

Ascaridia galli fatty acid-binding protein, a member of the nematode polyprotein allergens family

Anna Timanova¹, Sylke Müller², Thomas Marti², Ilia Bankov¹ and Rolf D. Walter²

¹Institute of Experimental Pathology and Parasitology, Bulgarian Academy of Science, Sofia, Bulgaria; ²Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany

A fatty acid-binding protein from the nematode *Ascaridia galli* was characterized. The gene was isolated and recombinantly expressed in *Escherichia coli*. According to the deduced amino acid sequence *A. galli* fatty acid-binding protein (AgFABP) belongs to the family of nematode polyprotein allergens, as shown by Western blotting and PCR analysis with genomic DNA and cDNA. Both native and recombinant proteins bind fatty acids and retinoids with high affinity. The fluorescent fatty acid analogue 11-[(5-dimethylaminonaphthalene-1-sulfonyl)-amino] undecanoic acid (DAUDA) shows substantial changes in its emission spectrum when bound to AgFABP; this binding is reversed by fatty acids such as oleate. Moreover, changes of the intrinsic fluorescence of retinol and retinoic acid confirm retinoid binding activity of AgFABP. Fluorescence titration experiments with DAUDA indicate stoichiometric binding to a single binding site per monomer unit with affinities (K_d) of 1.6 and 1.8×10^{-7} M for native and the recombinant protein, respectively. The apparent binding affinities of the nonfluorescent ligands were calculated in displacement experiments with DAUDA and values in the same range were obtained for myristic, palmitic, oleic, linoleic, arachidonic and retinoic acid. Additionally, the binding affinity of AgFABP for oleate and palmitate was determined by direct and indirect radiochemical analysis and the values obtained were similar to those from the fluorescent experiments. Both proteins show a preference for the binding of long-chain saturated and unsaturated fatty acids, but not for short chain (C3–C12) and branched fatty acids, cholesterol and tryptophan.

Keywords: *Ascaridia galli*, fatty acid-binding protein, nematode, polyprotein.

Parasitic nematodes have to adapt to different environmental conditions during their life cycle. The free-living stages utilize endogenous carbohydrate reserves and are also able to metabolize lipids aerobically for energy generation, whereas the adult parasitic stages are solely dependent on carbohydrate metabolism as an energy source [1]. However, due to a constant turnover of structural lipids the adult nematodes synthesize triacylglycerols and furthermore, certain lipid derivatives are metabolically active (prenoids, steroids, prostaglandins) [2].

In all stages of *Ascaris suum* and *Ascaris lumbricoides*, a low M_r antigen was identified and found to be secreted by the parasites *in vitro* [3]. In a variety of other nematode parasites similar antigens were identified which are distinguished by their abundance and immunodominance [4–7]. These proteins, termed according to their immunological importance and their protein organization ‘nematode polyprotein allergens’ (NPA) [8], have been found to bind a broad range of lipidic ligands with high affinity: fatty acids, retinol and retinoic acid [9–11]. Little is known about the precise function of the fatty acid-binding proteins (FABPs) in mammals and other organisms, but it is generally assumed that they are involved in fatty acid transport and metabolism [12–15].

We have focused our attention on *Ascaridia galli*, a prevalent and costly nematode parasite in chicken. This infection results in reduction of both the rate and efficiency of weight gain in the birds [16]. The significant amount of fatty acid present in the host's intestine, together with the fact that the parasite itself contains large amounts of lipids but possesses only limited ability to synthesize long chain fatty acids *de novo*, raises questions about the function of FABP in fatty acid transport and metabolism in *A. galli* [17].

MATERIALS AND METHODS

Chemicals

The fluorescent fatty acid analogue 11-((5-dimethylaminonaphthalene-1-sulfonyl)amino)undecanoic acid (DAUDA) was obtained from Molecular Probes Inc. [$1\text{-}^{14}\text{C}$]Palmitic acid ($54\text{ mCi}\cdot\text{mmol}^{-1}$) was from NEN-DuPont. All-*trans*-retinol, retinoic acid, all fatty acids, BSA, nitro-blue tetrazolium and 5'-bromo-4-chloro-3-indolyl phosphate were from Sigma.

Parasites

Adult *A. galli* parasites were isolated from the intestine of experimentally infected chicken, washed in NaCl/P_i (137 mM NaCl, 8 mM Na_2HPO_4 , 3 mM KCl, 2 mM KH_2PO_4 , pH 7.5) and stored at -70°C .

Purification of FABP

A. galli fatty acid-binding protein (AgFABP) was purified as described previously for FABP from *Ascaris suum* reproductive

Correspondence to R. D. Walter, Department of Biochemical Parasitology, Bernhard Nocht Institute for Tropical Medicine, Bernhard-Nocht-Str.74, 20359 Hamburg, Germany. Fax: + 49 40 31182418, Tel.: + 49 40 31182420, E-mail: walter@bni.uni-hamburg.de

Abbreviations: DAUDA, 11-((5-dimethylaminonaphthalene-1-sulfonyl)-amino) undecanoic acid; FABP, fatty acid-binding protein; NPA, nematode polyprotein allergens; RACE, rapid amplification of cDNA ends.

