Isolation and purification of a fatty acids binding protein in *Giardia*

Hasan, S.; Diaz de la Guardia, R.; Maasse, M.; Cordova, O.; Mascaró, M.C. & Osuna, A.
Instituto de Biotecnología Campus Universitario de Fuentenueva.
Facultad de Ciencias. Universidad de Granada. 18071 Granada. España.

Received: 02.05.01 Accepted: 11.12.01

Abstract: For the first time, a protein capable of bonding to fatty acids has been purified and described in a parasitic protozoan. This protein has a molecular weight of 8 to 10 kDa and an isoelectric point of 4.96. The Scatchard analysis of the equilibrium data revealed a K_d affinity constant 3.12 x 10^{-8} M, when the labelled oleic acid was displaced by unmarked oleic acid with a concentration 10-fold greater than that of marked.

Key words: *Giardia lamblia;* fatty-acid-bonding protein.

Resumen: Se ha purificado y descrito por primera vez en un protozoo parásito, *Giardia lamblia,* la existencia de una proteína capaz de ligar ácidos grasos, dicha proteína tiene un peso molecular de 8 a 10 kDa y un punto isoeléctrico de 4.96. El ácido oleico marcado fue desplazado con oleico no marcado con una concentración 10 veces superior a la de oleico marcado. Los análisis de Scatchard de los datos en equilibrio revelan una constante de afinidad K_d 3.12 x 10^{-8} M.

Palabras Clave: *Giardia lamblia;* proteína capaz de ligar ácidos grasos.

1. Introduction

Giardia lamblia is a flagellate protozoan which infects the upper intestinal tract in many animal species, including humans, in which this pathogen is the most frequent cause of gastrointestinal infection by protozoa (Heyworth, 1996). *Giardia* can cause diarrhoea, steatorrhea, retarded growth and malnutrition. The course of the infection is variable, from asymptomatic infections to severe diarrhoea (Hopkins and Juanek, 1991).

Like other intestinal protozoa, *Giardia* is incapable of *de novo* fatty-acid synthesis. Practically all eukaryotic cells studied in this regard have a series of proteins which bond to and transport fatty acids. These are the so-called fatty-acid-bonding proteins (FABP), in many of which the sequence and structure is known and can be classified into families. One family of low molecular weight (≤ 14 kDa) is found in many cells of higher animals and of helminths, where this family constitutes 2-3% of the total cytosol protein active in fatty-acid metabolism (Frolov and Schroeder, 1997). Although apparently located in the cytosol, this protein is related to microsomal and mitochondrial membranes, providing an interaction that facilitates its ability to synthesize phospholipids (Murphy *et al., 1999*). This FABP bonds to free fatty acids and acyl-CoA, and also bonds to several hydrophobic molecules, including the haemo group. The antigen values of these proteins constitute one of the most important characteristics currently under consideration for use in vaccination against infections by helminths.

In this study, we present the first evidence of a protein capable of bonding to fatty acids in a protozoan.

Corresponding author: A. Osuna
Instituto de Biotecnología. Facultad de Ciencias. Universidad de Granada. 18071 Granada. España. Tlfno.: +34958244163
Fax:+34958243174. Email: aosuna@ugr.es

2. Materials and Methods

2.1) Trophozoite culture of Giardia lamblia

Trophozoites of *G. lamblia* strains ATCC 30888 and 30957 were cultured in TYI-S 33 culture (Keister,1983) enriched with 10% bovine serum and composed of K_2HPO_4 (1.0 g), KH_2PO_4 (0.6 g) NaCl (2.0 g), 30 g Biosate peptone (BBL), 0.5 g bovine bile (Sigma), 22.8 mg ammonium iron citrate. The pH was adjusted to 7.2 with NaOH 0.1 N and autoclaved (121°C, 15 min) in 880 ml of deioninzed water. Afterwards, 20ml of a solution containing 2.2 g of chlorohydrate of L-cisteine, 0.2 g of ascorbic acid, 10 g of glucose were added after sterilizing by filtration through a membrane (0.22 μm in diameter). Finally, 100 ml of foetal bovine serum was

added, previously inactivated at 56°C for 30 min. The parasites were cultured in glass flasks at 37°C for at least 72 h. On reaching the logarithmic growth phase, the parasites were centrifuged at 250 g for 10 min at 4°C. The pellet containing the flagellates was washed in saline phosphate buffer (PBS 0.15M) at pH 7.4, to eliminate the remains of the culture medium and serum.

2.3) Preparation of the homogenate

The pellet from the last wash was resuspended in phosphate buffer (pH 7.4) containing 1mM EDTA, 1mM DTT, 1% Triton X-100 (Campbell *et al.*,1994) with a mixture of protease inhibitors (Complete Mini, Boehringer Mannheim GmbH). This mixture contained 3.0 mg Antipain-HCl, 0.5 mg bestatin, 1.0 mg cimostatin, 3.0 mg E-64, 0.5 mg leupeptin, 0.5 mg pepstatin, 3.0 mg phosphoramidon, 20.0 mg pefabloc SC, 0.5 mg aprotinin and 10.0 mg EDTA (inhibitors of serinproteases, cisteinproteases and metaloproteases, respectively).

