by SmARG. As reported, cercariae and other life stages of *S. mansoni* express a light chain family member of heterodimeric amino acid permeases, which resembles the mammalian y⁺ transporter system (Skelly et al., 1999). As recently shown, classically and alternatively activated murine macrophages need to up-regulate the expression of the y⁺ cationic transporter system for the sufficient uptake of L-arginine feeding into NO or L-ornithine production (Yeramian et al., 2006). Microarray data from cercariae and schistosomula show a transiently enhanced expression of schistosome permease during the first 24 h after transformation (data not shown). Therefore, further comparative studies of host and schistosome L-arginine uptake are necessary to elucidate whether schistosomes can control the L-arginine metabolism of mammalian macrophages.

Upregulation of arginase by all life stages of *S. mansoni* with close contact to the human host indicates that the enzyme likely has a general function, which must be related to the specific

living conditions in the mammalian host. This could include energy metabolism, osmosis, cell proliferation, extracellular matrix production (Jenkinson et al., 1996), nitrogen metabolism as in *F. gigantica* (Mohamed et al., 2005) or controlling the dilatation of parasitised blood vessels by reducing the production of NO from endothelial NOS-3 (Berkowitz et al., 2003).

The complicated life cycle of *Schistosoma* requires long-lasting infections within their definitive host. During infection schistosomes have to cross physical barriers such as skin or mucosa, they have to avoid destruction by the host's immune system and they have to compete successfully with their host for metabolic resources. Importantly, they also have to keep their host alive for a long time. Therefore, these pathogens are only successful when they are able to adapt extremely well to all specific environments encountered during their life cycle. Hence, it can be expected that enzymes for important metabolic pathways of these parasites share many structural and functional similarities with their host-derived orthologs. Indeed, our sequence data, the homology model for SmARG, the relative position in a phylogenetic tree and a number of enzymatic data display many similarities between SmARG and human arginase.

In contrast to the relative close distance between schistosome and mammalian arginase, the similarity between human arginases and arginases from other human pathogens in our phylogenetic tree (*Leishmania major*, *Trypanosoma brucei* and *S. aureus*) is much lower. Perhaps it reflects that the arginase family members in these organisms have evolved under different evolutionary pressure. All three of these microbial pathogens are highly vulnerable to NO-mediated killing by host macrophages. There is evidence that at least the two protozoan parasites benefit from host arginase activity by preventing NOS-2 activity and generating polyamines, which are essential metabolites for these organisms (McInnes et al., 1998; Gobert et al., 2000; Iniesta et al., 2001; Kropf et al., 2005). Hence, these microbial arginases may avoid highly effective competition with human arginase, which could explain the larger differences in their amino acid sequence that contributes to their different functional properties. Together, our results indicate that SmARG is highly adapted to the conditions found in the mammalian host. Nevertheless, we noted several important differences between schistosome and human arginase.

The molecular weight of native SmARG indicates a hexamer as the natural state for the enzyme. Hexamer formation has been reported mostly for bacterial arginases, where two trimeric arginase units interact to form a dimer of trimers (Bewley et al., 1999). Most invertebrate arginases form large multimers or exist as large monomeric enzymes. The molecular weight for *F. gigantica* arginase is 92 kDa and it was isolated as monomer (Mohamed et al., 2005). In contrast, mammalian arginases, such as of human and rat origin, form homotrimers (Jenkinson et al., 1996). The amino acid sequence of both schistosome arginases reveals that the essential amino acids for trimerisation are all conserved between humans and schistosomes. In HsARG-1 the 14 residues at the C-terminus are considered an oligomerisation motif (Kanyo et al., 1996), which is absent in bacterial arginases (Bewley et al., 1999). Although both schistosome arginases possess an even longer C-terminus, similar to HsARG-2, there is no sequence similarity between human and schistosome arginases in this area. Hence, the mechanism of schistosome arginase oligomerisation and possible functional consequences require further investigation.

When we tested the sensitivity of SmARG to known inhibitors of human arginase we discovered some surprising differences, such as the failure to inhibit SmARG with branched-chain amino acids and norNOHA or the inability to replace Mn^{2+} by other divalent cations. Strikingly, reducing agents strongly inhibited SmARG activity, while mammalian and many other arginases are not sensitive to the blockage of disulphide-bonds (Greenberg et al., 1956; Mora et al., 1966; Soru, 1983). On the contrary, a recent study showed that S-nitrosylation of cysteine residue 303 in HsARG-1 causes arginase activation (Santhanam et al., 2007). The

importance of cysteines for the function of SmARG is also shown by the inhibition of its enzymatic activity when L-cysteine was added. Of the six cysteine residues in SmARG only residues 66, 291 and 336 are exclusively found in schistosome arginases and not in either human isoform. Therefore, these residues are the candidates to form specific disulphide bonds in SmARG. Our structural model for SmARG indicates that C291 and C332 come in close proximity near the catalytic site, predicting a disulphide bond between these two cysteine residues, which does not exist in human arginase. In summary, these data demonstrate that SmARG has certain structural features, which are clearly distinct from mammalian host arginase.

These unique properties of SmARG are important. If we are to successfully target SmARG for elimination of the parasite in infected humans, it will be essential to avoid inhibiting host arginase activity, particularly in hepatocytes. Otherwise the urea cycle and the protective immune response against parasite eggs could be compromised. Our data indicate that it might be possible to exploit the differences between human and schistosome arginase and generate a parasite-arginase-specific inhibitor. Such a treatment could offer an alternative to the current treatment with praziquantel and might even prove to be more effective since all life stages in the mammalian host express arginase and could be targeted. Therefore, determining the functional role of arginase for the biology of the parasite will be the next logical step to elucidate the value of arginase as potential drug target.

