The occurrence of free D-alanine and an alanine racemase activity in *Leishmania amazonensis*

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Abstract

Free D-amino acids are implicated in several biological functions. This study examined the presence of D-alanine in *Leishmania amazonensis*. Measuring chiral amino acid content by high-performance liquid chromatography we detected a significant amount of free D-alanine in promastigotes of these parasites. D-Alanine accounts for 8.9% of total free alanine and is found primarily in the soluble fraction. Specific racemization of L-alanine to D-alanine was detected in cell lysates and this enzyme activity was inhibited by D-cycloserine, an alanine racemase inhibitor. Furthermore, we were able to decrease this pool of D-amino acid by treating our cultures with D-cycloserine. We demonstrate for the first time the existence of a significant amount of free D-alanine in *L. amazonensis* and an alanine racemase activity present in cell lysates. The restriction of D-alanine to bacteria, some fungi and now in *L. amazonensis* opens a new perspective on treatment of diseases caused by these microorganisms.

Introduction

D-Amino acids have long been described in the cell wall of Gram-positive and especially Gram-negative bacteria, where they constitute essential elements of peptidoglycan and act as substitutes for cell wall techoic acids (Lamzin *et al.*, 1995; Chamond *et al.*, 2003). Several D-amino acids have been discovered in small peptides that function mainly as antibiotic agents and are produced by a variety of microorganisms through nonribosomal protein synthesis (Kleinkauf & von Dohren, 1987; Chamond *et al.*, 2003). Although D-amino acids and D-amino acid peptides are usually stereotyped as unnatural, they are present in prokaryotes and eukaryotes. An increasing number of studies have reported the presence of D-amino acids, either protein-bound (Nagata *et al.*, 1998) or free (Nagata *et al.*, 1999), in a wide variety of organisms, including mammals. D-Alanine, however, has never been found in either mammals or protozoans.

Alanine is usually available as the L-stereoisomer and the conversion to D-alanine by the cytoplasmic enzyme alanine racemase is required for the initial step in the alanine branch of peptidoglycan biosynthesis (Nagata *et al.*, 1998). All known bacteria require D-alanine, while L-alanine is the isomer required for eukaryotic protein synthesis. Alanine represents more than 30% of the intracellular pool of free amino acids in *Leishmania* (Simon *et al.*, 1983) and is also the major free amino acid in several other species of protozoan (Knodler *et al.*, 1994).

Leishmaniasis leads to a complex set of symptoms that range in severity from self-healing cutaneous lesions and mucocutaneous commitment to fatal visceral disease (Roberts *et al.*, 2000). It has been estimated that 10% of the world population is at risk of infection; two million new cases are reported every year. Infection results from transmission to humans and other mammals of the protozoan *Leishmania* sp. by a phlebotomine sand fly, producing parasitization of tissue macrophages that can persist for a lifetime. The immune response can be overwhelmed by sophisticated strategies employed by the parasite to avoid, subvert or nullify macrophage protective responses in order to establish an intracellular milieu suitable for survival and multiplication. Among the biochemical tactics, both extra- and intracellular developmental forms express a great variety of surface molecules that can act in a coordinated manner (Vannier-Santos *et al.*, 2002).

The great inter- and intra-specific variability of *Leishmania* makes it difficult to establish common virulence factors, but the phosphorylated disaccharide-containing conjugates

such as lipophosphoglycan and proteophosphoglycan are putative candidates (Ilgoutz & McConville, 2001). In the present study, we report for the first time the occurrence of the free D-enantiomer of alanine in this eukaryotic organism. An enzyme activity that racemizes L-alanine to D-alanine and is inhibited by D-cycloserine (DCS), an alanine racemase inhibitor (Helmy, 1970) is also described. Furthermore, the presence of DCS in the growth medium was able to reduce D-alanine levels in *Leishmania* promastigote cultures. The restriction of D-alanine to bacteria, some fungi and now *Leishmania amazonensis* suggests new targets for treatment of diseases caused by these microorganisms.