The pellet containing the protozoa was frozen and thawed 3 times, followed by sonication (1 min at 0°C) to break up the cells. The sonicated fraction was centrifuged at 22,000 g for 30 min and the resulting supernatant collected.

Prior to treatments, the supernatant was submitted to chromography through a Bio-Beads™ SM-2 (BIO-RAD) column in order to eliminate the detergent used in the lysis buffer. The detergent-free extract was passed through a Lipidex-1000 column (Sigma) for an effective elimination of the free and protein-bonded fatty acids. For elution, 10 mM of phosphate buffer was used at pH 7.4 and a flow of 20 ml/h, following the method of Glatz *et al.* (1984); Jiao *et al.*(1998). Once delipidized, the sample was submitted to chromography through an affinity column prepared with Sepharose Epoxy 6B (Pharmacia Biotech) as a matrix to which the stearic acid had previously been bonded.

For the preparation and bonding of the stearic acid to the gel, the instructions provided by Pharmacia LKB Biotechnology were followed. After the column was prepared, it was balanced with PBS at pH 7.4 and a flow rate of 10 ml/h; the baseline was fixed, and the sample was submitted to chromography, passing it through at a flow rate of 0.1 ml/min. Afterwards the column was washed with PBS (pH 7.4) to eliminate the unbonded fraction.

The bonded fraction was eluted using 0.1 M Tris HCl buffer containing 1 M NaCl and/or 7M urea, and the eluted fraction was dialysed with distilled water. The protein concentration was determined by the Bradford method. The sample was distributed in aliquots for liophilization. For the electrophoretic separation, the Phast System (Pharmacia) was used with polyacrylamide gels at 12.5% (12.5 Homogeneous PhastGel) and 20% (20 Homogeneous PhastGel) under denatured conditions with buffer containing 0.112 M acetate, 0.112 M Tris at pH 6.5. For buffer strips separation pills were used (PhastGel SDS Buffer Strips). The samples were dissolved in a sample buffer and heated for 5 min at 100°C. The sample buffer was that described by Laemmli (1970), with 10 mM of Tris/HCl; 1 mM EDTA at pH 8.0, 2.5% SDS and 17% glycerine (to augment the density of the samples), and bromophenol blue at 0.01% to label the buffer and make its migration in the gel visible. For easier solubilization of the purified proteins, the following sample buffer was used: 10 mM of Tris/HCl at pH 8.0 with 2.5% SDS, 2.5 M urea, and bromophenol blue at 0.01%. The samples were diluted to a final concentration of 0.1mg/ml and then centrifuged to eliminate the insoluble aggregates (Dunn, 1993). The gels were stained well with Coomassie blue or silver nitrate (Heukeshoven and Dernick, 1985). For the determination of the isoelectric point of the proteins purified, the isoelectrofocusing was performed (Andrews, 1986). The separation and staining was made in the same PhastSystem ™ (Pharmacia). Polyacrylamide gels were used within the pH range of 3.0-9.0 (PhastGel IEF 3-9, marketed by Pharmacia Biotech). The samples were dissolved in Tris buffer (10 mM) pH 8.0 and urea. The separation involved a pre-focus of 2.5 mA 75 Vh and the separation at the same amperage and 410 Vh. At the end of the process, the gel was stained with silver nitrate.

As a means of demonstrating the capacity of the purified protein to bond to the fatty acids, the protein was separated in polyacrylamide gels in SDS-PAGE and transferred to nitrocellulose, and then incubated with oleic acid [^{14}C(U)] NEN™ Dupont (USA) at a concentration of 0.1 μCi/ μg protein (Campbell *et al.*, *1994*), performing an autoradiography on Kodak X-OMAT film, and incubation on a Cronex Dupont intensifier screen for 4-5 days.

The specificity study of the bonding between the protein and the labelled oleic acid used the technique of labelled-product displacement, incubating with increasing concentrations of unlabelled oleic acid at 37°C. Afterwards the proteins were separated with SDS-PAGE under non-reducing conditions, and autoradiography was performed as described above.

Similarly, the labelled-product displacement was performed by incubating with other substances, such as α-tocopherol, testosterone, butiric or retinoic acid as well as agents used in anti-*Giardia* chemotherapy,

such as metronidazol. Then the proteins were separated and submitted to autoradiography.

