A novel lipid-binding protein from the cestode *Moniezia expansa*

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A lipid-binding protein (LBP) has been purified from the cytosol of the cestode *Moniezia expansa*. The native LBP was found to be an oligomer of approx. 250 kDa, consisting of 11 kDa monomers. The LBP bound saturated and unsaturated fatty acids, but not their CoA derivatives, with K_D values in the range 0.68–7.8 μ M. Cholesterol, dihydroergosterol, bilirubin and retinoids were also bound, but α -tocopherol, bile acids, alk-2-enals and alka-2,4-dienals were not. Evidence suggests that there are two binding sites per subunit, each with different specificities. The fluorescent fatty acid 11-[(5-dimethylaminonaphthalene-1-sulphonyl)amino]undecanoic acid (DAUDA) and retinol both showed an additional high-affinity binding site with a density of approximately 0.1 per subunit, suggesting specific binding to the oligomer. The amino acid composition of *Moniezia* LBP was distinct from that of previously characterized fatty acid-binding

proteins (FABPs). The protein was not N-terminally blocked and yielded a unique amino acid sequence, unrelated to that of any known FABP; there was also evidence of microheterogeneity. Polyclonal antibodies raised to the *Moniezia* protein did not cross-react with mammalian, nematode or digenean FABP. The Gibbs free energy for protein folding (13.02 kJ/mol; 3.1 kcal/mol), determined by urea denaturation, was identical for both the native and ligand-bound *Moniezia* LBP. CD spectra showed that the *Moniezia* protein contained 36% α -helix and that the secondary structure underwent only minor changes on ligand binding. *Moniezia* LBP binds a range of anthelmintics, with K_D values again in the range 0.66–7.3 μ M. It is possible that, in helminths, binding proteins may play a role in determining the specificity and site of action of anthelmintics.

INTRODUCTION

The intracellular fatty acid-binding proteins (FABPs) are members of a family of widely distributed low-molecular-mass (14–15 kDa) proteins that bind, amongst other things, fatty acids, their acyl-CoA esters, sterols and non-polar organic ions such as haem, bilirubin and bromosulphophthalein. Some FABPs, like the one from mammalian liver, bind a wide range of different ligands; others are more restricted in their interactions. A variety of functions, including fatty acid uptake, transport and storage, targeting of fatty acids to specific organelles and pathways, sequestration of toxic compounds and regulation of gene action have been proposed for FABPs, but their exact function remains unclear [1,2]. Substrates bound to binding proteins may have a kinetic advantage and be preferentially metabolized. This could involve targeted transport and substrate channelling or modified enzyme activity [3–5].

In this paper we describe the purification of a lipid-binding protein (LBP) from the cytosol of the tapeworm *Moniezia expansa*. The protein is present in high amounts and appears to represent a new class of FABP. The interaction of parasite LBP with anthelmintics may be important in determining drug specificity and site of action and could be involved in the development of drug resistance.

MATERIALS AND METHODS

Materials

11-[(5-Dimethylaminonaphthalene-1-sulphonyl)amino]undecanoic acid (DAUDA) was obtained from Molecular Probes. Sephadex G-25M (PD10 columns), G-75, G-200, and DEAE-A50, were obtained from Pharmacia. Alka-2,4-dienals and alk-2-enals were obtained from Aldrich. Western Blue stabilized

substrate for alkaline phosphatase was obtained from Promega, poly(vinylidene difluoride) membrane (Immobilon-P) from Millipore and Titremax from CYTRX. Goat anti-rabbit IgG (alkaline phosphatase-conjugated), Lipidex 1000, bilirubin, cholesterol, dihydroergosterol, cholic acid, lithocholic acid, chenodeoxycholic acid, retinoic acid, retinal, retinol, α -tocopherol, all saturated and unsaturated fatty acids and their acyl-CoA esters were from Sigma. All anthelmintics were from commercial sources (Wellcome, Bayer, Hoechst, Ciba Geigy, Janssen Pharmaceutica).

Purification of *M. expansa* LBP

Adult *M. expansa* were obtained from the intestines of freshly slaughtered sheep at a local abattoir. The cestode tissue was homogenized at 4 °C in extraction buffer (1:1, w/v) [10 mM phosphate buffer (pH 7.4)/0.2 mM EDTA/0.2 mM dithiothreitol/1 mM PMSF]. The homogenate was centrifuged at 12000 *g* for 5 min at 4 °C. The resulting supernatant fraction was centrifuged at 105000 *g* for 90 min at 4 °C, and the supernatant was designated the cytosol. *M. expansa* LBP was partially purified from the cytosol by adding $(\text{NH}_4)_2\text{SO}_4$ to 70% saturation. After incubation (overnight at 4 °C) and centrifugation (30 min at 12000 *g*), the supernatant was aspirated and desalted using a Sephadex G-25 column, equilibrated with 10 mM potassium phosphate buffer, pH 7.4 (buffer A). The proteins, eluted with buffer A, were concentrated by ultrafiltration, using an Amicon PM30 membrane; the 30 kDa cut-off filtration membrane did not let any appreciable fatty acid-binding activity through. The concentrate was loaded on to a Sephadex G-75 column (60 cm $\times$ 2.5 cm), previously equilibrated with buffer A. The first protein peak (void volume), which was found to contain the bulk of the fatty-acid binding activity, was then exchanged into

Abbreviations used: DAUDA, 11-[(5-dimethylaminonaphthalene-1-sulphonyl)amino]undecanoic acid; LBP, lipid-binding protein; FABP, fatty acid-binding protein; Ex, excitation; Em, emission.