We believe our study is the first to comprehensively characterise the properties of an arginase family member in a helminth parasite infecting humans. It demonstrates clear evidence for co-evolution between host and parasite arginase family members. Many structural and biochemical features are well conserved between human and schistosome arginase. Nevertheless, our data also display important differences between human and schistosome arginase indicating the possibility to develop SmARG-specific inhibitors. These results significantly expand our knowledge regarding the metabolism of pathogenic schistosome species and improve the relative poor characterisation of arginases in invertebrates.

Acknowledgements

We thank Dr. Fred Lewis and his colleagues at the Biomedical Research Institute (Rockville, MD) for providing parasite material used in this study. We further thank Mary Leusink (LPD, Bethesda, MD) and Fae Tompkins (Cornell University, Ithaca, NY) for technical assistance. Funding was provided by grants PRI06B124 (Junta de Extremadura) and PI070400 (FIS, ISCIII, MSC) to JM Fuentes and KF Hoffmann is supported by grants received from the Wellcome Trust (RG35177 and RG35522).

References

- Ahmed SF, Oswald IP, Caspar P, Hieny S, Keefer L, Sher A, James SL. Developmental differences determine larval susceptibility to nitric oxide-mediated killing in a murine model of vaccination against *Schistosoma mansoni*. Infect. Immun 1997;65:219–226. [PubMed: 8975915]
- Angeli V, Faveeuw C, Delerive P, Fontaine J, Barriera Y, Franchimont N, Staels B, Capron M, Trottein F. *Schistosoma mansoni* induces the synthesis of IL-6 in pulmonary microvascular endothelial cells: role of IL-6 in the control of lung eosinophilia during infection. Eur. J. Immunol 2001a;31:2751–2761. [PubMed: 11536174]
- Angeli V, Faveeuw C, Roye O, Fontaine J, Teissier E, Capron A, Wolowczuk I, Capron M, Trottein F. Role of the parasite-derived prostaglandin D2 in the inhibition of epidermal Langerhans cell migration during schistosomiasis infection. J. Exp. Med 2001b;193:1135–1147. [PubMed: 11369785]
- Baggio R, Elbaum D, Kanyo ZF, Carroll PJ, Cavalli RC, Ash DE, Christianson DW. Inhibition of Mn-2 (2+)-arginase by borate leads to the design of a transition state analogue inhibitor, 2(S)-amino-6-boronohexanoic acid. J. Am. Chem. Soc 1997;119:8107–8108.
- Berkowitz DE, White R, Li D, Minhas KM, Cernetich A, Kim S, Burke S, Shoukas AA, Nyhan D, Champion HC, Hare JM. Arginase reciprocally regulates nitric oxide synthase activity and contributes

to endothelial dysfunction in aging blood vessels. *Circulation* 2003;108:2000–2006. [PubMed: 14517171]