For the study of the specificity of the bonding between the protein and oleic acid as a function of time, the protein was incubated with labelled oleic acid with the isotope ^{14}C, at 37° C, and the bonding was studied at time intervals of 0, 5, 10, 20, 30, 60 and 120 min. The protein samples corresponding to the incubation intervals were separated in SDS-PAGE under non-reducing conditions, and afterwards subjected to gel autoradiography, followed by the study of the area of the autoradiography, using the Quantiscan program (Biostat, UK).

To show the specificity of the bonding, the protein bonded to the labelled oleic was incubated with excess non-labelled oleic protein (100 x), at 37°C, and its movement over time was studied at intervals 0, 15, 30 and 60 min. Afterwards, the proteins were separated and autoradiography was performed, followed by the study of the areas of the autoradiographs corresponding to the different time intervals.

In all the above cases, after electrophoresis and subsequent autoradiography, the lanes of the gels were sliced and placed in scintillation vials, with 3 ml of scintillation liquid (EcoLite^{TM}), and afterwards the beta particles were counted (Beckman, Palo alto, CA, USA) in order to calculate the number of DPM. The scintillation liquid was composed of alkylbenzene, phenylxylethane, non-ionic surfactants, 2,5-diphenloxazole, and p-bis(o-methylstyril) benzene. These DPM were used to calculate the association (K+1) and dissociation constants (K-1), the quotient of which gave the affinity constant (K_D). These calculations were made with the program LIGAND-PC (Biosoft, UK)

3. Results

The electrophoresis in SDS-PAGE under non-reducing conditions of the fraction obtained after the specific elution of the affinity column gave at least 13 protein bands capable of bonding hydrophobically to the column (Fig. 1). Nevertheless, the autoradiographs made after the incubation of the proteins with labelled oleic acid showed that only one of the bands — the one with a relative molecular weight of 8-10 kDa — was able to bond with the fatty acid (Figure 2). This figure also shows the capacity of the protein to bond to oleic acid and the displacement of the labelled product when incubated with α-tocopherol or substances used in anti-protozoal chemotherapy (metronidazol and nifurtimox).

The specificity of the bonding between the protein and oleic acid was examined by incubating the protein

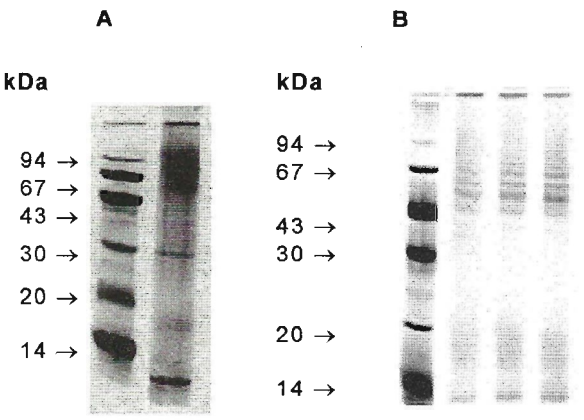

Fig. 1. SDS-PAGE (under non-reducing conditions) of the purified proteins of *Giardia lamblia*. (A) gel 20%; (B) gel 12.5%. In both cases the lane on the left shows the bands corresponding to the pattern proteins.

with radioactive oleic acid at increasing concentrations of non-radioactive oleic acid.

The kinetics of the bonding between the protein and oleic acid was studied over time, incubating the protein with oleic acid ^{14}C at 37°C and studying the bonding at time intervals of 0, 5, 10, 20, 30, 60 and 120 min. The samples of the protein corresponding to the incubation intervals were separated in SDS-PAGE under non-reducing conditions, and afterwards an autoradiography was made from the gel. The bonding curve (Fig. 3) showed the presence of the bonding site of the saturable protein at 90 min.

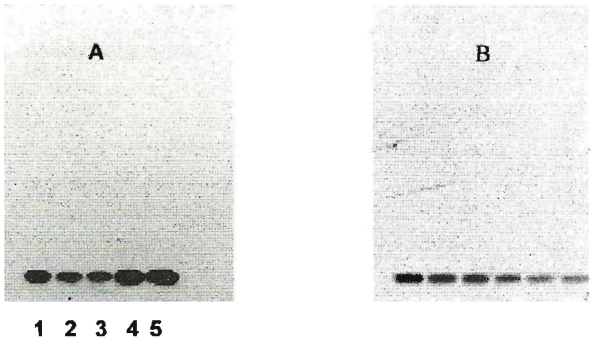

Fig. 2.
A. Autoradiography (gel 12.5%) that shows the displacement of the labelled oleic acid by: (1) α-tocopherol, (2) nifurtimox, (3) metronidazol, and (4, 5) control.
B. Autoradiography (gel 12.5%) showing the displacement of labelled oleic acid by non-labelled oleic

To study the characteristics of the bonding, we incubated the protein bonded to labelled oleic acid with excess non-marked oleic acid (10 x) a 37° C, and examined its movement over time at the same intervals as in the foregoing experiments. The study of the areas of the autoradiographs corresponding to the different time intervals showed that the bonding was competitive (Fig. 3B and C). The Scatchard analysis of the equilibrium data revealed an K_d affinity constant of 3.12×10^{-8} M.