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30 mM Tris/HCl buffer (pH 9.0) containing 60 mM NaCl (buffer B) using a Sephadex G-25 column previously equilibrated with buffer B. The eluate was loaded on to a Sephadex DEAE-A50 column (12 cm × 2 cm), equilibrated with buffer B. After adsorption, the column was washed with 2 bed vol. of buffer B, and the LBP was eluted with a linear gradient of 60–500 mM NaCl in buffer B. Fractions (5 ml) were collected at a rate of 20 ml/h. The absorbance of the column fractions was determined at 280 nm. The fractions eluting in 200 mM NaCl were pooled and concentrated by ultrafiltration (Amicon PM30 membrane). The purified LBP was subjected to delipidation using Lipidex 1000 at 37 °C as described by Glatz et al. [6]. LBP samples were loaded on a column of Lipidex 1000 (5 cm × 1 cm) equilibrated with 30 mM Tris/HCl buffer, pH 7.4, containing 1 mM dithiothreitol. The delipidated LBP eluted in the void volume. The purity of the preparation was monitored by 8–25 % gradient PAGE under denaturing and reducing conditions, using a FASTsystem (Pharmacia). The gels were silver stained according to the Bio-Rad procedure.

Assays of fatty acid-binding activity using fluorescent ligands

Detection of fatty acid-binding capacity in cytosolic extracts and during the purification procedure was determined using DAUDA [7]. This fluorescent lipid shows a blue shift of emission wavelength maxima from 530 nm to 480 nm when it binds to protein. In practice, fractions were added to 1 μ M DAUDA in 50 mM phosphate buffer, pH 7.2, in a final volume of 1 ml. After 10 min equilibration at 25 °C, fluorescence measurements were made at 500 nm and excitation wavelength at 350 nm, and the fluorescence enhancement that occurred with the probe binding to LBP was compared with the amount of fluorescence of the same probe in buffer alone. Furthermore, the fluorescent probe appeared to bind to the fatty acid-binding site of LBP, as most non-esterified fatty acids tested caused displacement of the probe; this was detected as a concomitant decrease in fluorescence intensity with increasing fatty acid concentration. The DAUDA-binding sites were characterized as outlined by Wilton [8]: 2 μ l samples of methanol containing 0.2 and 1.0 mM DAUDA were added to 2 ml of LBP (0.87 μ M) in phosphate buffer (50 mM, pH 7.2), and the fluorescence was recorded. Measurements of fluorescence were done, adding the same amounts of DAUDA to the buffer without LBP, as well as to buffer containing an excess concentration of LBP (40 μ M). Assuming that these latter data represent, respectively, 0 and 100 % binding, and that the amount of DAUDA bound is proportional to the relative fluorescent intensity [8], the proportion of bound (mol of probe/mol of LBP) and free probe was calculated and the data subjected to Scatchard analysis [9]. In the calculations, the molecular mass of LBP was taken to be that of the monomer (11 kDa); protein was determined using the Coomassie G-250 binding assay with BSA as standard [10] and calibrated with purified LBP. In all cases, the maximum final methanol concentration in the assays was 1 % (v/v). Other fluorescent substances that were studied in this way were retinol [excitation (Ex)_{max.} 350 nm, emission (Em)_{max.} 464 nm] and dansyl-DL- α -aminocaprylic acid (Ex_{max.} 328 nm, Em_{max.} 514–533 nm).

Binding studies using non-fluorescent ligands

The binding to *M. expansa* LBP of putative ligands that themselves show no fluorescence was evaluated by measuring the displacement of DAUDA bound to LBP. The apparent dis-

sociation constant (K_D) for competing ligands was calculated by standard analysis [11]:

$$F_{\text{obs.}} = \frac{F_{\text{max.}}}{1 + \frac{K_D}{L} \left(1 + \frac{i}{K_i} \right)}$$

where L is the concentration (1 μ M) of ligand producing the fluorescent signal (F), i the concentration of competing ligand and K_i its dissociation constant. A plot of $F_{\text{max.}}/F_{\text{obs.}}$ against i yields a straight line, with intercept $[1 + (K_D/L)]$ on the ordinate and a slope $(K_D/L) \times (1/K_i)$. The K_D for DAUDA on LBP was measured by separate titration and Scatchard analysis as shown above, and the K_D value of 7.72 μ M, together with the values obtained for the slope of the straight line for each ligand i , were used to calculate the apparent K_D , or K_i , from the equation $7.72/[\text{slope value}]$. The range for competing ligands was 0.1 to 5 μ M. Fatty acids were dissolved in ethanol, except caproic (C_6) and caprylic (C_8), which were dissolved in methanol; behenic (C_{22}) and lignoceric (C_{24}) were dissolved in ethanol after prolonged sonication. The final maximum ethanol/methanol concentration in the assays was 1 % (v/v). K_D values are given as means S.E.M.

Alternatively, binding of non-fluorescent ligands was studied by measuring changes in the fluorescence of LBP tryptophan. Fluorescence spectra of LBP between 300 and 500 nm (Ex_{max.} 284 nm) were recorded on a Perkin-Elmer LS-5B luminescence spectrometer. The binding of fatty acids and other ligands was measured by following the decrease of intrinsic protein tryptophan fluorescence at 317 nm. Typically, to 2 ml of LBP solution (0.87 μ M) in a cuvette, small increments (0.1 μ M as 2 μ l samples) of the ligand were injected into the cuvette with a micropipette, and the fluorescence intensity changes for every titration point were noted. Since differences in protein-tryptophan fluorescence, caused by binding ligands, are generally not proportional to the amount of bound ligand, no attempt was made to estimate the binding affinity and number of binding sites using this method.