Materials and methods

Parasites and preparation of *Leishmania* membrane and soluble fractions

The MHOM/BR/75/Josefa strain of *Leishmania amazonensis* was isolated from a human case of diffuse cutaneous leishmaniasis in Brazil by Dr C. A. Cuba-Cuba (Universidade de Brasília, Brazil) (Martiny *et al.*, 1996). Since then it has been maintained in our laboratory, in axenic culture and by hamster footpad inoculation. Promastigote forms were maintained at 22 °C in Warren's growth medium with 10% heat-inactivated fetal bovine serum, and harvested after 6 days, when the cells reached late-logarithmic phase (*c.* 9×10^7 cells mL^{-1}). Membrane and soluble fractions were obtained as described previously by Cohen *et al.* (1986). Briefly, the cells were washed twice with 75 mM Tris-HCl at pH 7.6 containing 140 mM NaCl and 10 mM KCl (buffer A) and once with an hyperosmotic solution containing 400 mM mannitol, 10 mM HCl, 1 mM magnesium acetate, 1 mM phenylmethylsulfonyl fluoride, soybean trypsin (0.15 mg mL^{-1}) and 10 mM Hepes at pH 7.4 (buffer B). The ice-cold pellet of washed cells was mixed with acid-washed glass beads of 75–150 μm (Sigma Chemical Co., St Louis, MO) in a 4 : 1 ratio. The mixture was ground by gentle rotation for 5–10 min while the progressive rupture of cells was monitored using a phase contrast microscope. This procedure was stopped when no more than one to three intact cells per field could be seen in the microscope. After grinding, 25 mL of buffer B was added and the suspension was then centrifuged at 1000 ***g*** for 10 min to remove glass beads and unruptured cells. The supernatant from this centrifugation was centrifuged at 5000 ***g*** for 20 min and the supernatant from this was further centrifuged at 16 000 ***g*** for 40 min. Finally, a microsomal fraction was obtained from the last supernatant by centrifugation at 40 000 ***g*** for 60 min. The membrane fraction and the supernatant (soluble fraction) from this last centrifugation were immediately used for determination of the D-alanine content.

Chemicals

Amino acids were obtained from Sigma Chemical Co., D-amino acid oxidase from pig kidney (EC 1.4.3.3) was from Roche Molecular Biochemicals (Indianapolis, IN) and other reagents were of analytical grade.

Analytical procedure

Reverse-phase high-performance liquid chromatography (HPLC) was used to detect amino acid enantiomers after fluorescent derivatization by the method of Hashimoto *et al.* (1992). Briefly, samples were first deproteinized by addition of trichloroacetic acid (TCA) [5% weight in volume (w/v), final concentration]. The suspension was centrifuged at 20 000 ***g*** for 5 min, and the supernatant was analyzed by HPLC after removal of trichloroacetic acid by four extractions with water-saturated diethyl ether. The amino acids were rendered fluorescent by pre-column derivatization with *N*-tert-butyloxycarbonyl-L-cysteine (Boc-L-Cys) and *o*-phthaldialdehyde (OPA). A mobile phase gradient of 120 min from 7% to 47% acetonitrile in sodium acetate buffer (0.1 M, pH 6.2) with 3% tetrahydrofuran was applied on a reverse-phase C-18 column for HPLC separation with fluorescence detection at an excitation wavelength of 344 nm and emission wavelength of 433 nm D-Alanine and L-alanine were recognized by their characteristic retention times.

Assay of alanine racemase activity

D-alanine formation was monitored by the HPLC method described. Promastigotes were lysed by successive freeze–thaw cycles. Racemase activity was measured in cell lysates in the presence of 20 mM Tris-HCl pH 8.5 and 200 mM L-alanine. After incubation at 37 °C, the reaction was terminated by the addition of TCA to a final concentration of 5%. The precipitated protein was removed by centrifugation, and the supernatant was prepared as described for HPLC analysis. Cell lysates boiled for 5 min were used as blanks. The protein concentrations in the cell lysates were measured by the procedure described by Lowry *et al.* (1951).

Hypotonic stress

The hypotonic challenge to *L. amazonensis* promastigotes was performed during 10 min in the medium described by Vieira *et al.* (1996). All experiments were performed in triplicate, with similar results obtained in at least three separate cell suspensions.

Statistical analysis

Statistical significance was determined by Students' *t*-test. Significance was considered as $P < 0.05$.

Results

The HPLC method was applied to the determination of free amino acids in the promastigotes of *Leishmania amazonensis*. A substantial peak co-eluted with the Boc-L-Cys-OPA derivative of authentic D-alanine (Fig. 1a, c). The identity of the D-alanine peak was confirmed by specific degradation with D-amino acid oxidase (DAAO) (Fig. 1b).