Figure 2 and 4 shows the displacement of the labelled oleic bonded to the protein by metronidazol at a molar concentration 10-fold higher than that used in the bonding of oleic at 37°C. In the study of the movement of labelled oleic acid in the different time intervals, Figure 4A shows the kinetics of the movement of an extremely hydrophobic drug, nifurtimox, which is used in this case in the chemotherapy against *Trypanosoma cruzi* but which shows no direct effects on *Giardia*. Similarly, the kinetics of the displacement of marked oleic was studied as a function of time for butiric acid, α-tocopherol and testosterone in the same time intervals (Fig. 5).

4. Discussion

The intestinal protozoan parasite *Giardia lamblia* afflicts children worldwide with intestinal maladies ranging in seriousness, including diarrhoea followed by constipation in addition to a number of syndromes that decapacitate digestion and interfere with the uptake of proteins, sugars and especially fats, giving rise to the most characteristic disorder, steatorrhea (Korman *et al.*, 1990). All of these processes of poor uptake induce weight loss in general and slow development in children (Farthing *et al.*, 1986a).

The energy metabolism in this flagellate is related to a number of amino acids such as arginine, which constitutes a potentially significant energy source, or alanine, which is the main end product of *Giardia* metabolism (Edwards *et al.*, 1989). Although the trophozoites have a transport system for the entry of alanine, they can produce alanine from pyruvate through alanine-aminotransferase. Alanine, under certain conditions, can be a major energy source. Trophozoites of *Giardia* appear to depend on preformed lipids more than on *de*

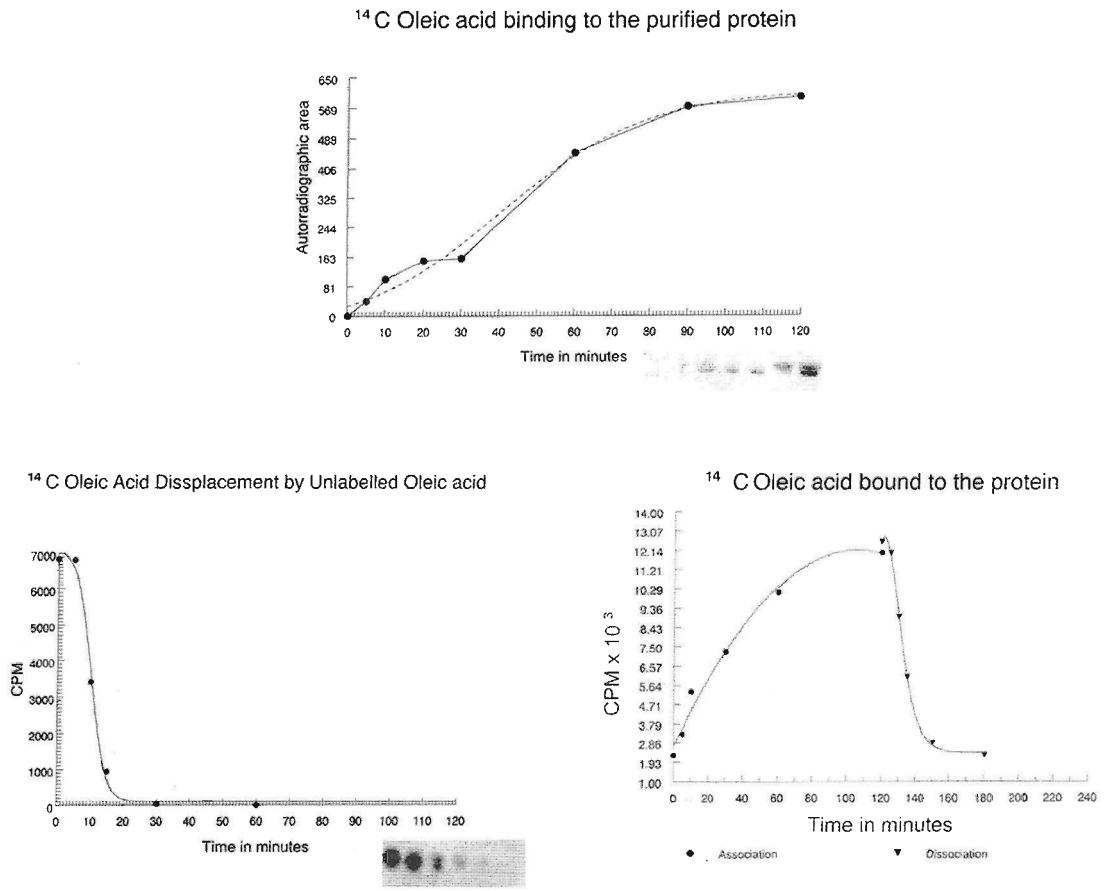

Fig. 3. Association and dissociation of labelled oleic acid over time. (A) shows the kinetics of the association, measured by the area of the autoradiograph. (B) shows the displacement (dissociation) of the labelled oleic acid by unlabelled oleic over time, measured by the CPM. (C) shows the kinetics of the association and dissociation over time.