Radiolabelled fatty acid-binding assay

The radiolabelled fatty acid-binding assay with Lipidex 1000 was carried out as described by Nemezc et al. [12]. The assay mixture contained 40 μ l of 185 kBq/ml fatty acid ([1- 14 C]palmitate or [1- 14 C]linoleic) in 25 % (w/v) ethanol, 960 μ l of 50 μ M potassium phosphate buffer, pH 7.2, with or without 0.8 μ M *Moniezia* LBP. Fatty acids were added to the tubes in 50 % ethanol, such that the final concentration ranged from 0.1 to 10 μ M. The final ethanol concentration was the same in each tube and did not exceed 1 %. After incubation at 25 °C for 20 min, the tubes were chilled in ice for 10 min and 150 μ l of 50 % (v/v) Lipidex-1000 in 0.1 M phosphate buffer, pH 7.2, was added to each tube. After stirring vigorously, the tubes were incubated at 4 °C for 10 min, then centrifuged at 16000 g for 4 min. Aliquots of the supernatant were removed, and radioactivity was determined by liquid-scintillation counting in an LKB 1215 Rack Beta using Emulsifier-Safe (Packard) scintillant. Blank assays (without LBP) were performed in parallel for each fatty acid concentration. The data were plotted as a Scatchard plot after correction for non-specific binding [13].

Physico-chemical characterization of *M. expansa* LBP

The conformational stability of the purified protein was estimated in solutions containing LBP (0.35 μ M), potassium phosphate buffer (0.1 M), pH 4.5, NaCl (0.2 M) with or without oleic acid

(1 μ M) or DAUDA (1 μ M). The linear extrapolation method [14] was used to estimate the unfolding free-energy changes of the protein in the presence of different concentrations (0–8 M) of urea, as measured by spectrofluorimetry (of DAUDA or intrinsic tryptophan). CD spectra of LBP [0.5 mg/ml in Tris/HCl buffer (20 mM), pH 7.0] were measured at 0.2 nm intervals between 190 and 240 nm using a JASCO J-600 spectropolarimeter, in 0.02 cm pathlength cells. For comparison, measurements were also carried out with the same samples in Tris/HCl buffer (20 mM), pH 7.0, containing 33 mM Na_2SO_4 . The data were converted to molar ellipticity values ($\text{degrees} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$) using a mean residue weight of 110. An estimate of the secondary structure was based on the CONTIN program [15]. For amino acid composition and N-terminal sequencing, the purified LBP was subjected to SDS/PAGE and electrophoretically transferred to a poly(vinylidene difluoride) membrane (Immobilon-P) and stained with Coomassie Blue. The protein band was cut from the membrane and subjected to amino acid analysis and N-terminal sequencing. Amino acid analysis was carried out by Dr. K. Bailey, Queen's Medical Centre, Nottingham and N-terminal sequencing by Eurosequence, Groningen, The Netherlands.

Western blot

A rabbit was injected intramuscularly with a suspension of 250 μ g of *M. expansa* LBP in 250 μ l of PBS and 250 μ l of Titremax. The IgG from pre-immune and immune sera was purified in a two-step procedure using octanoic acid and $(\text{NH}_4)_2\text{SO}_4$ [16]. The titre of pre-immune and immune sera was estimated by dot-blot ELISA: spots containing 1 μ g of LBP, or BSA (as control), were applied to Immobilon-P membranes; the membranes were further incubated with a solution of 1% BSA in PBS (pH 7.4); after 3 h the membranes were washed and further incubated with different concentrations of rabbit IgG (pre-immune and immune sera). Goat anti-rabbit IgG alkaline phosphatase conjugate (1:20000) was used as the secondary antibody. The blots were developed with Western Blue stabilized

substrate for alkaline phosphatase. The cross-antigenicity of LBP with other tissues was studied by preparing cytosolic extracts of the cestode *Hymenolepis diminuta*, digenean *Fasciola hepatica*, nematode *Ascaris suum*, as well as cytosolic extracts from rat liver and sheep intestinal tissue. Extracts were made in extraction buffer at 4 °C (1:1, w/v) [10 mM phosphate buffer (pH 7.4)/0.2 mM EDTA/0.2 mM dithiothreitol/1 mM PMSF], and stored at –80 °C before use. Samples of these extracts and that of *M. expansa* were separated on 8–25% gradient gels by SDS/PAGE using the FASTsystem. The separated proteins were

Figure 2 PAGE of *M. expansa* LBP

(a) 8 μ g of purified LBP (P) and high-molecular-mass standards (M) run on a native gradient gel, 8–25% acrylamide. (b) 5 μ g of purified LBP (P), 30 μ g of total cytosol (T) and low-molecular-mass markers (M) run on a SDS/20% polyacrylamide gel.

Figure 1 DEAE-Sephadex chromatography of *M. expansa* LBP

The void volume obtained after G-75 gel filtration was pooled, concentrated and separated on a DEAE-Sephadex A50 column (12 cm $\times$ 2 cm), equilibrated with 30 mM Tris/HCl buffer/60 mM NaCl (pH 9.0). The flow rate was 30 ml/h and the fraction volume was 4.8 ml. The proteins were eluted in a step-wise gradient (from 60 to 500 mM NaCl) as shown on the Figure. Absorbance at 280 nm (---); DAUDA-binding activity (—) (for details see the Materials and methods section); NaCl concentration (·····).