- Bewley MC, Jeffrey PD, Patchett ML, Kanyo ZF, Baker EN. Crystal structures of *Bacillus caldovelox* arginase in complex with substrate and inhibitors reveal new insights into activation, inhibition and catalysis in the arginase superfamily. *Structure* 1999;7:435–448. [PubMed: 10196128]
- Boucher JL, Custot J, Vadon S, Delaforge M, Lepoivre M, Tenu JP, Yapo A, Mansuy D. *N* omega-hydroxyl-L-arginine, an intermediate in the L-arginine to nitric oxide pathway, is a strong inhibitor of liver and macrophage arginase. *Biochem. Biophys. Res. Commun* 1994;203:1614–1621. [PubMed: 7945311]
- Buga G, Singh R, Pervin S, Rogers N, Schmitz D, Jenkinson C, Cederbaum S, Ignarro L. Arginase activity in endothelial cells: inhibition by NG-hydroxy-L-arginine during high-output NO production. *Am. J. Physiol* 1996;271:H1988–H1998. [PubMed: 8945918]
- Carvajal N, Cederbaum SD. Kinetics of inhibition of rat liver and kidney arginases by proline and branched-chain amino acids. *Biochim. Biophys. Acta* 1986;870:181–184. [PubMed: 3955053]
- Carvajal N, Torres C, Uribe E, Salas M. Interaction of arginase with metal ions: studies of the enzyme from human liver and comparison with other arginases. *Comp. Biochem. Physiol. B Biochem. Mol. Biol* 1995;112:153–159. [PubMed: 7584844]
- Carvajal N, Salas M, Lopez V, Uribe E, Herrera P, Cerpa J, Fuentes M. Manganese-dependent inhibition of human liver arginase by borate. *J. Inorg. Biochem* 1999;77:163–167. [PubMed: 10643656]
- Castresana J. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol* 2000;17:540–552. [PubMed: 10742046]
- Chenais B, Yapo A, Lepoivre M, Tenu JP. *N* omega-hydroxy-L-arginine, a reactional intermediate in nitric oxide biosynthesis, induces cytostasis in human and murine tumor cells. *Biochem. Biophys. Res. Commun* 1993;196:1558–1565. [PubMed: 7504481]
- Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* 2003;31:3497–3500. [PubMed: 12824352]
- Christianson DW. Arginase: structure, mechanism, and physiological role in male and female sexual arousal. *Acc. Chem. Res* 2005;38:191–201. [PubMed: 15766238]
- Cioli D, Botros SS, Wheatcroft-Francklow K, Mbaye A, Southgate V, Tchuente LA, Pica-Mattoccia L, Troiani AR, El-Din SH, Sabra AN, Albin J, Engels D, Doenhoff MJ. Determination of ED50 values for praziquantel in praziquantel-resistant and -susceptible *Schistosoma mansoni* isolates. *Int. J. Parasitol* 2004;34:979–987. [PubMed: 15217737]
- Colleluori DM, Ash DE. Classical and slow-binding inhibitors of human type II arginase. *Biochemistry* 2001;40:9356–9362. [PubMed: 11478904]
- Colleluori DM, Morris SM Jr, Ash DE. Expression, purification, and characterization of human type II arginase. *Arch. Biochem. Biophys* 2001;389:135–143. [PubMed: 11370664]
- Cox JD, Cama E, Colleluori DM, Pethe S, Boucher JL, Mansuy D, Ash DE, Christianson DW. Mechanistic and metabolic inferences from the binding of substrate analogues and products to arginase. *Biochemistry* 2001;40:2689–2701. [PubMed: 11258880]
- Daghigh F, Fukuto JM, Ash DE. Inhibition of rat liver arginase by an intermediate in NO biosynthesis, NG-hydroxy-L-arginine: implications for the regulation of nitric oxide biosynthesis by arginase. *Biochem. Biophys. Res. Commun* 1994;202:174–180. [PubMed: 8037711]
- Di Costanzo L, Sabio G, Mora A, Rodriguez PC, Ochoa AC, Centeno F, Christianson DW. Crystal structure of human arginase I at 1.29-Å resolution and exploration of inhibition in the immune response. *Proc. Natl. Acad. Sci. USA* 2005;102:13058–13063. [PubMed: 16141327]
- Diez A, Fuentes JM, Prada F, Campo ML, Soler G. Immunological identity of the two different molecular mass constitutive subunits of liver arginase. *Biol. Chem. Hoppe Seyler* 1994;375:537–541. [PubMed: 7811393]
- Doenhoff MJ, Kusel JR, Coles GC, Cioli D. Resistance of *Schistosoma mansoni* to praziquantel: is there a problem? *Trans. R. Soc. Trop. Med. Hyg* 2002;96:465–469. [PubMed: 12474468]
- Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004;32:1792–1797. [PubMed: 15034147]

- El-Gayar S, Thuring-Nahler H, Pfeilschifter J, Rollinghoff M, Bogdan C. Translational control of inducible nitric oxide synthase by IL-13 and arginine availability in inflammatory macrophages. *J. Immunol* 2003;171:4561–4568. [PubMed: 14568929]
- Fitzpatrick JM, Johnston DA, Williams GW, Williams DJ, Freeman TC, Dunne DW, Hoffmann KF. An oligonucleotide microarray for transcriptome analysis of *Schistosoma mansoni* and its application/ use to investigate gender-associated gene expression. *Mol. Biochem. Parasitol* 2005;141:1–13. [PubMed: 15811522]
- Fitzpatrick JM, Hirai Y, Hirai H, Hoffmann KF. Schistosome egg production is dependent upon the activities of two developmentally regulated tyrosinases. *FASEB J* 2007;21:823–835. [PubMed: 17167065]
- Fuentes JM, Campo ML, Soler G. Physico-chemical properties of hepatocyte plasma-membrane-bound arginase. *Arch. Int. Physiol. Biochim. Biophys* 1991;99:413–417. [PubMed: 1725742]
- Fuentes JM, Campo ML, Soler G. Kinetics and inhibition by some aminoacids of lactating rat mammary gland arginase. *Arch. Int. Physiol. Biochim. Biophys* 1994a;102:255–258. [PubMed: 7849272]
- Fuentes JM, Campo ML, Soler G. Kinetics of manganese reconstitution and thiol group exposition in dialyzed rat mammary gland arginase. *Int. J. Biochem* 1994b;26:653–659. [PubMed: 8005350]
- Fuentes JM, Lompre AM, Moller JV, Falson P, le Maire M. Clean Western blots of membrane proteins after yeast heterologous expression following a shortened version of the method of Perini et al. *Anal. Biochem* 2000;285:276–278. [PubMed: 11017716]
- Gobert AP, Daulouede S, Lepoivre M, Boucher JL, Bouteille B, Buguet A, Cespuglio R, Veyret B, Vincendeau P. L-Arginine availability modulates local nitric oxide production and parasite killing in experimental trypanosomiasis. *Infect. Immun* 2000;68:4653–4657. [PubMed: 10899869]
- Gobert AP, McGee DJ, Akhtar M, Mendz GL, Newton JC, Cheng Y, Mobley HL, Wilson KT. *Helicobacter pylori* arginase inhibits nitric oxide production by eukaryotic cells: a strategy for bacterial survival. *Proc. Natl. Acad. Sci. USA* 2001;98:13844–13849. [PubMed: 11717441]
- Greenberg DM, Bagot AE, Roholt OA Jr. Liver arginase. III. Properties of highly purified arginase. *Arch. Biochem. Biophys* 1956;62:446–453. [PubMed: 13328133]
- Haas W, Grabe K, Geis C, Pach T, Stoll K, Fuchs M, Haberl B, Loy C. Recognition and invasion of human skin by *Schistosoma mansoni* cercariae: the key-role of L-arginine. *Parasitology* 2002;124:153–167. [PubMed: 11860033]
- He YX, Salafsky B, Ramaswamy K. Comparison of skin invasion among three major species of *Schistosoma*. *Trends Parasitol* 2005;21:201–203. [PubMed: 15837605]
- Hecker M, Nematollahi H, Hey C, Busse R, Racke K. Inhibition of arginase by NG-hydroxy-L-arginine in alveolar macrophages: implications for the utilization of L-arginine for nitric oxide synthesis. *FEBS Lett* 1995;359:251–254. [PubMed: 7532597]
- Herbert DR, Holscher C, Mohrs M, Arendse B, Schwegmann A, Radwanska M, Leeto M, Kirsch R, Hall P, Mossmann H, Claussen B, Forster I, Brombacher F. Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. *Immunity* 2004;20:623–635. [PubMed: 15142530]
- Herve M, Angeli V, Pinzar E, Wintjens R, Faveeuw C, Narumiya S, Capron A, Urade Y, Capron M, Riveau G, Trottein F. Pivotal roles of the parasite PGD2 synthase and of the host D prostanoid receptor 1 in schistosome immune evasion. *Eur. J. Immunol* 2003;33:2764–2772. [PubMed: 14515260]
- Hesse M, Modolell M, La Flamme AC, Schito M, Fuentes JM, Cheever AW, Pearce EJ, Wynn TA. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J. Immunol* 2001;167:6533–6544. [PubMed: 11714822]
- Hoffmann KF, James SL, Cheever AW, Wynn TA. Studies with double cytokine-deficient mice reveal that highly polarized Th1- and Th2-type cytokine and antibody responses contribute equally to vaccine-induced immunity to *Schistosoma mansoni*. *J. Immunol* 1999;163:927–938. [PubMed: 10395689]
- Hokke CH, Fitzpatrick JM, Hoffmann KF. Integrating transcriptome, proteome and glycome analyses of *Schistosoma* biology. *Trends Parasitol* 2007;23:165–174. [PubMed: 17336161]
- Huelsenbeck JP, Ronquist F. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 2001;17:754–755. [PubMed: 11524383]