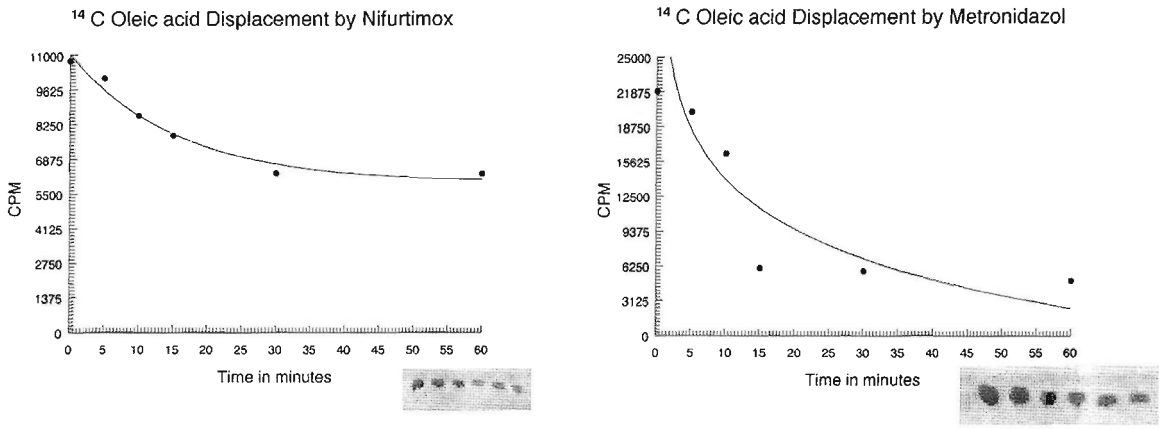

Fig. 4. Displacement of labelled oleic over time measured by CPM in (A) nifurtimox and (B) metronidazol

novo synthesized ones and can incorporate acids such as arachidonic and palmitic into phospholipids and neutral lipids within the cell (Blair and Weller, 1987). These pathogens are also capable of incorporating palmitic acid by glycosilation and palmitosilation of membrane proteins (VSP), proteins responsible for the antigen variations in *Giardia* (Hiltpold *et al.* 2000).

In all eukaryotic cells investigated in this sense, there is a number of proteins, both in the membrane and in the cytosol, which can bond and transport fatty acids. These are called "fatty-acid binding proteins" (FABP).