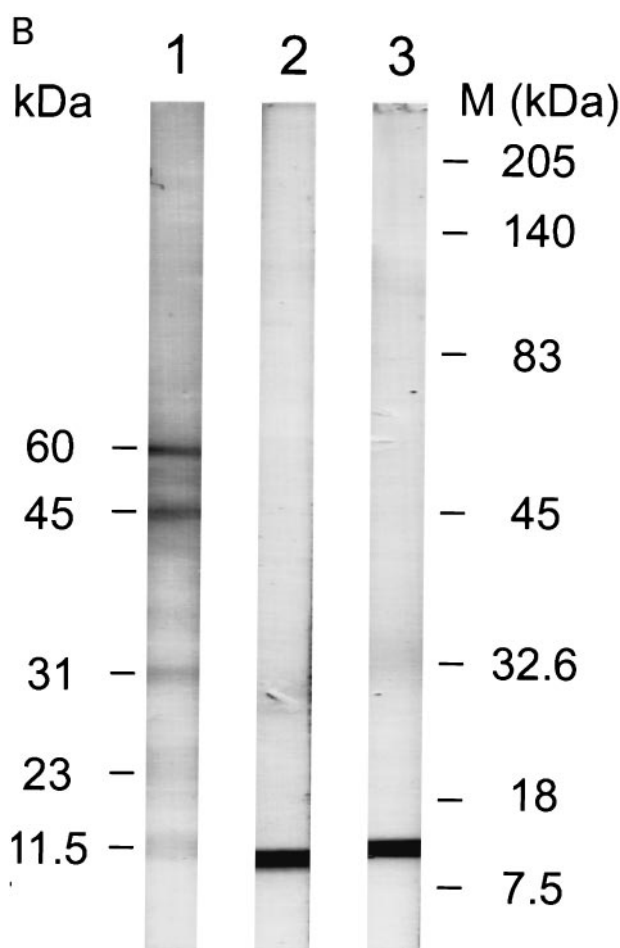
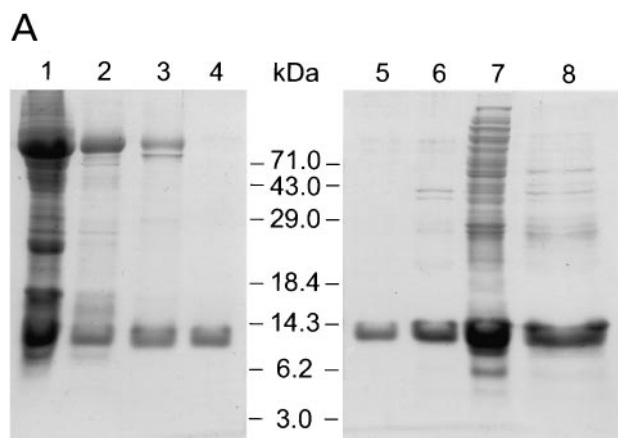
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tissue [18] and summarized in Table 1 and Fig. 1A. The fatty acid binding activity during the purification procedure was determined using the fluorescent fatty acid analogue DAUDA [19]. The relative fluorescent intensity proportional to the amount of DAUDA bound was determined as outlined by Wilton [20]. The specific activity is expressed as pmol bound ligand (DAUDA) per μg of protein. The M_r of the native protein was estimated by gel filtration on a calibrated FPLC-Superdex 75 column (1.6×60 cm). The purity and the size of a single transcriptional unit of AgFABP were demonstrated by SDS/PAGE (Fig. 1A). The N-terminal sequence of the purified AgFABP was determined by automated Edman degradation on an ABI 473A protein sequencer (Applied Biosystems).

Table 1. Purification of *A. galli* FABP. The specific activity determination is described in Materials and methods section.

Purification step	Native AgFABP		Recombinant AgFABP	
	Specific activity (pmol· μg^{-1})	Total protein (mg)	Specific activity (pmol· μg^{-1})	Total protein (mg)
Cytosol	6.3 ^a	308.0	4.1	180.0
(NH ₄) ₂ SO ₄	10.3 ^a	48.0	6.7	102.6
DEAE	19.2 ^a	8.0	12.6	37.8
Superdex 75	31.7	4.0	29.7	14.4

^aAgFABP was accompanied by a 50-kDa protein binding DAUDA. By direct N-terminal sequencing (data not shown) it was confirmed that it represents an entirely different FABP from the one investigated in this study.



Identification of AgFABP gene and message by PCR

Genomic DNA and total RNA from *A. galli* were isolated from whole worm homogenate using the guanidinium-thiocyanate extraction procedure as described previously [21].

Based on the N-terminal sequence of the native AgFABP (EQKEELKQM), the degenerate sense oligonucleotide [5'-GA(A/G)CA(A/G)AA(A/G)GA(A/G)GA(A/G)(C/T)TNA(A/G)CA(A/G)ATG-3'] was designed. The antisense oligonucleotide [5'-TA(G/A/T)AT(T/C)TT(T/C)TT(A/G)CAN(A/G)CNGG-3'] was based on a conserved region (YIKKC/VAP) of known FABP sequences of other helminths [10,18]. The PCR was performed on either genomic DNA or reverse transcribed RNA of *A. galli*, using the following protocol: 95 °C, 2 min; 48 °C, 1.5 min; 72 °C, 1.5 min for 30 cycles. The PCR products were separated on a 1% agarose gel, excised from the gel and purified (GeneClean, BIO 101). Subsequently, the products were cloned into a TA modified vector (pCR II, Invitrogen) and both strands were sequenced using the dideoxynucleotide chain termination method [22].