- Iniesta V, Gomez-Nieto LC, Corraliza I. The inhibition of arginase by *N*(omega)-hydroxy-L-arginine controls the growth of *Leishmania* inside macrophages. *J. Exp. Med* 2001;193:777–784. [PubMed: 11257143]
- James ER, Taylor MG. Transformation of cercariae to schistosomula: a quantitative comparison of transformation techniques and of infectivity by different injection routes of the organisms produced. *J. Helminthol* 1976;50:223–233. [PubMed: 1010921]
- James S, Glaven J. Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* involves arginine-dependent production of reactive nitrogen intermediates. *J. Immunol* 1989;143:4208–4212. [PubMed: 2592772]
- James SL, Cheever AW, Caspar P, Wynn TA. Inducible nitric oxide synthase-deficient mice develop enhanced type 1 cytokine-associated cellular and humoral immune responses after vaccination with attenuated *Schistosoma mansoni* cercariae but display partially reduced resistance. *Infect. Immun* 1998;66:3510–3518. [PubMed: 9673227]
- Jenkins SJ, Hewitson JP, Jenkins GR, Mountford AP. Modulation of the host's immune response by schistosome larvae. *Parasite Immunol* 2005;27:385–393. [PubMed: 16179032]
- Jenkinson CP, Grody WW, Cederbaum SD. Comparative properties of arginases. *Comp. Biochem. Physiol. B Biochem. Mol. Biol* 1996;114:107–132. [PubMed: 8759304]
- Jolly ER, Chin CS, Miller S, Bahgat MM, Lim KC, DeRisi J, McKerrow JH. Gene expression patterns during adaptation of a helminth parasite to different environmental niches. *Genome Biol* 2007;8:R65. [PubMed: 17456242]
- Kanyo ZF, Scolnick LR, Ash DE, Christianson DW. Structure of a unique binuclear manganese cluster in arginase. *Nature* 1996;383:554–557. [PubMed: 8849731]
- Klatt P, Schmidt K, Uray G, Mayer B. Multiple catalytic functions of brain nitric oxide synthase. Biochemical characterization, cofactor-requirement, and the role of *N* omega-hydroxy-L-arginine as an intermediate. *J. Biol. Chem* 1993;268:14781–14787. [PubMed: 7686905]
- Knudsen GM, Medzihradsky KF, Lim KC, Hansell E, McKerrow JH. Proteomic analysis of *Schistosoma mansoni* cercarial secretions. *Mol. Cell. Proteomics* 2005;4:1862–1875. [PubMed: 16112986]
- Kropf P, Herath S, Weber V, Modolell M, Muller I. Factors influencing *Leishmania major* infection in IL-4-deficient BALB/c mice. *Parasite Immunol* 2003;25:439–447. [PubMed: 14651591]
- Kropf P, Fuentes JM, Fahnrich E, Arpa L, Herath S, Weber V, Soler G, Celada A, Modolell M, Muller I. Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis in vivo. *FASEB J* 2005;19:1000–1002. [PubMed: 15811879]
- Lavulo LT, Sossong TM Jr, Brigham-Burke MR, Doyle ML, Cox JD, Christianson DW, Ash DE. Subunit-subunit interactions in trimeric arginase. Generation of active monomers by mutation of a single amino acid. *J. Biol. Chem* 2001;276:14242–14248. [PubMed: 11278703]
- Lewis F, John Wiley and Sons, Inc. Schistosomiasis. *Curr. Protoc. Immunol* 2001;(Suppl 28):19.1.1–19.1.28.
- Marti-Renom MA, Stuart AC, Fiser A, Sanchez R, Melo F, Sali A. Comparative protein structure modeling of genes and genomes. *Annu. Rev. Biophys. Biomol. Struct* 2000;29:291–325. [PubMed: 10940251]
- McGee DJ, Zabaleta J, Viator RJ, Testerman TL, Ochoa AC, Mendz GL. Purification and characterization of *Helicobacter pylori* arginase, RocF: unique features among the arginase superfamily. *Eur. J. Biochem* 2004;271:1952–1962. [PubMed: 15128304]
- McInnes IB, Leung B, Wei XQ, Gemmell CC, Liew FY. Septic arthritis following *Staphylococcus aureus* infection in mice lacking inducible nitric oxide synthase. *J. Immunol* 1998;160:308–315. [PubMed: 9551985]
- Moali C, Brollo M, Custot J, Sari MA, Boucher JL, Stuehr DJ, Mansuy D. Recognition of alpha-amino acids bearing various C@NOH functions by nitric oxide synthase and arginase involves very different structural determinants. *Biochemistry* 2000;39:8208–8218. [PubMed: 10889028]
- Modolell M, Corraliza I, Link F, Soler G, Eichmann K. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. *Eur. J. Immunol* 1995;25:1101–1104. [PubMed: 7537672]