DAAO is a highly selective enzyme that degrades only neutral D-amino acids and its preferential substrates *in vitro* are D-alanine and D-serine (D'Aniello *et al*., 1993). The purified enzyme used in this study exhibited a high rate of degradation in a solution of pure D-alanine (data not shown). The incubation of samples of promastigotes of *Leishmania amazonensis* with DAAO (0.3 mg mL^{-1} for 12 h at 37 °C) was followed by marked reduction of the D-alanine peak, with no effect on the other constituents in the chromatogram (Fig. 1b).

The concentration of free D-alanine in the cellular extract was estimated to be 514 ± 84 pmol mg^{-1} dry cell weight. The free L-alanine content was in accordance with a previous report (Table 1) (Simon *et al*., 1983). The ratio of free D-alanine/total alanine was 0.09 ± 0.02 (Table 1). As shown in Table 1, DAAO treatment reduced the D-alanine levels by 90% while exerting no influence on the level of L-alanine.

To establish the cellular localization of free D-alanine, the D-amino acid content was quantified in both membrane and soluble fractions. The majority of free D-alanine (80.6%) was found in the soluble fraction (Fig. 2). A minor amount of D-alanine found in the membrane fraction may be related to the presence of vesicles in our membrane fraction (Benaim *et al*., 1991) which could carry free D-alanine.

Table 1. Free alanine contents in *Leishmania amazonensis* promastigotes and the selective DAAO degradation of the D-form

	Content, pmol mg^{-1} dry cell weight		Effect of DAAO, % of control
	Before DAAO	After DAAO	
D-alanine	514 ± 84	50 ± 9	9.7
L-alanine	5370 ± 410	5423 ± 891	100.9
Total alanine	5884 ± 494	ND	
Ratio	0.09 ± 0.02	ND	

Values are expressed as means ± SEM of three (before DAAO) and three (after DAAO) different cultures.
DAAO, D-amino acid oxidase; ND, not done.

Alanine is the major amino acid involved in tonicity control in *Leishmania* sp. To assess whether the D-form of alanine participates in this process the *Leishmania amazonensis* promastigotes were exposed to a hypotonic challenge. This exposure was followed by release of both L- and D-forms of alanine (data not shown).

Similarly to putative alanine racemase described in *Trypanosoma cruzi* (El-Sayed *et al*., 2005) one hypothetical protein containing a sequence conserved in *Leishmania major* with a significant similarity to alanine racemase was deposited in the GENE Bank (gi: 68126573). D-alanine formation was measured in cell lysates incubated with L-alanine (Fig. 3a) indicating the presence of an alanine racemase in *Leishmania amazonensis*. The observed racemization was abolished when the cell lysates were boiled (data not shown). The time course of D-alanine formation was linear for at least 60 min ($r^2 = 0.9663$) (Fig. 3b).

DCS, a structural analogue of D-alanine, is a known inhibitor of alanine racemases (Cáceres *et al*., 1997). The