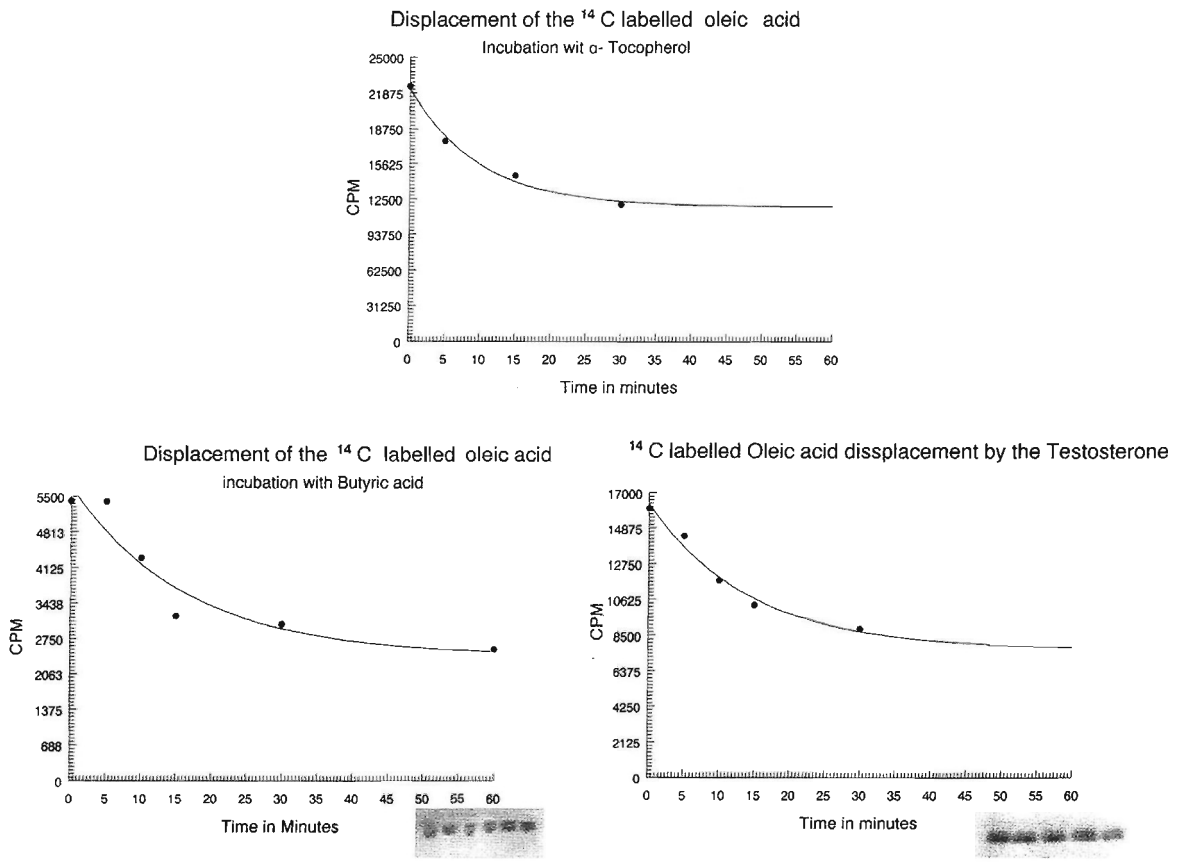

Fig. 5. Displacement of labelled oleic over time by (A) α-tocopherol. (B) butiric acid. (C) testosterone.

The total of proteins described with these properties are 5 in the membranes and 9 in the cytosol (Glatz *et al.*, 1996). For some of these the sequence and structure are known, and all present a number of aspects in common, such as short sequences and structures formed by a series of 10 non-parallel β chains forming a barrel, and two α chains buffering the open end of the structure. By this end, it bonds to the fatty acid, which is inserted, thus crossing the cell membrane.

The FABP cytosol proteins, apart from fatty-acid transport, have shown the ability to facilitate β-oxidation, acting as cofactors for the reactions where the fatty acids are involved, either as regulators or as substrates (Lalonde *et al.*, 1994). These can bond to and transport other hydrophobic ligands such as the haemo group, bilirubin, retinoic acid or hormones that are not readily water soluble (Bass *et al.*, 1993). The ligands are bonded to a protein by an ionic interaction between the carboxyl group of the ligand and arginine or tirosine at the bonding site, in addition to a network of hydrogen bridges and hydrophobic interactions, which serve to stabilize the orientation of the ligand. Therefore, any change in the hydrophobic quality of the bonding site can alter the affinity of the different ligands (Londraville *et al.*, 1996).

The affinity of the ligands appears to be related to the presence of a single amino acid, arginine. Methods of mutagenesis to replace arginine by another residue, or chemical modifications to remove the charge reduce the bonding capacity by 65% (Buelt and Bernlohr, 1990; Sha *et al.*, 1993). Arginine is one of the 7 amino acids (Arg, Asp, Cys, Glu, His, Lys and Tyr) that conserve a lateral ionizable chain when it forms proteins with a pK defined for each. Arginine shows the highest pK (12.84) of the 7 amino acids mentioned (Hardy,1985), which can contribute to its high affinity with fatty acids. In addition, the specificity to the ligands is related to the presence of arginine, and thus when it changes to glutamine this affinity for fatty acids diminishes while its ability to bond to retinoic-acid increases.

Given the location of the FABP in membranes, and the sequence variation in different species, these proteins play an important role in the diagnosis and vaccination of some parasitic agents such as *Schistosoma* spp, *Echinococcus granulosus* and *Ascaris lumbricoides*, (Chabalgoity *et al., 1997*; Gobert, 1998; Timanova *et al., 1999*).

In the present study, we provide the first description of a protein capable of bonding to fatty acids in a protozoal parasite, *Giardia lamblia*. For this, the purification method, as described in Materials and Methods, consisted of affinity chromatography by a column composed of epoxy-sepharose groups bonded to stearic acid.

However, when the ligand-organic acid complex is strongly bonded (as in our case), it is necessary to interrupt the elution flow, leaving the absorbent to be in contact with the complex for a minimum time of 30 min (maximum 120 min) before continuing the separation process. This allows the dissociation and recovery of the proteins bonded in the least volume possible of elution buffer (Pharmacia LKB).

In this way, by a prior homogenization (lysis) treatment with detergent, sonication of the trophozoites and subsequent elimination of the detergent and of the free fatty acids present, chromatography with Biobead and Lipidex, we achieved an efficient purification in practically a single pass, avoiding the problematic chromatographs based on changes in the ionic force and separation by molecular weight. The analysis of the proteins purified by electrophoresis in SDS-PAGE enabled the identification of a total of 13 bands that corresponded to proteins with relative molecular weights of between 120 and 10-8 kDa (Fig. 1 A and B).