- Mohamed SA, Fahmy AS, Mohamed TM, Hamdy SM. Urea cycle of *Fasciola gigantica*: purification and characterization of arginase. *Comp. Biochem. Physiol. B Biochem. Mol. Biol* 2005;142:308–316. [PubMed: 16125991]
- Mora J, Tarrab R, Bojalil LF. On the structure and function of different arginases. *Biochim. Biophys. Acta* 1966;118:206–209. [PubMed: 5954062]
- Munder M, Eichmann K, Modolell M. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by CD4+ T cells correlates with Th1/Th2 phenotype. *J. Immunol* 1998;160:5347–5354. [PubMed: 9605134]
- Munder M, Mollinedo F, Calafat J, Canchado J, Gil-Lamaignere C, Fuentes JM, Luckner C, Doschko G, Soler G, Eichmann K, Muller FM, Ho AD, Goerner M, Modolell M. Arginase I is constitutively expressed in human granulocytes and participates in fungicidal activity. *Blood* 2005;105:2549–2556. [PubMed: 15546957]
- Oswald I, Wynn T, Sher A, James S. NO as an effector molecule of parasite killing: modulation of its synthesis by cytokines. *Comp. Biochem. Physiol. Pharmacol. Toxicol. Endocrinol* 1994;108:11–18. [PubMed: 7520338]
- Ouzounis CA, Kyrpides NC. On the evolution of arginases and related enzymes. *J. Mol. Evol* 1994;39:101–104. [PubMed: 8064866]
- Page RD. TreeView: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci* 1996;12:357–358. [PubMed: 8902363]
- Perozich J, Hempel J, Morris SM Jr. Roles of conserved residues in the arginase family. *Biochim. Biophys. Acta* 1998;1382:23–37. [PubMed: 9507056]
- Pesce J, Kaviratne M, Ramalingam TR, Thompson RW, Urban JF Jr. Cheever AW, Young DA, Collins M, Grusby MJ, Wynn TA. The IL-21 receptor augments Th2 effector function and alternative macrophage activation. *J. Clin. Invest* 2006;116:2044–2055. [PubMed: 16778988]
- Roberts SC, Tancer MJ, Polinsky MR, Gibson KM, Heby O, Ullman B. Arginase plays a pivotal role in polyamine precursor metabolism in *Leishmania*. Characterization of gene deletion mutants. *J. Biol. Chem* 2004;279:23668–23678. [PubMed: 15023992]
- Rodriguez R, Chinae G, Lopez N, Pons T, Vriend G. Homology modeling, model and software evaluation: three related resources. *Bioinformatics* 1998;14:523–528. [PubMed: 9694991]
- Rutschman R, Lang R, Hesse M, Ihle JN, Wynn TA, Murray PJ. Cutting edge: Stat6-dependent substrate depletion regulates nitric oxide production. *J. Immunol* 2001;166:2173–2177. [PubMed: 11160269]
- Sabio G, Mora A, Rangel MA, Quesada A, Marcos CF, Alonso JC, Soler G, Centeno F. Glu-256 is a main structural determinant for oligomerisation of human arginase I. *FEBS Lett* 2001;501:161–165. [PubMed: 11470277]
- Santhanam L, Lim HK, Miriel V, Brown T, Patel M, Balanson S, Ryoo S, Anderson M, Irani K, Khanday F, Di Costanzo L, Nyhan D, Hare JM, Christianson, Rivers R, Shoukas A, Berkowitz DE. Inducible NO synthase dependent S-nitrosylation and activation of arginase1 contribute to age-related endothelial dysfunction. *Circ. Res* 2007;101(7):692–702. [PubMed: 17704205]
- Savioli L, Stansfield S, Bundy DA, Mitchell A, Bhatia R, Engels D, Montresor A, Neira M, Shein AM. Schistosomiasis and soil-transmitted helminth infections: forging control efforts. *Trans. R. Soc. Trop. Med. Hyg* 2002;96:577–579. [PubMed: 12625126]
- Sekowska A, Danchin A, Risler JL. Phylogeny of related functions: the case of polyamine biosynthetic enzymes. *Microbiology* 2000;146(Pt 8):1815–1828. [PubMed: 10931887]
- Senft AW. Studies in arginine metabolism by schistosomes. I. Arginine uptake and lysis by *Schistosoma mansoni*. *Comp. Biochem. Physiol* 1966;18:209–216. [PubMed: 6007424]
- Skelly PJ, Stein LD, Shoemaker CB. Expression of *Schistosoma mansoni* genes involved in anaerobic and oxidative glucose metabolism during the cercaria to adult transformation. *Mol. Biochem. Parasitol* 1993;60:93–104. [PubMed: 8396206]
- Skelly PJ, Pfeiffer R, Verrey F, Shoemaker CB. SPRM11c, a heterodimeric amino acid permease light chain of the human parasitic platyhelminth, *Schistosoma mansoni*. *Parasitology* 1999;119(Pt 6):569–576. [PubMed: 10633918]
- Smithers SR, Terry RJ. The infection of laboratory hosts with cercariae of *Schistosoma mansoni* and the recovery of the adult worms. *Parasitology* 1965;55:695–700. [PubMed: 4957633]