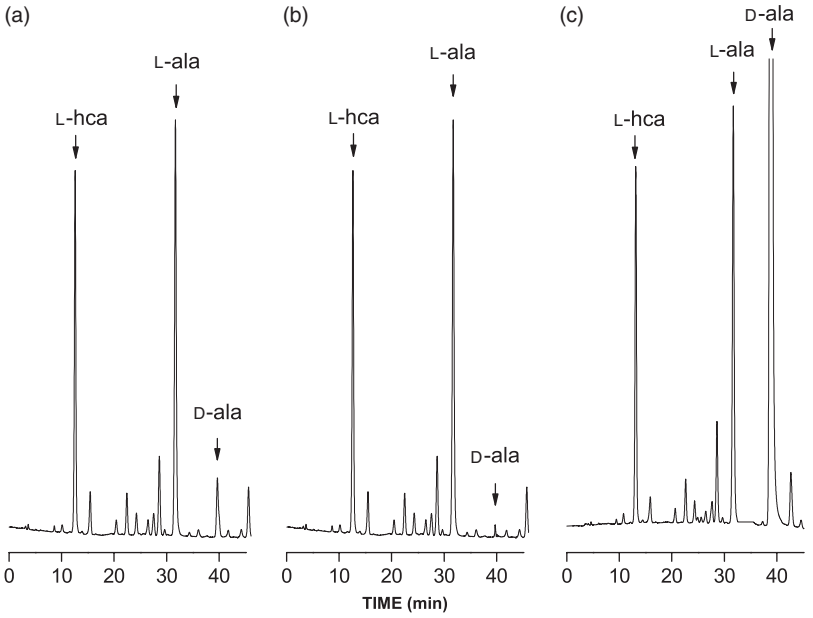

Fig. 1. Representative high-performance liquid chromatography (HPLC) chromatograms of *Leishmania amazonensis* extracts. Deproteinized parasite extracts were analyzed by HPLC as described in Materials and methods. L-Homocysteic acid (L-HCA) was used as an internal standard. (a) D-Alanine (D-ala) is eluted after L-alanine (L-ala). (b) Extract treatment with D-amino acid oxidase (0.3 mg mL^{-1} for 12 h at 37 °C) markedly decreased this peak. (c) Authentic D-alanine added to the extracts co-eluted with the original D-alanine peak.

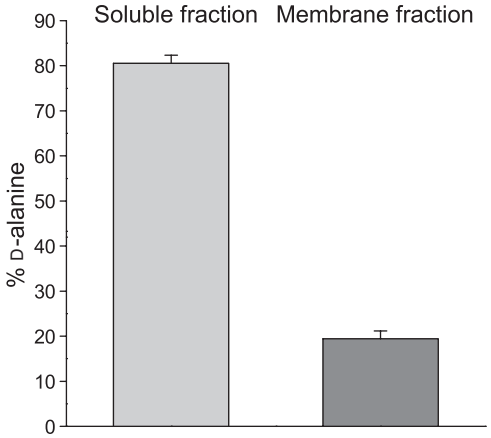

Fig. 2. D-Alanine distribution in the parasites. The free D-alanine contents were analyzed by high-performance liquid chromatography (HPLC), as described. D-alanine contents of promastigotes grown for 6 days in culture were measured in both membrane and soluble fractions. Percentages of D-amino acid found in the two different fractions in relation to total D-alanine content are shown. The values are means ± SEM of three different cultures.

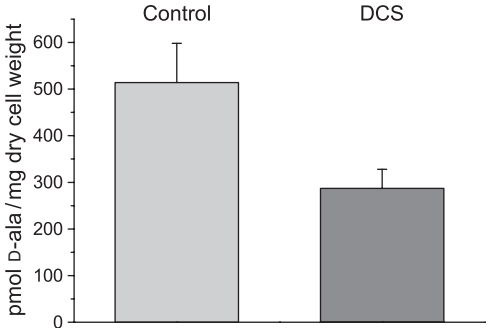

Fig. 4. D-Cycloserine effect on D-alanine content. Total D-alanine content of parasites grown for 6 days in the regular medium supplemented (DCS) or not (control) with D-cycloserine (600 μg mL^{-1}). The values shown are means ± SEM of five (control) and three (treated) different cultures. $P < 0.05$, compared with control.

only inhibitor that has been marketed for clinical purposes, it is mainly used as a second-line anti-tuberculosis agent, although side effects, especially toxicity, have limited its use (Helmy, 1970). Addition of DCS strongly inhibited D-alanine formation measured in cell lysates (Fig. 3a). To ascertain the presence of endogenous racemization activity in the growth parasites they were grown in the presence of 600 μg mL^{-1} of DCS. In this condition there was a significant decrease in the measured free D-alanine pool in the stationary growth phase (day 6) (Fig. 4), with no effect on the total amount of alanine (data not shown).

Discussion

The present study demonstrates for the first time the existence of a significant amount of free D-alanine in *Leishmania amazonensis*, as well as the presence of alanine racemase activity in the cell lysates. The D-amino acid is produced from the L-form by a racemization reaction which is inhibited by DCS, a specific alanine racemase inhibitor (Fig. 3a). D-alanine contents are also reduced in parasites grown with DCS (Fig. 4). The possibility cannot be excluded that a part of the free D-alanine detected in the present study was racemized from the L-form during preparation and assay processes, but this is unlikely because no racemization of L-alanine was observed during the present derivatization process (data not shown).