Rapid amplification of cDNA ends (RACE)

In order to obtain the 5' and 3' ends of the AgFABP mRNA, RACE was performed according to the manufacturer's instructions (Gibco-BRL). Total RNA of *A. galli* was reverse transcribed with either an oligo d(T) adapter primer (5'-GGCCACGCGTCGACTAGTACTT₁₈-3') (3'RACE) or a specific antisense primer (5'RACE) (Fig. 2). The single-stranded cDNA was then tailed (5'RACE) or used directly for a PCR using specific primers directed to the adapters and the specific nucleotide sequence of AgFABP. The PCR products were

Fig. 1. SDS/PAGE and Western blot of AgFABP. (A) Purification of recombinant (r) (lanes 1–4) and native (n) (lanes 5–8) AgFABP (15% SDS/PAGE). Lane 1, cytosol of pJC20-AgFABP-transformed *E. coli* after induction (17 μg protein); lane 2, supernatant after 70% ammonium sulphate saturation (9 μg protein); lane 3, rAgFABP fraction after DEAE cellulose chromatography (4.1 μg protein); lane 4, rAgFABP eluted from a Superdex 75 column (1 μg protein); lane 5, nAgFABP eluted from an FPLC Superdex 75 column (0.8 μg protein); lane 6, nAgFABP fraction after DEAE cellulose chromatography (3.5 μg protein); lane 7, supernatant obtained after 70% ammonium sulphate saturation of *A. galli* cytosol (6.2 μg protein); lane 8, *A. galli* cytosol (14 μg protein). Proteins were visualized by Coomassie blue staining. (B) Immunoblot of anti-AgFABP raised against recombinant AgFABP. Forty μg of cytosolic protein (lane 1) or 2 μg of purified *A. galli* native (lane 2) and recombinant (lane 3) protein were resolved by 10% SDS/PAGE, blotted onto nitrocellulose, incubated with rabbit anti-rAgFABP serum, followed by anti-rabbit IgG, conjugated with alkaline phosphatase, and developed.