- Soler G, Mataix FJ, Ruiz-Amil M. Physico-chemical properties of guinea pig liver arginase (author's transl). *Rev. Esp. Fisiol* 1981;37:37–44. [PubMed: 7244325]
- Soru E. Chemical and immunological properties of *B. athracis* arginase and its metabolic involvement. *Mol. Cell. Biochem* 1983;50:173–183. [PubMed: 6406830]
- Stempin C, Giordanengo L, Gea S, Cerban F. Alternative activation and increase of *Trypanosoma cruzi* survival in murine macrophages stimulated by cruzipain, a parasite antigen. *J. Leukoc. Biol* 2002;72:727–734. [PubMed: 12377942]
- Stirewalt MA. *Schistosoma mansoni*: cercaria to schistosomule. *Adv. Parasitol* 1974;12:115–182. [PubMed: 4141581]
- Takiguchi M, Matsubasa T, Amaya Y, Mori M. Evolutionary aspects of urea cycle enzyme genes. *Bioessays* 1989;10:163–166. [PubMed: 2662961]
- Tenu JP, Lepoivre M, Moali C, Brollo M, Mansuy D, Boucher JL. Effects of the new arginase inhibitor N(omega)-hydroxy-nor-l-arginine on NO synthase activity in murine macrophages. *Nitric Oxide* 1999;3:427–438. [PubMed: 10637120]
- Whelan S, Goldman N. A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol. Biol. Evol* 2001;18:691–699. [PubMed: 11319253]
- Wiederstein M, Sippl MJ. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. *Nucleic Acids Res* 2007;35:W407–410. [PubMed: 17517781]
- Wynn TA, Eltouni I, Cheever AW, Lewis FA, Gause WC, Sher A. Analysis of cytokine mRNA expression during primary granuloma formation induced by eggs of *Schistosoma mansoni*. *J. Immunol* 1993;151:1430–1440. [PubMed: 8335939]
- Wynn TA, Oswald IP, Eltouni IA, Caspar P, Lowenstein CJ, Lewis FA, James SL, Sher A. Elevated expression of Th1 cytokines and nitric oxide synthase in the lungs of vaccinated mice after challenge infection with *Schistosoma mansoni*. *J. Immunol* 1994;153:5200–5209. [PubMed: 7525727]
- Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN. Living with water stress: evolution of osmolyte systems. *Science* 1982;217:1214–1222. [PubMed: 7112124]
- Yeremian A, Martin L, Serrat N, Arpa L, Soler C, Bertran J, McLeod C, Palacin M, Modolell M, Lloberas J, Celada A. Arginine transport via cationic amino acid transporter 2 plays a critical regulatory role in classical or alternative activation of macrophages. *J. Immunol* 2006;176:5918–5924. [PubMed: 16670299]
- Yoshino TP, Laursen JR. Production of *Schistosoma mansoni* daughter sporocysts from mother sporocysts maintained in synxenic culture with *Biomphalaria glabrata* embryonic (Bge) cells. *J. Parasitol* 1995;81:714–722. [PubMed: 7472861]
- Zabaleta J, McGee DJ, Zea AH, Hernandez CP, Rodriguez PC, Sierra RA, Correa P, Ochoa AC. *Helicobacter pylori* arginase inhibits T cell proliferation and reduces the expression of the TCR zeta-chain (CD3zeta). *J. Immunol* 2004;173:586–593. [PubMed: 15210820]

Fig. 1.