Figure 3 Sephadex G-200 gel filtration of *M. expansa* LBP

Purified LBP (10 mg), previously incubated with 2 μ mol of DAUDA, was loaded on to a Sephadex G-200 column (90 cm $\times$ 1.5 cm) equilibrated with 20 mM potassium phosphate buffer/0.1 M NaCl (pH 7.2). The protein was eluted at a flow rate of 6 ml/h, 3 ml fractions were collected and the fluorescence was measured. The elution profile is expressed in the graph as the ratio of the eluted volume, V_e , over the void volume, V_0 . Molecular-mass markers were: (a) catalase, 250 kDa; (b) β -amylase, 200 kDa; (c) BSA, 55 kDa; (d) horseradish peroxidase, 40 kDa; (e) trypsinogen, 24 kDa.

then transferred to an Immobilon-P membrane using the PhastTransfer. The Western blot was incubated with anti-LBP IgG (1 : 1000) and developed as described for the dot-blot ELISA.

RESULTS

Purification of *M. expansa* LBP

Fractionation of *M. expansa* cytosol with 70% $(\text{NH}_4)_2\text{SO}_4$ yielded a supernatant that contained almost all of the proteins that interacted with the fluorescent DAUDA probe. Further fractionation of this supernatant by Sephadex G-75 gel filtration revealed virtually no peaks of DAUDA-binding activity in the low-molecular-mass-protein fraction; instead, all the activity was found in the void volume. After separating this fraction by DEAE-anion-exchange chromatography, a single DAUDA-binding-activity peak eluted at 200 mM NaCl (Figure 1); this final preparation showed a single band on native and SDS/PAGE of approx. 165 and 11 kDa respectively (Figure 2). However, when the purified protein was loaded on to a Sephadex G-200 column, the molecular mass was estimated to be approx. 250 kDa (Figure 3). The mobility of a protein under native PAGE depends on other factors besides size, such as its charge, conformation, and degree of hydration. Therefore, a native molecular mass of

250 kDa, based on Sephadex G-200 gel filtration, is a more reliable estimate. The purification procedure resulted in a recovery of 60% of the DAUDA-binding activity (Table 1).

The low-molecular-mass form of the protein found by SDS/PAGE depended solely on the presence of SDS in the electrophoresis buffer, and was independent of whether the protein was previously denatured with SDS, β -mercaptoethanol or heat. Treatment of the delipidated high-molecular-mass protein with a range of concentrations of linoleic and oleic acids (ligand to protein ratio 0.2 : 5) again resulted in no dissociation into subunits as judged by Sephadex G75 chromatography or by ultrafiltration through an Amicon 30 kDa cut-off filter.

Physico-chemical characterization of *M. expansa* LBP

The UV/visible spectrum of purified LBP showed a peak at 280.5 nm (results not shown), and the $\epsilon_{280}^{1\%}$ was estimated to be 9.858. When purified LBP was subjected to isoelectric focusing (IEF) on Pharmacia IEF gels, a single band was observed at approx. pH 6.4 (results not shown).

Binding of DAUDA to the protein was dependent on the pH and the ionic strength of the incubation medium, showing optima at about 100 mM NaCl and at pH 5–6 (Figure 4). This is a rather low pH value for optimum binding to a lipid-binding protein. The subsequent binding assays were carried out at pH 7.2, since this represents a more realistic physiological pH and many of the substrates tested show only limited solubility in acid solution.

In order to obtain information on the conformational stability of the protein, unfolding by urea was measured. The Gibbs free energies for protein folding ($\Delta G_{\text{app}}^\circ$) were essentially identical for native and ligand-bound LBP, being about 13.02 kJ/mol (3.1 kcal/mol), based on measurements in the presence of urea between 0 and 2 M (Figure 5); at higher urea concentrations the unfolding was complete. The CD spectrum of *M. expansa* LBP is shown in Figure 6. When the data were analysed through the CONTIN program [9], the secondary structure of the native protein in 20 mM Tris/HCl, pH 7.0, was estimated to contain $36 \pm 1\%$ of α -helix and $64 \pm 4\%$ of β -sheet and remainder secondary structure. In the presence of cholesterol, the percentage of α -helix remained unchanged (α -helix $35 \pm 1\%$, β -sheet and remainder $65 \pm 3\%$), whereas in the presence of linoleic acid the percentage of α -helix was reduced to $30 \pm 1\%$ and the β -sheet and remainder increased to $70 \pm 3\%$. When 33 mM Na_2SO_4 , which has an ionic strength comparable with 100 mM of NaCl, was present in the buffer, virtually no changes in the secondary structure were observed after adding linoleic acid or cholesterol to the LBP (LBP in the presence of 33 mM Na_2SO_4 : $36 \pm 0.7\%$ α -helix, $64 \pm 0.7\%$ β -sheet and remainder; plus linoleic acid: $37 \pm 0.8\%$ α -helix, $63 \pm 0.8\%$ β -sheet and remainder; plus cholesterol: $36 \pm 1.5\%$ α -helix, $64 \pm 1.6\%$ β -sheet and remainder).