The concentration of free D-alanine appears to be higher than that of other important free amino acids that have been measured in *Leishmania* sp. promastigotes, such as glutamic acid, valine, tyrosine and lysine (Simon *et al.*, 1983). Alanine is the major constituent of the intracellular pool of amino acids in *Leishmania* (Simon *et al.*, 1983) and in several other species of parasitic protozoan (Knodler *et al.*, 1994). The alanine concentrations reported in the literature lie in the same range as our data (Table 1). The biological significance

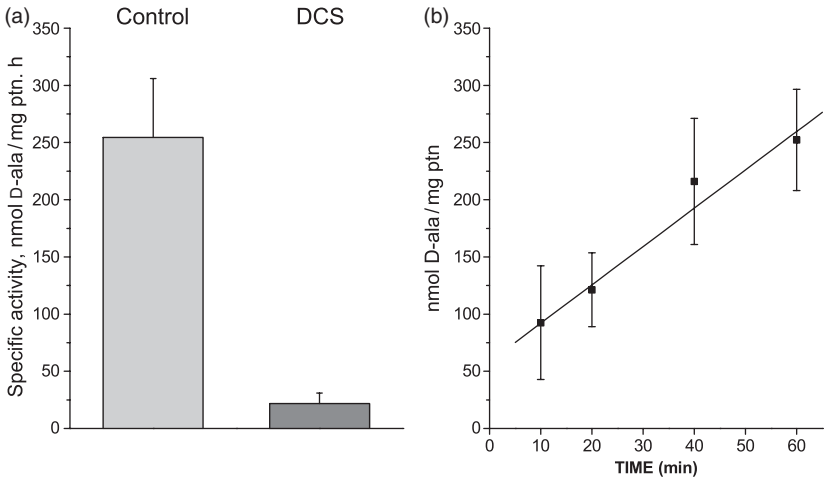

Fig. 3. Alanine racemase activity in *Leishmania amazonensis*. (a) Specific alanine racemase activity was measured in cell lysates, incubated for 60 min in medium supplemented (DCS) or not (control) with D-cycloserine (1 mM). $P < 0.05$, compared with control. (b) Time course of D-alanine formation measured in cell lysates. The experiments were replicated three times using different cell cultures and the values shown are means ± SEM. Blanks employed boiled cell lysates.

of such free D-alanine contents in *Leishmania amazonensis* remains to be elucidated. Alanine plays an important role as an osmolyte in many organisms and cells, including *Giardia intestinalis* (Blum, 1996). In *Leishmania donovani* alanine is the key osmolyte and enters the cell via a transport system (Bonay & Cohen, 1983).

Promastigotes develop during the *Leishmania* life cycle in the digestive tract of the sand fly, where they are challenged by chemical fluctuations. In these conditions, the parasites need homeostatic mechanisms to maintain their integrity. Changes in the intracellular alanine content have been described in response to variations in medium tonicity. When exposed to hyperosmotic stress, *Leishmania* promastigotes increase their alanine content (Burrows & Blum, 1991; Walsh & Blum, 1992), while hypotonic challenges elicit substantial alanine release (Darling & Blum, 1990). It seems that D-alanine participates in this process since the exposure of *Leishmania amazonensis* to hypotonic medium was followed by a substantial release of D-alanine simultaneously with the L-form (data not shown).

The presence of hypothetical sequences for alanine racemase in *Leishmania spp*. and the activity of conversion of L-alanine to D-alanine measured in cell lysates suggest the presence of a racemase enzyme in the parasites.

The major part of the alanine content in *Leishmania amazonensis* was found in the L-form. Considering that most racemases have an equilibrium constant of 1, one could expect half of the alanine in each form. However, L-alanine participates in different metabolic routes being generated from pyruvate by alanine aminotransferase. This production of the L-form through racemase-independent processes permits the large difference observed in the chiral amino acid contents. It is noteworthy that similar differences are observed in other systems studied (Hashimoto *et al*., 1992; Nagata *et al*., 1998, 1999).

The transformation of L-alanine to D-alanine in cell lysates might have involved multiple steps rather than any direct enzymatic racemization. But the fact that DCS, an alanine analog that specifically inhibits alanine racemase in several organisms, can specifically reduce both the alanine racemase activity measured in cell lysates and the D-alanine concentration in *Leishmania* cultures strengthens the evidence for a specific biosynthesis pathway. D-Alanine is a constituent of the peptidoglycan fraction of bacterial cell walls and DCS has been marketed for treatment of mycobacterial infections, although it has side effects (Cáceres *et al*., 1997). Bacterial strains that are unable to synthesize D-alanine show abnormalities in the cell wall and do not grow in cultures without D-alanine supplementation (Thompson *et al*., 1998; Palumbo *et al*., 2004). The free D-alanine present in the *Leishmania amazonensis* cytoplasm may be a constituent of molecules that are relevant to protection of the parasites against insults. More potent inhibitors of D-alanine synthesis could be useful as a strategy in leishmaniasis therapy.

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