Infection of mice with *Schistosoma mansoni* cercariae elevates arginase activity but not mouse arginase-1 expression in the skin. C57BL/6Ai mice were infected with 500 cercariae/ear. At indicated time points after infection the mice were euthanised and the ears removed. Ears were processed and arginase activity measured as described in Section 2. (A) Arginase activity for individual mice at different time points collected from two separate experiments. The bar displays the median activity for each group. Significance of differences between uninfected mice and infected groups was determined by one-way analysis of covariance followed by Dunnett's post-test using GraphPad Prism v. 4.0b. Five ears from each group were pooled and total RNA isolated. After subsequent purification of mRNA and its reverse transcription into

cDNA, the relative expression of murine arginase-1 was determined by quantitative PCR. The data were normalised to the expression of hypoxanthine-phosphoribosyltransferase (HPRT) and they are depicted in (B) as fold-change in expression (median $\pm$ SEM) compared with ears from non-infected mice (dotted line). Shown are the representative results for one out of two similar experiments. (C) Cercariae and adult worms were isolated, lysed and arginase activity was determined according to Section 2. Data (median $\pm$ SEM) are representative for three separate experiments.

Hs	ARG1	MSAKS-----RTIGIGIAPFSKQPPRGVEEGPTVLRKAGGLEKLKE	42
Hs	ARG2	MSLRGSLRLLQTRVHSILKKSVHSVAVIGAPFSQQKRRKGVHEGPAAIREAGLMKRLS	60
	<u>Sm</u> ARG	-MLKSVATPYPIQD-EKPKLLYTSANFLGIPITNRGQPKI GTYQGPFLIRKSNFQVLAE	58
	Sj	ARG -MLKSVATPYYPVQNGETPKLLYPHNVFLGIPVNVKQGPKLTGQGDPFIRKSNFQVLAE	59
		* * * * *	
		* * * * *	
		* * * * *	
Hs	ARG1	QECDDVKYDGLFPFADIPN-DSFPQ-IVKNPRSVKGASEQLAGKVAEVKKNGR-----	92
Hs	ARG2	LGCHLKD FGDLSFTTPVK-DBLYNNLIVNPRSVGLANQELAEVVSRAVSDGY-----	111
	<u>Sm</u> ARG	DGIQLTDCGDII PVEINEAEDPQRFGMKWSRSFSLTTLIRAEVVELMKQSNKHTVELSG	118
	Sj	ARG DGIQLTDCGDVIVPVELSEDPERCGMKWSRSFTQTTLIADRVQLVKGSNKHSIESNN	119
		* * * * * * * * * *	
		* * * * * * * * * *	
		* * * * * * * * * *	
Hs	ARG1	----ISLVLGGRSLAIGISGHARVHPDLGVIVVDAITNTPLTTSSGNLRHQPVSFL	148
Hs	ARG2	-----SCVTLGGRSLAIGTISGHARHCPDLCVWVVDAAITNTPLTTSSGNLRHQPVSFL	167
	<u>Sm</u> ARG	SKSTPLVIVGGDLSMATGTLIGHAEKAPDLCLVWIDAAITNTPLNSGNNRHMPLSFL	178
	Sj	ARG SKPSPLVIVGGDLSMATGTLIGHARAKPDCVCIWVDAITNTPPNSTGNIRHMPLSFL	179
		* * * * * * * * * *	
		* * * * * * * * * *	
		* * * * * * * * * *	
Hs	ARG1	LKELKGKIPDVPGFWSVTPCISAKDVIYVIGLRDVPDGEHYILKTLGIKYFSMTEVRLGI	208
Hs	ARG2	LRELQKVPVLQPGFSWIKEPCISASIVYIGLRDVPDPPEHFLKKNYDIQYFSMRDIRGI	227
	<u>Sm</u> ARG	VKELQDQIPWLDDEFGIKPCPLNASNIAYI GLRDLDAHETHDIRKHGAIYFTMLDVRMGI	238
	Sj	ARG VKELQDQIPWLDDEHFSIKPCPLDASNLYI GLRDLDVYETDIRKHAIYFTMLDVRMGM	239
		* * * * * * * * * *	
		* * * * * * * * * *	
		* * * * * * * * * *	
Hs	ARG1	GKVMEEITSYLLGRKKRPIHLSFPIVGLDPFSFTPATGTPVVGGLTYRGLYIRPEIYKTG	268
Hs	ARG2	QKVMERTFDLLIGKRQRPIHLSFIIDAFPTLPAATGTPVVGGLTYRGLYIRPEIHNHTG	287
	<u>Sm</u> ARG	EAVIKEALLAVNPRLEKAIHLSFIIDALDPLVAPSTGTAVPGGLTIRAGLRIPEVSATG	298
	Sj	ARG EAVIKEALQAVNPRLEKPIHLSFIIDALDPIAPSTGTAVPGGLTIRAGLRIPEISATG	299
		* * * * * * * * * *	
		* * * * * * * * * *	
		* * * * * * * * * *	
Hs	ARG1	LLSGDIMEVNPGLSGKTPEEVTRTVNTAVATVIALPGLAEIGNHKPIIDYLLNPPK-----	322
Hs	ARG2	LLSALDIVEVNPQLATSEEEKATNTAVNTAVIASFGQTIPEGGHIVFDYLLPTSSPDESE	347
	<u>Sm</u> ARG	KLVSVELAEVNLPLIG-SQEDVYLTQSSAVHILRALGHGSSGLPKYVRNLITDQIMISRA	357
	Sj	ARG KLSIVELAEVNLPLIG-SKEDVEKTSASVHILRALGHGSSGLPKMKNNSNTNTIVRQA	358
		* * * * * * * * * *	
		* * * * * * * * * *	
		* * * * * * * * * *	
Hs	ARG1	-----	
Hs	ARG2	NQARVRI 354	
	<u>Sm</u> ARG	AHMQTQK 364	
	Sj	ARG ERIQIK 364	

Fig. 2.