Table 1 Purification of LBP from *M. expansa*

Sample	Protein (mg/ml)*	Total volume (ml)	Total protein (mg)	Protein (%)	Specific DAUDA binding (pmol/ μ g)†	Total activity	Yield
Cytosol	7.05	75.0	528.8	100.0	126	66525	100.0
$(\text{NH}_4)_2\text{SO}_4$	18.50	8.0	148.0	28.0	334	49364	74.2
G-75	7.43	10.5	78.0	14.7	584	45505	68.4
DEAE	7.39	4.5	35.5	6.7	1125	39943	60.0

* Based on a Coomassie G-250-binding assay [10].

† Determined by Scatchard analysis; see the Materials and methods section for details.

Figure 4 Dependence of DAUDA binding to *Moniezia* LBP on pH and ionic strength

The effect of pH of the incubation medium on the binding of DAUDA (1 μ M) to *M. expansa* LBP (0.87 μ M) was studied in acetate and Tris/HCl buffers covering the pH range 3–9.5 (○). In order to study the effect of ionic strength on binding, the protein and ligand were incubated in a saline Tris/HCl buffer, pH 7.2, containing 0–4.0 M NaCl (●). Measurements were taken at Em_{500} and Ex_{350} nm.

Figure 5 Estimation of Gibbs free energy of *M. expansa* LBP

LBP (0.35 μ M) alone (●), or equilibrated with oleic acid (1 μ M) (▲) or DAUDA (1 μ M) (■), was denatured with urea at concentrations between 0 and 8 M. Fluorescence data transformed according to Pace [14] are shown in the Figure, and were used to evaluate the unfolding free energy changes of the protein (see the Materials and methods section for details). One cal/mol is equal to 4.2 J/mol.

The amino acid composition of *M. expansa* LBP showed that the protein was rich in glutamine/glutamic acid, and in the non-polar amino acids alanine, isoleucine and lysine. Cysteine and tryptophan were destroyed by the methods used to hydrolyse the

Figure 6 CD spectrum of *M. expansa* LBP

This shows the spectrum between 190 and 240 nm of LBP (0.5 mg/ml) in Tris/HCl buffer (20 mM, pH 7.0).

protein. However, since the protein also displayed a fluorescence with Ex_{max} 287 and Em_{max} 317 nm, the presence of at least one tryptophan is probable. In Table 2, the amino acid composition

Abbreviations: Asx, Asp + Asn; Glx, Glu + Gln; n.d., not determined.

Amino acid	<i>M. expansa</i>	Rat			EgDII§	Sm14†
	LBP*	(L)-FABP†	(I)-FABP†	(H)-FABP‡		
Asx	5.5	11	16	9	10	16
Glx	18.4	17	16	14	15	11
Ser	4.4	6	4	4	5	13
Gly	5.5	12	12	10	11	8
His	0.0	2	1	2	2	1
Arg	8.9	2	6	2	7	5
Thr	2.3	12	10	10	13	20
Ala	10.4	2	6	2	3	3
Pro	2.6	2	0	16	1	2
Tyr	5.2	3	5	2	3	1
Val	6.5	12	11	9	16	14
Met	1.0	7	4	4	7	6
Ile	9.1	9	7	7	4	3
Leu	8.4	6	10	5	7	7
Phe	2.8	6	8	5	9	7
Lys	8.9	17	15	14	16	13
Trp	n.d.	n.d.	n.d.	n.d.	1	2
Cys	n.d.	n.d.	n.d.	n.d.	2	1
Total	100	126	131	115	132	133

* Calculated from an average of two hydrolysates.

† According to Aipers et al. [17].

† Calculated from Glatz et al. [18].

§ Amino acid composition inferred from the nucleotide sequence of an FABP from *Echinococcus granulosus* [19].

¶ An FABP from *Schistosoma mansoni* [20].

Figure 7 Partial N-terminal amino acid sequence of *M. expansa* LBP

The N-terminal sequence showed that the protein is not N-terminally blocked. Amino acids at the first 30 positions were identified and are shown in Figure 7. At several positions two amino acids were detected, which may indicate microheterogeneity of the protein (position 23, Asp 10 pmol/Glu 21 pmol; position 24, Phe 16 pmol/Arg 9 pmol; position 27, Lys 11 pmol/Asn 6 pmol; position 29, Pro 14 pmol/Ala 7 pmol). Another indication of microheterogeneity (although less clear) was found at position 5, where besides Asn (approx. 95 pmol) a Lys residue (approx. 10 pmol) was detected (not shown in Figure 7).

Binding studies

Binding of DAUDA to the purified LBP from *Moniezia* was analysed by means of a Scatchard plot. This revealed a single class of saturable binding sites ($n = 0.93 \pm 0.09$) with a K_D of $7.72 \pm 1.2 \mu\text{M}$. At low molar ratios of DAUDA:LBP (up to