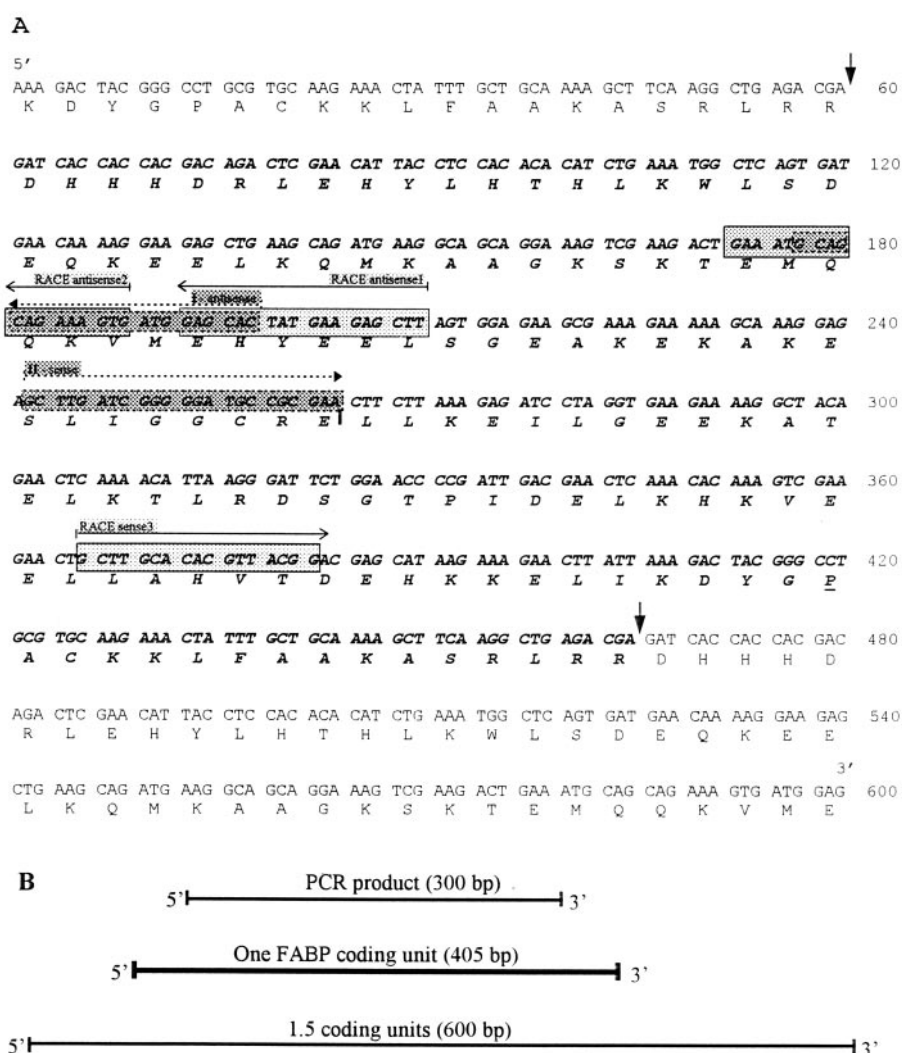


Fig. 2. Cloning strategy. (A) Nucleotide and derived amino acid sequence of AgFABP. To show the tandemly repeat organization of AgFABP, oligonucleotides I and II (light shading) were used. The primers used for 3' and 5' RACE are shown by dark shading. The vertical arrows indicate the putative cleavage sites used in processing the polypeptide. (B) Relationship of the PCR product, one transcriptional unit of AgFABP cDNA and 1.5 units of AgFABP.

analysed on a 1.4% agarose gel, excised, purified and subcloned into pCRII (Invitrogen), using T/A cloning. The nucleotide sequences were determined as described above.

Construction of expression plasmid

To synthesize the AgFABP coding region for expression in *E. coli*, the sense oligonucleotide FABPEX1 (encoding the first six amino acids of a single transcriptional AgFABP unit including the ATG start codon) and the antisense oligonucleotide FABPEX1 (encoding the last seven amino acids including the TAA-termination codon) were designed and used to perform a PCR with reverse transcribed RNA of *A. galli* as template. The sense primer contained an *Nde*I and the antisense primer a *Bam*HI restriction site to ensure directed cloning into the expression vector pJC20 [23]. To facilitate subcloning into pJC20 the PCR fragments were first subcloned into pCRII, using T/A cloning; this was followed by a preparative digestion with *Nde*I and *Bam*HI and isolation of the restricted AgFABP coding region which was then cloned into pJC20 previously digested with the same restriction enzymes. The construct was transformed into *E. coli* BL21 (DE3) and used for the expression of the recombinant protein.

Production of recombinant AgFABP

Transformed *E. coli* BL21 (DE3) were cultured in Luria-Bertani medium containing 50 µg ampicillin·mL⁻¹ overnight at

37 °C. The stock culture was diluted 1 : 100 in the same medium and the bacteria grown to *A*₆₀₀ 0.5 before induction with 1 mM isopropyl thio-β-D-galactoside. After 4 h at 37 °C, the cells were harvested and recombinant AgFABP was purified as described for the native protein.

Western blotting

One hundred microgrammes of purified rAgFABP were used to raise antibodies against the protein in a rabbit (BioScience). The final bleed was used for analysing the native protein in whole *A. galli* extracts. For immunoblotting, *A. galli* soluble protein was separated by 10 or 15% SDS/PAGE and transferred to nitrocellulose membranes [22,24]. Membranes were blocked with 2.5% BSA in NaCl/P_i and subsequently incubated with the primary antibody at a dilution of 1 : 5000. After extensive washing with NaCl/P_i/Tween the membranes were incubated with an alkaline phosphatase-conjugated goat anti-(rabbit IgG) secondary antibody (Sigma), and developed with nitro blue tetrazolium and 5'-bromo-4-chloro-3-indolyl phosphate.

Binding assays

The fatty acid binding of the protein with palmitic acid and its displacement by oleate were determined with the Lipidex method in 10 mM potassium-phosphate buffer, pH 7.4 using [¹⁴C] palmitate [25].

	10	20	30	40	50	60	70	80
AgFABP	DHHHD -RLEH	YLTHL KWLS	DE QKEEL KQM	KAAG K SKTEM	QQK VM EHYEE	LS G EAK EKA K	ESLIG G CREL	LKEIL G E EKA
TBA-1	HHF --TLEN	SLDTHL KWLS	HE QKEEL LQM	KKD GK SKKEL	QDKIMHYEH	LEG DAK HEAT	EQLK G GCREI	LKHVV G E EKA
ABA-1	-- HHF --TLES	SLDTHL KWLS	QE QKDE LLKM	KKD GK AKKEL	EAKILHYDE	LEG DAK KEAT	EHLK G GCREI	LKHVV G E EKA
LL20	DHH -EHN LDE	YFRTHLS WLT	DI QKDE IRKM	KEEG KP KADM	QK KIF DY ES	LT GDEK KEAS	EKLREG C RAL	LKQIV G DEKM
gp15/400	-- H --EHS LDD	YFRTHLS WLT	DA QKDE IRKM	KEEG KQ KMDM	QK KIF RYFES	LT GDGK KEAG	EKL RGG CREL	LRQIV G DEKM
Di5	----- S	YFQTYLS WLT	DA QKDE IKKM	KEEG KS KMDI	QK KIF RYFES	LT GDKK KAA	EELQ QG CLMA	LSEI I GN EKM
Oo-ESAg	DHH -EHN LEE	AMEKYLT WLN	DD QKEE VKKL	YGAG-DKQAM	YK KVME IYDS	VSGDV KEKA T	VELKA AC RHY	VKDSI G E EENA
DVA-1	DHHHE HN LEE	ALEKYLT WLN	EE QKSQ MKTI	YESG-DREAL	YK KVLE FFEA	AT GEV KE KA A	VELKS AC RHY	IKDYI G DE EKA