Schistosoma mansoni encodes a protein with high sequence similarity to *Homo sapiens* arginases. The cDNA for SmARG was cloned, sequenced and translated into an amino acid sequence according to Materials and methods. CLUSTALW was used to align the human and schistosome arginase sequences using default parameters where ‘*’ indicates identical residues (also indicated as bold amino acid residues), ‘:’ indicates conserved substitutions and ‘.’ indicates semi-conserved residues. It demonstrates that SmARG (AAP94031) and SjARG (AAQ16108) are highly similar to both *H. sapiens* arginase isoforms (NP_000036, NP_001163). Both parasite orthologs contain the six invariant metal coordinating amino acids and one functionally important histidine (H141, HsARG-1) residue (red shaded boxes). In addition, four amino acid residues essential for trimer formation of HsARG1 are indicated (blue shaded boxes). Finally, SmARG residues Cys291 and Cys332 (green shaded boxes), which potentially establish a disulphide bond, are also highlighted.

Fig. 3.

A structural model for SmARG demonstrates high homology to HsARG-1, particularly for the catalytic centre. Based on the crystal structure of HsARG-1, a virtual structural model for SmARG was generated according to Materials and methods. The two panels emphasize the active site residues and the binuclear Mn^{2+} centre of HsARG-1 (A) and SmARG (B). In (B), the position of two cysteine residues (C291, C332), potentially involved in a disulphide bond (gold), is shown.

Fig. 4.

The position of SmARG in a phylogenetic reconstruction of arginase family members indicates a close relation with human and mouse arginase-1. Phylogenetic analysis was inferred by MrBayes 3.1.1 and illustrated by Treeview (rooted tree) as described in Section 2. Numbers along nodes are Bayesian posterior probability values and indicate the degree of support for the inferred tree with the scale bar representing the number of amino acid changes calculated by the Whelan and Goldman (WAG) protein substitution model. GenBank Accession Nos. (and organism description) are given for each sequence used in the analysis.

Fig. 5.

Expression of SmARG is upregulated in all life stages with mammalian host interaction but not in life stages with snail contact. Total RNA was isolated from different parasite life stages, reverse transcribed into cDNA and then amplified by PCR using a specific primer pair for SmARG. The PCR-products were electrophoresed and stained with ethidium bromide. The intensity of the products was determined and normalised to the *Schistosoma mansoni* house-keeping gene α -tubulin. The Bar graph depicts the intensity of arginase amplicons from different life stages in arbitrary units, which is equivalent to relative expression of arginase mRNA. All data are representative of at least two repeats.

Fig. 6.

Arginase is predominantly located in the head of cercariae but is not secreted upon transformation into tissue-penetrating larvae. *Schistosoma mansoni* cercariae were harvested from infected snails and then separated into heads and tails. The two isolated body parts were analysed for arginase activity (A) and for arginase protein expression levels by Western blot (B). Recombinant HsARG-1 was used as a reference. In further experiments cercariae were activated by 2 h incubation with linoleic acid. The culture supernatant and the cercariae were separated by centrifugation, then analysed for arginase activity (C) and arginase protein expression levels by Western blot (D). Data are representative of two individual experiments.

Fig. 7.

The enzymatic activity of SmARG depends upon manganese cations and disulphide bonds and is inhibited by the biological arginase inhibitor *N*⁰-hydroxy-L-arginine (NOHA). Recombinant SmARG was analysed for its enzymatic properties in a series of assays, which are detailed in Section 2. (A) Relative activity of SmARG in the presence of several amino acids and borate compared with the activity of the enzyme without any additions (100% activity). The relative activity of SmARG in the presence of several divalent cations is compared in (B). The activity was determined from arginase either containing Mn²⁺ (not dialysed) or after removal of Mn²⁺ (dialysed). Arginase activity of non-dialysed enzyme without additional cations was set as 100% activity. (C) The effect of different sulfhydryl-group-modifying agents on SmARG activity. The recombinant enzyme was used directly (not dialysed) or after dialysis to remove Mn²⁺ (dialysed) and subsequent replacement of Mn²⁺ in selected samples (dialysed + 10 mM Mn²⁺). All results were compared with arginase activity of non-dialysed enzyme without the presence of reducing agents (100%). The relative SmARG activity in the presence of the arginase inhibitors NOHA and *N*⁰-hydroxy-nor-L-arginine (norNOHA) is shown in (D). Results are depicted as a percentage of control activity (no inhibitor) at 500 mM L-arginine (100%). Bars in all panels show the mean percentage activity $\pm$ SD. All data are representative for three individual assays.

Table 1Enzymatic properties of *Schistosoma mansoni* arginase (SmARG) compared with human arginase (HsARG) isoforms

Parameter	SmARG	HsARG-1	HsARG-2 ^c
K_m (mM)	17	26 ^a	0.3
K_{cat} (s ⁻¹)	537	447 ^a	231
Specific activity (UI/mg)	669	536 ^a	222
pH optimum	10	9.7 ^a	9.5
T_m (°C)	62	78 ^a	N/A
Mn ²⁺ saturation (mM)	1	1 ^a	1
K_i ornithine (mM)	1.9	2 ^b	>10
M_w subunit (kDa)	40	34 ^a	43

^aPublished by Sabio et al. (2001).^bPublished by Carvajal et al. (1999).^cAll published by Colleluori et al. (2001).