However, in all cases, the band capable of bonding to oleic acid had a molecular weight of 10-8 kDa and one isoelectric point (Ip) of 4.96. The rest of the proteins purified by their bond to the affinity column, and which do not bond to this fatty acid could be artefacts, from the formation of aggregates during the purification process. It is possible that, given the hydrophobic nature shown by these proteins in aqueous solution, they associated through hydrophobic groups, giving rise to erroneous molecular weights and being incapable of bonding the fatty acids, on finding the hydrophobic groups already occupied.

Although this protein appears to be related to a family of intracellular proteins able to bond with fatty acids, it may also be related to membrane proteins (Murphy *et al.*, 1999). In fact, the proteins described above have a molecular weight of some 14 kDa, with a high affinity with hydrophobic ligands (Bass, 1993). As indicated above, this series includes 9 different proteins identified in mammal cells (Van Nieuwenhoven, 1996).

To characterize our protein and its affinity for non-saturated fatty acids, we examined its ability to bond to oleic acid. This bond proved to be dependent on temperature and on the contact time, the bonding graph showing an apparent bonding site which saturates at 90 min (Fig. 3.A). Similar to that described for other FABP (Campbell *et al.*, 1994), this bond is competitive in the presence of excess unlabelled oleic acid. Its displacement over time is depicted in Figure

3 B. The Scatchard analyses of the equilibrium data revealed a K_d affinity constant of 3.12×10^{-8} M, when the labelled oleic acid was displaced with unlabelled oleic at a concentration 10-fold higher than that of labelled oleic acid. This indicates the high affinity of long-chain fatty acids. The affinity of the protein was fundamentally towards these fatty acids, although it can bond hydrophobic substances, as occurs with other proteins belonging to this family. The α-toco pherol revealed much slower displacement kinetics than observed with oleic acid. Figures 5 a, b and c show the displacement curves for the different hydrophobic substances assayed. These data, as in the case of the affinity for fatty acids, reflect the strong similarity with those found with other FABP described in other animal species (Maatman *et al.*, 1994).

The role of these FABP should not be considered only as the bonding and incorporation of fatty acids, but rather they also serve as proteins capable of bonding to any hydrophobic substance, including some agents used in anti-protozoal chemotherapy, such as metronidazol (Fig. 4A), the molecule chosen for the specific treatment of protozoa in the genera *Giardia*, *Trichomonas* and *Entamoeba* as well as for anaerobic bacteria. This protein bonds to a lesser degree to nifurtimox than to metronidazol (Fig. 4A and B), the specific agent and extremely hydrophobic for tissue protozoa such as *Trypanosoma cruzi*. In this sense, these proteins could constitute not the action target of these drugs but rather a transport mechanism to the cytosol for the enzymatic target. They might also be responsible for the resistance that some protozoan strains show towards certain drugs.

This displacement of oleic labelled by metronidazol showed rapid kinetics, these results perhaps serving as an important basis for future research into the role of this protein as a target for this drug or as a determinant of the study of parasite strains resistant to this drug.

5. References

Andrews, A.T. 1986. Electrophoresis, Theory, Techniques and Biochemical and Clinical Applications. Clarenden Press, Oxford.

Bass, N.M. 1993. Cellular Binding Proteins for Fatty Acids and Retinoids: Similar or Specialized Functions?, *Mol Cell Biochem*, 123, 191-202.

Blair, R.J. and Weller, P.F. 1987. Uptake and esterification of arachidonic acid by trofozoites of *Giardia lamblia*. *Mol Biochem Parasitol*, 25, 11-18.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72, 248-254.

Buelt, M.K. and Bernlohr, D.A. 1990. Modification of the adipocyte lipid binding protein by sulphydryl reagents and analysis of the fatty acid binding domain. *Biochem*, 29, 7408-7413.

Campbell, F.M.; Gordon, M.J. and Dutta-Roy, A.K. 1994. Plasma membrane fatty acid-binding protein (FABP) of the sheep placenta. *Biochimica et Biophysica Acta*. 1214, 187-192.

Chabalgoity, J.A.; Harrison, J. A.; Esteves, A.; Demarco de Hormaeche, R.; Ehrlich, R; Anjam Khan, C.M. and Hormaeche, C. E. 1997. Expression and Immunogenicity of an *Echinococcus granulosus* Fatty Acid-Binding Protein in Live Attenuated *Salmonella* Vaccine Strains, *Infection and Immunity*, 2402-2412.

Dunn, M.J. 1993 Gel electrophoresis: Proteins. (Graham, J.M. and Billington, D., Eds.). Bios Scientific Publishers Limited, UK.

Edwards, M.R.; Gilroy, F.V.; Jimenez, B.M. and O'Sullivan, W.J. 1989. Alanine is a major end product of metabolism by *Giardia lamblia*: a proton nuclear magnetic resonance study. *Mol Biochem Parasitol*, 37, 19-26.