about 0.1) a high-affinity binding site (K_D $0.41 \pm 0.09 \mu\text{M}$, $n = 0.12 \pm 0.02$) was found (Figure 8). When LBP was incubated with saturated or unsaturated fatty acids, as well as CoA derivatives, no quenching of intrinsic protein tryptophan fluorescence, as evidenced by light emission at 317 nm after excitation at 287 nm was observed. In contrast, when these fatty acids and CoA esters were incubated with LBP and DAUDA, they caused displacement of the fluorescent probe: of the saturated non-esterified fatty acids, stearic acid and arachidic acid had the highest affinity (K_D 0.68 and 0.69 μM respectively) (Table 3). No binding was observed for caproic, caprylic, behenic or lignoceric acid. In the case of caprylic acid, this was confirmed using its fluorescent derivative dansyl-DL- α -aminocaprylic acid. When this fluorescent lipid was incubated with a high concentration of BSA, the fluorescence maximum of the probe showed a blue shift from 533 to 514 nm, and a 5-fold increase of fluorescence intensity per μM ligand. In the presence of *M. expansa* LBP, no increase ($< 0.01\%$) was detected, suggesting that this ligand bound to BSA, but not to LBP (results not shown). All of the unsaturated fatty acids tested were found to cause displacement of DAUDA fluorescence, with K_D values of less than 1 μM , except for erucic and nervonic acid. The CoA derivatives of fatty acids showed less binding capacity than the non-esterified fatty acids: only palmitoyl- and palmiteoyl-CoA had binding affinities that were just measurable by displacement of DAUDA, both with a K_D of about 7 μM .

Using an isotopic assay, the $K_{D,app}$ for palmitate was found to be $0.42 \pm 0.03 \mu M$ ($n = 1.1 \pm 0.05$) and for linoleate $0.34 \pm 0.1 \mu M$ ($n = 1.5 \pm 0.77$). This is in reasonable agreement with the results from DAUDA displacement.

When the binding of a series of other putative ligands was tested, some of these ligands, namely bilirubin, retinoids (retinoic

Figure 8 Scatchard analysis of the binding of DAUDA by purified *M. expansa* LBP

Fixed amounts ($0.87 \mu\text{M}$) of purified LBP were incubated with $0.2\text{--}7 \mu\text{M}$ DAUDA in a total volume of 2 ml of buffer solution. Fluorescence was measured (Ex_{350} , Em_{500}) after equilibration. The data (as shown in the graph insert) were subjected to Scatchard analysis (main graph). It was assumed that the maximal fluorescence achieved in the presence of excess LBP represents 100% binding and that the amount of DAUDA bound is proportional to the relative intensity. As shown in the main graph, at low molar DAUDA/LBP ratios (up to about 0.1) a binding site with an apparent K_D of $0.41 \mu\text{M}$ ($n = 0.12$) (B) was identified, and at higher molar ratios a single binding site with an apparent K_D of $7.72 \mu\text{M}$ ($n = 0.93$) (A). Correlation coefficients: line A, $r = 0.94$; line B, $r = 0.99$.

Figure 9 Scatchard analysis of the binding of retinol by purified *M. expansa* LBP

Fixed amounts ($0.87 \mu\text{M}$) of purified LBP were incubated with $0.1\text{--}5 \mu\text{M}$ retinol in a total volume of 2 ml of buffer solution. Fluorescence was measured after equilibration (Ex_{350} , Em_{464}). The data, shown for concentrations up to $1 \mu\text{M}$ in the graph insert, were subjected to Scatchard analysis (main graph). It was assumed that the maximal fluorescence achieved in the presence of excess LBP represents 100% binding and that the amount of retinol bound is proportional to the relative intensity. As shown in the main graph, at low molar retinol/LBP ratios (up to about 0.1) a binding site with an apparent K_D of $0.5 \mu\text{M}$ ($n = 0.11$) (B) was identified, and at higher molar ratios a low-affinity binding site with an apparent K_D of $27 \mu\text{M}$ ($n = 0.98$) (A). Correlation coefficients: line A, $r = 0.66$; line B, $r = 0.95$.

Table 3 Binding of non-esterified fatty acids to *M. expansa* LBP

None of the lipids showed quenching of intrinsic tryptophan fluorescence. Apparent K_D values were obtained after measuring the quenching of bound DAUDA fluorescence, and are means $\pm$ S.E.M. of at least 3 experiments. —, no binding detected.

Ligand	Apparent K_D (μM)
Saturated fatty acids	
Caproic acid (6:0)	—
Caprylic acid (8:0)	—
Capric acid (10:0)	7.80 ± 4.7
Lauric acid (12:0)	2.99 ± 0.08
Myristic acid (14:0)	1.21 ± 0.13
Palmitic acid (16:0)	0.95 ± 0.01
Stearic acid (18:0)	0.68 ± 0.01
Arachidic acid (20:0)	0.69 ± 0.01
Behenic acid (22:0)	—
Lignoceric acid (24:0)	—
Unsaturated fatty acids	
Oleic acid (18:1)	0.76 ± 0.01
Arachidonic acid (20:4)	0.82 ± 0.01
Linoleic acid (18:2)	0.72 ± 0.01
Linolenic acid (18:3)	0.76 ± 0.01
Palmitoleic acid (16:1)	0.78 ± 0.01
Docosahexaenoic acid (22:6)	0.58 ± 0.1
Petroselenic acid (18:1)	0.79 ± 0.01
Elaidic acid (18:1)	0.88 ± 0.01
Nervonic acid (24:1)	2.16 ± 0.01
Erucic acid (22:1)	1.31 ± 0.01
CoA derivatives	
<i>n</i> -Octanoyl-CoA (8:0)	—
Palmitoyl-CoA (16:0)	7.28 ± 0.14
Palmitoyl-CoA (16:1)	7.15 ± 0.11
Stearoyl-CoA (18:0)	—
Linoleoyl-CoA (18:2)	—
Behenoyl-CoA (22:0)	—