	90	100	110	120	130	140
AgFABP	TEL KTLR D SG	TPIDEL KHKV	EELLAHV TDE	H KKELI KDYG	PACK KL FAAK	AS RLRR
TBA-1	AEI KALK D SG	ASKDEL KAKV	EEALHAV TDE	E KKQ HIAEFG	PACK KI YGV	AS RRRR
ABA-1	AEL KNL KD SG	ASKEEL KAKV	EEALHAV TDE	E KKQ YIADFG	PACK KI YGVH	TS RRRR
LL20	AEL KQM KD SG	VGIEEL IAKV	DHMLEHV TDE	P KKEKI QYEG	PACH KI YGTP	VM RNKR
gp15/400	AEL KQM KESG	LGQEEL RAKV	DEM LEHV TDE	A KKQKI HEYG	PAC RKI YED-	-- RHKR
Di 5	LML KEIK D SG	ADPEQIR MKV	EDMLKL VVDK	E KKKR IDEYA	PVCR KI YAA-	- MNRR
Oo-ESAg	EKL KEMK ESG	ATPEAIA AKV	EEFIAA ITDE	K KKQA ERAA	VAC KKI YGV-	AR RLKR
DVA-1	EKI KEMK ESG	VSTEEIS KKV	DEFIAM ITDD	E KKAKA LRAS	NAC KKI YGV-	AK RLRR

Fig. 3. Alignment of the amino acid sequence deduced from AgFABP cDNA (GenBank accession number AF065383) with FABP homologues of seven other genera of parasitic nematodes. TBA-1 precursor of *Toxocara canis* (EMBL/GenBank accession number D89753); ABA-1 allergen from *Ascaris suum* (L03211); LL20 15 kDa ladder allergen from the filarial nematode *Loa loa* (U03103); gp15/400 glycoprotein from *Brugia pahangi* (X68190); Di5 cuticle antigen from *Diofilaria immitis* (M95173); excretory/secretory antigen from *Ostertagia ostertagi* (Z46800) and DvA-1 polypeptide precursor from *Dictyocaulus viviparus* (U02568). The conserved residues are indicated in bold. The amino-terminal sequence determined by direct sequencing of native AgFABP is boxed.

The binding of DAUDA, retinol and retinoic acid of AgFABP was determined with a spectrofluorescence assay [26] at an excitation wavelength of 350 nm (SFM 25, Kontron). The fluorescence was analysed for 1 μ M DAUDA, 1 μ M retinoic acid or 0.5 μ M retinol in the absence or presence of 0.56 μ M native or 0.58 μ M recombinant AgFABP. The emission spectra between 400 and 600 nm were determined. The fluorescent maxima for the other ligands with or without protein were obtained by scanning between 450 and 550 nm at the excitation wavelength indicated.

Displacement studies with DAUDA were performed by the addition of 0.5 μ M DAUDA with or without 0.5 μ M competitor to 0.2 μ M FABP. Emission spectra (450–550 nm) were recorded at an excitation wavelength of 350 nm because the various competitors cause a different wavelength shift of the fluorescence emission maximum.

The K_d for DAUDA was estimated according to Bagshaw and Harris [26]. The fluorescent measurements were corrected for dilution and the fluorescent contribution of the unbound ligand and the data (bound ligand vs. unbound ligand) were either fitted by standard nonlinear regression methods or subjected to Scatchard analysis and the K_d and number of binding sites (n) for each AgFABP monomer estimated.

The binding of nonfluorescent ligands to AgFABP and rAgFABP was evaluated by measuring the displacement of DAUDA bound to FABP. The K_d for competing ligands were calculated by standard analysis [26].

RESULTS

AgFABP DNA sequences

The 300-bp PCR AgFABP genomic and cDNA fragments containing part of the coding region of the FABP from *A. galli* were cloned initially. To obtain the entire 5' and 3' regions of the AgFABP mRNA, RACE was performed (Fig. 2). The cDNA

obtained contained neither the 5' nor the 3' noncoding regions of AgFABP, but showed parts of another unit of the coding region (Fig. 2) suggesting that AgFABP is transcribed from a gene encoding a protein that consists of multiple tandemly repeated units, with basal subunits of 405 bp. To investigate this further, several PCRs were performed using oligonucleotides I and II (Fig. 2). The fragments obtained encoded a single open reading frame which translated into 1.5 partial subunits of AgFABP. The same results were obtained with genomic DNA and reverse transcribed RNA, indicating that the AgFABP gene does not contain introns.

Deduced amino acid sequence and native protein

The deduced amino acid sequence of one unit encoded a protein of 135 amino acids with a typical protease recognition site of polyproteins and prohormones at the C-terminus (RRLR) of each unit [8,27,28]. In contrast to the FABP of *Brugia malayi*, the AgFABP does not contain a consensus site for N-glycosylation. This is in accordance with the predominant occurrence of the native AgFABP in the cytosolic fraction of *A. galli*.