Farthing, M.J.G.; Mata, L.J.; Urrutia, J.J. and Kornmal, R.A. 1986a. Giardiasis: Impact on child growth. In: *Diarrhoea and Malnutrition in Childhood* (Waiker-Smith, J.A. and McNeish, A.S. Eds.) Butterworths, London, 68-78.

Frolov A. and Schroeder F. 1997. Time-resolved fluorescence of intestinal and liver fatty acid binding proteins: Role of fatty acyl CoA and fatty acid. *Biochemistry*, 36, 505-517.

Glatz, J.F.C.; Baerwaldt, C.C.F. and Veerkamp, J.H. 1984. Diurnal Variation of cytosolic fatty acid-binding protein content and of palmitate oxidation in rat liver and heart. *Amer Soc Biol Chem Inc*, 259, 4295-4300.

Glatz, J.F.C. and Van der Vusse, G.J. 1996. Cellular fatty acid-binding proteins: Their function and physiological significance. *Prog Lipid Res*, 35, 3, 243-282.

Gobert, G.N. 1998. Immunolocalization of Schistosome Proteins, *Mic Res and Tech*, 42, 167-185.

Hardy, P.M. 1985. The protein amino acids. In: *Chemistry and Biochemistry of the Amino Acids* (Barrett, G.C., Ed.) Chapman & Hall, London, 6-24.

Heukeshoven, J. and Dernick, R. 1985. Simplified method for silver staining of proteins in polyacrylamide gels and mechanism of silver staining. *Electrophoresis*, 6, 103-112.

Heyworth, M.F. 1996. *Giardia* infections. In: *Enteric infections and immunity*. (Paradise L.J. *et al.*, Eds.) New York: Plenum Press, 227-38.

Hiltpold, A.; Frey, M.; Hülsmeier, A. and Köhler, P. 2000. Glycosylation and palmitoylation are common modifications of *Giardia* variant surface proteins. *Mol. Biochem. Parasitol*, 109, 61-65.

Hopkins, R.S. and Juranek, D.D. 1991. Acute giardiasis: an improved clinical case definition for epidemiologic studies. Amer. J. of Epid. 133, 402-407.

Keister, D.B. 1983. Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. *Trans R Soc Trop*

Med Hyg, 77, 487-488.

Korman, S.H.; Bar-Oz, B.; Mandelberg, A. and Matoth, I. 1990. Giardiasis with protein-losing enteropathy: Diagnosis by fecal al-antitrypsin determination. *J of Pediat Gastr and Nut*, 10, 249-252.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Lalonde J.M.; Bernlohr D.A. and Banaszak L. 1994. Adipocyte lipid-binding protein coplexed arachidonic acid: titration calorimetry and x-ray crystallographic studies. *J Biol Chem* 269, 339-347.

Lee, J.; Jiao, X.; Hejtmancik, J.F.; Kaiser-Kupfer, M. and Chader, G.J. 1998. Identification, isolation and characterization of a 32-kDa fatty acid-binding protein missing from lymphocytes in humans with Bietti crystalline dystrophy (BCD). *Mol Genet Metab*, 65, 143-54.

Londraville, R.L.; Storch, J. and Sidell, B.D. 1996. Binding site polarity and ligand affinity of homologous fatty acid-binding proteins from animals with different body temperatures. Molecular and Cellular Biochemistry 159, 39-45.

Maatman, R.G.H.J.; van Moerkerk, H.T.B.; Nooren, I.M.A.; van Zoelen, E.J.J. and Veerkamp, J.H. 1994. Expression of human liver fatty acid binding protein in *Escherichia coli* and comparative analysis of its binding characteristics with muscle fatty acid binding protein. *Biochi Biophy Acta*, 1214, 1-10.

Murphy, E.J.; Edmondson, R.D.; Russell, D.H.; Colles, S. Y and Schroeder, F. 1998. Isolation and characterization of two distinct forms of liver fatty acid binding protein from rat. *Bioch Biophy Acta*, 1436, 413-425.

Sha, R.S.; Kane, C.D.; Xu, Z.; Banaszak, L.J. and Bernlohr, D.A. 1993. Modulation of ligand binding affinity of the adipocyte lipid-binding protein by selective mutation. Analysis *in vitro* and *in situ*. *J Biol Chem*, 268, 7885-7892.

Timanova, A.; Muller, S.; Marti, T.; Bancov, I. and Walter, R.D. 1999. *Ascaridia galli* fatty acid-binding protein, a member of nematode polyprotein allergens family. *Eur J Biochem*, 261, 569-76.

Van Nieuwenhoven, F.A.; Van der Vusse, G.J. and Glatz, J.F.C. 1996. Membrane-associated and cytoplasmic fatty acid-binding proteins. *Lipids*, 31, S223-S227.