acid, retinal, retinol), cholesterol and dihydroergosterol, caused quenching of the intrinsic tryptophan fluorescence. With the exception of the sterols, all of these ligands also caused displacement of DAUDA. The apparent K_D values (μM) determined by DAUDA displacement were bilirubin 0.32 ± 0.004 , retinoic acid 1.46 ± 0.04 and retinal 0.57 ± 0.01 . The binding of retinol was studied by measuring fluorescence at 464 nm, and the data were subjected to Scatchard analysis. This revealed a high-affinity site with a $K_{D,app}$ of $0.5 \pm 0.2 \mu\text{M}$ ($n = 0.11 \pm 0.01$) at low concentration ratios of retinol to protein. At higher concentrations of retinol, a single ($n = 0.98 \pm 0.45$) low-affinity site with a $K_{D,app}$ of $27 \pm 6.7 \mu\text{M}$ was found (Figure 9). Analysis of the fluorescent binding data for DAUDA and retinol by the method of van Zoelen et al. [21] gave non-linear plots, confirming the presence of more than one binding site. Bile acids (cholic acid, lithocholic acid, chenodeoxycholic acid), α -tocopherol, alka-2,4-dienals (*trans,trans*-2,4-nonadienal, *trans,trans*-2,4-decadienal, *trans,trans*-2,4-heptadienal and *trans,trans*-2,4-hexadienal), alk-2-enals (*trans*-2-hexenal, *trans*-2-nonenal and *trans*-2-octenal) and 4-hydroxynonenal did not show either quenching of tryptophan fluorescence or displacement of bound DAUDA.

Of a series of common anthelmintics tested, praziquantel and benzimidazole carbamates showed no evidence of binding to the LBP. In contrast, other drugs caused both fluorescence quenching of tryptophan and displacement of bound DAUDA, with K_D values down to $0.47 \mu\text{M}$ (hexachlorophene) and $0.66 \mu\text{M}$ (bithionol) (Table 4).

Western blotting of LBP

A rabbit polyclonal immune serum against *M. expansa* LBP was obtained with a titre of 1:3500 as estimated by dot-blot ELISA, compared with a titre of 1:200 in pre-immune serum. The antibodies recognized a single band in purified LBP preparations

Table 4 Binding of anthelmintics to *M. expansa* LBP

Apparent K_D values were obtained after measuring the quenching of bound DAUDA fluorescence, and are the means $\pm$ S.E.M. of at least 3 experiments. The statistical error was less than 10% of the mean. —, no binding detected; +, quenching of tryptophan fluorescence observed.

Ligand	Tryptophan quenching	Apparent K_D (μ M)
Albendazole	—	—
Bithionol	+	0.66 ± 0.01
Dichlorophene	+	7.35 ± 0.06
Fendbendazole	—	—
Hexachlorophene	+	0.47 ± 0.06
Niclosamide	+	0.76 ± 0.01
Nitroscanate	+	0.81 ± 0.01
Oxybendazole	—	—
Oxyclozanide	+	0.79 ± 0.01
Praziquantel	—	—
Rafoxanide	+	0.75 ± 0.04
Resorantel	+	4.36 ± 0.17
Scolaban	—	—
Trichlobendazole	—	—

Figure 10 SDS/PAGE and immunoblot of anti-LBP serum against *M. expansa* LBP and non-*M. expansa* extracts

About 30 μ g of extract protein or 3 μ g of purified LBP were run on SDS/PAGE (gradient 8–25% polyacrylamide) (lanes a), then blotted, incubated with rabbit anti-LBP serum, followed by anti-rabbit IgG, conjugated with alkaline phosphatase, and developed with a colour reagent (lanes b). Shown are extracts from sheep intestine (S), rat liver (R), *Ascaris suum* (A), *Fasciola hepatica* (F), *Hymenolepis diminuta* (H), *M. expansa* (M) and purified LBP from *M. expansa* (L).

and in cytosolic fractions of homogenized whole *M. expansa*. No obvious cross-reactivity was found with extracts from *F. hepatica*, *A. suum*, rat liver or sheep intestine. Only in extracts from *H. diminuta* were a faint protein band in the 11–13 kDa region and a series of stronger reacting bands in the regions of 30–40 kDa and higher revealed (Figure 10).

DISCUSSION

Parasitic helminths contain large amounts of lipid, but possess only limited ability to synthesize long-chain fatty acids *de novo*, so must rely heavily on uptake of non-esterified fatty acids from the host [22].

In vertebrates some seven cytoplasmic FABP types with amino acid sequence similarities of 25–65% have been identified, namely liver, intestinal, heart/muscle, adipocyte, myelin, ileal and epi-

dermal FABP types [23–27]. Invertebrate FABPs showing sequence similarity with mammalian FABP have been isolated from a variety of sources, including *Schistosoma mansoni* [20], *Echinococcus granulosus* protoscoleces [19], *Schistocerca gregaria* [28], *Locusta migratoria* [29,30] and *Manduca sexta* [31]. In general FABPs preferentially bind unsaturated fatty acids with K_D values in the μ M range [12,32]; only liver FABP and heart FABP bind both fatty acids and their CoA derivatives [25]. Liver FABP and heart FABP bind approximately one fatty acid per protein molecule [18]. Binding of *Moniezia* LBP to the fluorescent fatty acid DAUDA revealed a single binding site per protein molecule, with a K_D of 7.7 μ M. At low DAUDA-to-protein ratios a high-affinity site (K_D 0.41 μ M) with a density of about 0.1 per protein molecule was also apparent. Retinol similarly showed low- and high-affinity sites with a density of 1 and 0.1 per protein molecule respectively. The *Moniezia* LBP bound saturated and unsaturated fatty acids with K_D values in the μ M range, with a preference for unsaturated acids; fatty acyl-CoA derivatives were not effectively bound. Nor did the protein bind α -tocopherol, bile acids, alk-2-enals or alka-2,4-dienals. The latter are secondary products of lipid peroxidation; lack of binding of carbonyls to *Moniezia* LBP suggests that the protein is not involved in protection against oxidative stress.