The alignment of AgFABP with FABPs of other helminths shows a high percentage of identity (41–64%) particularly with those of *Ascaris suum* ABA-1, *B. malayi*, *Diofilaria immitis*, *Toxocara canis*, *Loa loa*, *Dictyocaulus viviparus* and *Ostertagia ostertagi* (Fig. 3) [7,9–11,29–33], but not with the retinol binding protein from *Onchocerca volvulus* Ov-20, the lipid binding protein from the cestode *Moniziesia expansa* and those isolated from trematodes which seem to represent different classes of parasitic FABPs [34–38].

The molecular mass of AgFABP based on the deduced amino acid sequence is 15.69 kDa. The sizes of the native and recombinant proteins are in good agreement with the predicted molecular mass (12 and 13 kDa) as shown by SDS/PAGE (Fig. 1A) and subsequent Western blot analysis (Fig. 1B). Further, the occurrence of translated multiple repeated subunits of AgFABP was shown by Western blotting (Fig. 1B). At least

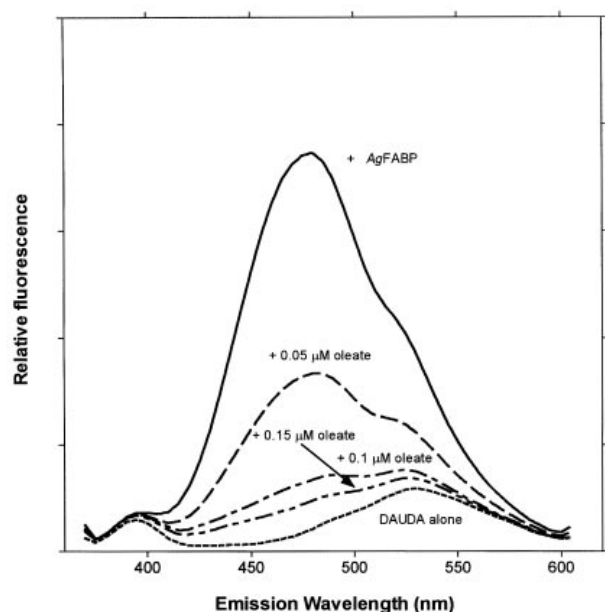


Fig. 4. Fatty acid binding activity of native AgFABP. Fluorescence emission spectra (uncorrected, $\lambda_{\text{exc}} = 350$ nm) of $1 \mu\text{M}$ DAUDA in 50 mM potassium phosphate buffer, pH 7.2 or with $0.56 \mu\text{M}$ AgFABP. Also shown is the reversal of changes in DAUDA emission by competition with oleic acid (0.05 – $0.15 \mu\text{M}$) added to the AgFABP/DAUDA mixture.

five units (12 kDa, 23 kDa, 31 kDa, 45 kDa and 60 kDa) were detectable when a $100\,000\text{ g}$ supernatant of *A. galli* prepared in NaCl/ P_i was separated by 10% SDS/PAGE.

Purification of native AgFABP and production of recombinant rAg-FABP

DNA encoding one unit of AgFABP (including the ATG start codon and the TAA stop codon) was amplified by PCR using Elongase (Gibco-BRL), cloned into the expression vector pJC20 and *E. coli* BL 21 (DE3) were transformed. Bacteria transformed with the expression plasmid pJC20-AgFABP produced high amounts of recombinant protein: $36\text{ mg pure rAgFABP}\cdot\text{L}^{-1}$ bacterial culture was obtained. After gel filtration the proteins were eluted in a single peak of ≈ 23 – 24 kDa, suggesting that AgFABP may form a dimer.

Ligand binding

Both, native and recombinant AgFABPs bind the fluorescent fatty acid analogue DAUDA. Upon binding to AgFABP the emission is strongly increased (8–10-fold) and the fluorescence emission spectrum is characterized by a marked blue shift of ≈ 50 nm (Fig. 4). Titration of both native and recombinant proteins from *A. galli* resulted in apparent dissociation constants

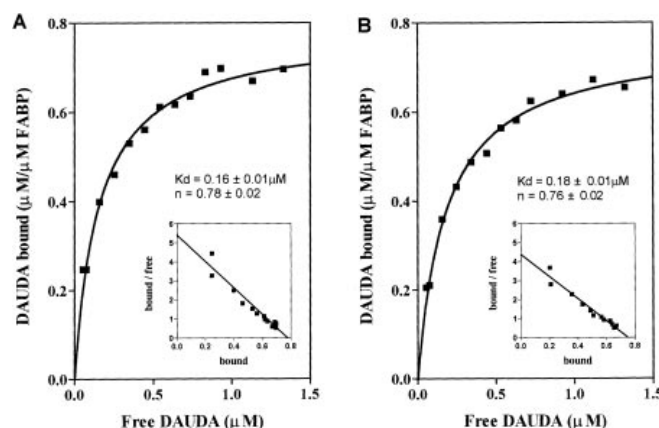


Fig. 5. Analyses of DAUDA binding to purified native (A) and recombinant (B) AgFABP. DAUDA was added in small aliquots to fixed amounts of protein ($\approx 0.3 \mu\text{M}$) in 50 mM potassium phosphate buffer, pH 7.2. The fluorescent measurements ($\lambda_{\text{exc}} = 350$ nm, $\lambda_{\text{ems}} = 490$ nm) were corrected for dilution and contribution of unbound ligand. It was assumed that the maximal fluorescence determined according to the method described by Bagshaw and Harris [26] by titration of a fixed amount of DAUDA with increasing concentrations FABP, represents 100% binding and that the amount of the fluorescent probe bound is proportional to the relative intensity. The lines derived were fitted by nonlinear regression techniques to a single binding-site model to give estimates of K_d and n (main graph). The data were also subjected to Scatchard plots (inserts). Correlation coefficients: (A) $r^2 = 0.98$ and $r^2 = 0.93$, (B) $r^2 = 0.99$ and $r^2 = 0.95$, for the main graph and the insert, respectively.

of $0.16 \pm 0.01 \mu\text{M}$ and $0.18 \pm 0.01 \mu\text{M}$ (means $\pm$ SD of four experiments), respectively, with one binding site ($n = 0.78 \pm 0.02$ and 0.76 ± 0.02) per protein monomer (Fig. 5A and B).

Retinoids also appear to have an intrinsic fluorescence when bound to AgFABP as shown for both retinol and retinoic acid (Fig. 6A and B). The binding can be reversed by the addition of oleic acid, indicating that the binding sites for retinol and fatty acids coincide or interact competitively (Figs 4, 6A and B). The K_d values for retinol, calculated from titration experiments, were $0.56 \pm 0.15 \mu\text{M}$ for both native and recombinant protein ($n = 0.80 \pm 0.12$).

Binding of fatty acid

Binding activities of fatty acids to AgFABP were analysed by competition experiments in which DAUDA bound to the AgFABP was displaced by nonfluorescent compounds. The percentage of displacement obtained for fatty acids with varying chain length and saturation, as well as the K_i values are summarized in Table 2. The K_i values describing the binding affinity of both native and recombinant AgFABP for retinoic acid were calculated to be 0.20 ± 0.03 and 0.23 ± 0.04 and the displacement percentage of DAUDA by this ligand was found to be $66.2 \pm 7.3\%$ and $65.0 \pm 14.3\%$, respectively. The data show

Table 2. Displacement of DAUDA and binding affinity of AgFABP for various fatty acids.

Ligand	Native AgFABP		Recombinant AgFABP	
	K_i (μM)	Displacement (%)	K_i (μM)	Displacement (%)
Lauric acid (11 : 0)	1.15 ± 0.17	48.6 ± 7.4	1.11 ± 0.18	53.8 ± 4.6
Myristic acid (12 : 0)	0.37 ± 0.08	65.0 ± 8.3	0.34 ± 0.07	62.9 ± 4.7
Palmitic acid (16 : 0)	0.18 ± 0.06	88.6 ± 3.4	0.17 ± 0.04	92.0 ± 2.2
Oleic acid (18 : 1)	0.08 ± 0.02	94.5 ± 3.5	0.08 ± 0.01	95.6 ± 2.4
Linoleic acid (18 : 2)	0.21 ± 0.01	65.1 ± 10.1	0.19 ± 0.01	73.8 ± 12.8
Arachidonic acid (20 : 4)	0.20 ± 0.02	68.5 ± 5.9	0.24 ± 0.05	62.5 ± 9.8

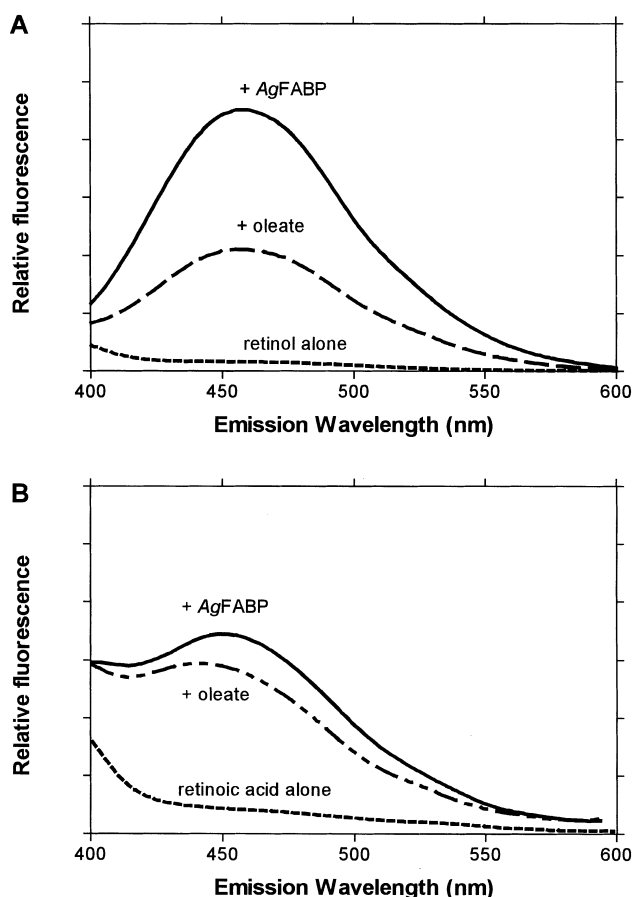


Fig. 6. Binding of retinol (A) and retinoic acid (B) to AgFABP. Fluorescence emission spectra (uncorrected, $\lambda_{exc} = 350$ nm) of $0.5 \mu\text{M}$ retinol and $1 \mu\text{M}$ retinoic acid, respectively, in 50 mM potassium phosphate buffer, pH 7.2 or with $0.56 \mu\text{M}$ AgFABP. Also shown is the reversal of changes in retinoid emission by competition with oleic acid ($0.2 \mu\text{M}$ and $0.5 \mu\text{M}$, respectively) added to the AgFABP/retinol or AgFABP/retinoic acid mixture.