Of the ligands tested, cholesterol and dihydroergosterol caused considerable quenching of intrinsic tryptophan fluorescence, but not displacement of DAUDA. In contrast, saturated and unsaturated fatty acids displaced DAUDA but showed no tryptophan quenching. This would suggest two binding sites: one that is sensitive to tryptophan quenching and is preferentially occupied by sterols, the other that does not cause tryptophan quenching and is preferentially occupied by fatty acids. However, the binding of bilirubin and retinoid compounds to *Moniezia* LBP caused both tryptophan quenching and DAUDA displacement. Possibly these compounds bind in such a way that they overlap both binding sites.

Moniezia LBP showed a single band of about 11 kDa on SDS/PAGE. Gel filtration of the native protein gave a molecular mass of 250 kDa, suggesting an oligomer of about 22 subunits. Aggregation seemed to be due entirely to hydrophobic interactions; it did not involve thiol links, and there was no evidence that ligand binding led to dissociation of the oligomer. Under the conditions used for the binding assays, the *Moniezia* LBP would be in the oligomeric form. The demonstration with DAUDA and retinol of high-affinity binding sites with a density of 0.1 per protein molecule suggests that these ligands may bind to the oligomer as a whole, possibly in a non-specific manner, as well as to the subunit binding sites. A less likely explanation is that, in the oligomeric state, one of the oligomers shows a high-affinity site. Differences in binding between the free and oligomeric forms of *Moniezia* LBP could be a way of regulating the sequestration of ligands *in vivo*, as has been suggested by Fournier et al. [33] for the concentration-dependent reversible aggregation of heart FABP.

Urea denaturation studies gave a consistent Gibbs free energy value of about 13.02 kJ/mol (3.1 kcal/mol) for *Moniezia* LBP, irrespective of whether it was obtained using DAUDA binding or tryptophan fluorescence. This is similar to the values of 16.38 kJ/mol (3.9 kcal/mol) and 13.86 kJ/mol (3.3 kcal/mol) reported for phospho and dephospho 3T3L1 adipocyte FABP denatured with guanidinium chloride [34], but is significantly different from the value of 8.4 kJ/mol (2 kcal/mol) reported for recombinant rat liver FABP using urea denaturation [35], and the 42 kJ/mol (10 kcal/mol) and 52.92 kJ/mol (12.6 kcal/mol) reported for apo and holo recombinant rat intestinal FABP, again using urea denaturation [36].

The CD spectrum of *Moniezia* LBP showed a large negative ellipticity at about 222 nm, and the overall spectrum predicted that the protein contained about 36% α -helix. This is a little higher than the 31% α -helix predicted for delipidated rat liver FABP [37]. On binding oleic acid the percentage of α -helix in rat liver FABP decreased to 17%. In contrast, *Moniezia* LBP showed only a small or insignificant decrease in α -helix content after binding linoleic acid (30% α -helix) or cholesterol (35% α -helix). When the binding studies were carried out in the presence of 33 mM Na₂SO₄, no significant change in secondary structure was detectable. The amino acid composition of *Moniezia* LBP is markedly different from that of the other FABPs. Unlike the majority of FABPs, the *Moniezia* protein was not N-terminally blocked and the N-terminal sequence showed no match with any protein in the Swissprot or EMBL database, suggesting a novel sequence. Interestingly there were indications of microvariation in the amino acid sequence of *Moniezia* LBP, despite the fact that the protein appeared to be chromatographically and electrophoretically homogenous.

The partial sequence shows no recognition site for protein kinase. However, unpublished experiments have shown that incubation of *Moniezia* LBP with protein kinase C results in protein phosphorylation and changes in the binding affinity for DAUDA (D. Janssen and J. Barrett, unpublished work).

A polyclonal antibody against *Moniezia* LBP failed to recognize any antigens in extracts of *F. hepatica*, *A. suum*, rat liver or sheep intestine. However, a faint protein band in the 11–13 kDa region was found in *H. diminuta* extracts, with a series of stronger bands at 30–40 kDa and higher. This suggests that *Moniezia* LBP is a unique protein, antigenically different from mammalian, nematode or digenian FABPs, but with similarities to other cestode LBPs.

Moniezia LBP was found to interact with a number of anthelmintics and bound dichlorophene, hexachlorophene, niclosamide, oxclozanide, resorantel, bithionol, rafoxanide and nitroscanate with K_D values in the μ M range. Benzimidazole carbamates (albendazole, fendbendazole, oxybendazole) and praziquantel were not bound. The anthelmintics that did bind are all structurally phenol derivatives with Cl⁻, I⁻ or Br⁻ substitutions. By interacting with binding proteins these anthelmintics could disrupt the protein's normal physiological function. Sequestration of anthelmintics by binding proteins may also be an important detoxification mechanism. Alternatively, binding proteins could act as transport proteins for anthelmintics facilitating drug movement from the cell surface to specific target organelles, thus playing a role in determining drug specificity and site of action.

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