that both native and recombinant AgFABP have similar binding properties, indicating that the recombinant protein is correctly folded. In order to confirm the results from the DAUDA binding experiments as well as the similarity in the binding characteristics of native and recombinant AgFABP, the radiochemical Lipidex procedure in combination with the competitive method were used for analysis of ligand–protein binding. The binding affinity of rAgFABP for palmitic acid was determined to be $0.14 \pm 0.2 \mu\text{M}$ (Table 2). One binding site (1.03 ± 0.05) per monomer AgFABP was found. Furthermore, the binding affinity for oleate was investigated in displacement experiments using [^{14}C]palmitate. For oleic acid, a K_i of $0.08 \pm 0.01 \mu\text{M}$ was calculated, in excellent agreement with those obtained by DAUDA displacement studies (Table 2). AgFABP shows a preference for binding oleic and palmitic acid. Further, increasing the chain length of saturated fatty acids favours binding to the protein. Under standard assay conditions $0.5 \mu\text{M}$ butyric, 2-methyl-butyric, valeric, 2-methyl-valeric, caproic, caprylic or capric acid did not displace the fluorescent lipid analogue from the binding site of AgFABP.

DISCUSSION

A cytosolic fatty acid binding protein was purified from *A. galli*. The N-terminal sequence of the native protein was determined and degenerate oligonucleotides were used to isolate parts of the

gene and cDNA of AgFABP. The protein is transcribed from a gene composed of multiple tandemly repeated units as shown by PCR analyses using genomic DNA and cDNA. In accordance, native AgFABP is derived from a large precursor of at least 60 kDa to form units of 12–13 kDa as shown by Western blot analysis. These results are in agreement with data reported for the other nematode polyprotein allergens from *Ascaris suum*, *T. canis*, *B. malayi*, *L. loa*, *Dictyocaulus viviparus*, *Dirofilaria immitis* and *O. ostertagi* [7,29–33]. Both native and recombinant AgFABPs appear to form dimers as suggested by single 23 or 24 kDa activity peaks after gel-filtration, respectively. Similar results were reported for gp15/400 from *B. malayi*, ABA-1 from *Ascaris suum* and DvA-1 from *Dictyocaulus viviparus* [9–11].

The slight size difference observed between native and recombinant proteins may be due to the proposed proteolytic cleavage of the polyprotein-precursor, which only occurs with the native FABP [8,27]. Further, a secondary action of an amidopeptidase considered as exopeptidase is maybe used to trim the primary endoproteolytic cleavage products of polyproteins [39].

The ligand binding properties of the native and recombinant AgFABP are similar for both proteins and comparable to those reported for NPA units from other nematode species e.g. ABA-1, gp15/400 and DvA-1 [9–11]. Although the amino acid sequence and gene organization of Ov-20 appear to be different from that of the AgFABP, both proteins bind a similar range of substrates such as retinol, retinoic acid, DAUDA and oleate with comparable affinity [38] suggesting similarities in their specific functions in the nematodes. Another lipid binding protein isolated from *Moniezia expansa* is different from all those described above but it still has similar binding characteristics to the NPA and occurs in the cytosol as oligomer [37,40].

Upon binding to the FABPs the emission spectrum of the fluorescent lipid analogue DAUDA changes considerably. During the interaction the fluorescence emission intensity of DAUDA is enhanced and the peak of the emission spectrum is shifted to the shorter wavelength [19]. Similar results were obtained with AgFABP: the intensity of DAUDA fluorescence was increased by 8–10-fold and the emission spectrum peak was shifted to the blue range by ≈ 50 nm. The blue shift is greater than those observed with BSA and rat liver FABP [19] but is comparable with those reported for ABA-1, gp15/400 and DvA-1 [9–11]. These data support the existence of a remarkable degree of apolarity within the binding site of AgFABP suggesting that during transport the retinoids and free fatty acids are isolated from the polar solvent in the cytosol of the cell to prevent their oxidation [9].

The binding of hydrophobic, nonfluorescent fatty acids and retinoids was determined in competitive experiments by displacement of DAUDA from the AgFABP binding site. In addition, by direct and competitive radiochemical analysis, respectively, the binding affinity of palmitic and oleic acid to AgFABP were determined. Oleate, palmitate, arachidonic acid, linoleic acid and retinoic acid competed strongly with DAUDA binding, whereas no effect was obtained with short chain fatty acids (C3–C10), cholesterol or tryptophan. The branched chain fatty acids 2-methyl valeric and 2-methyl-butyric acid, excreted by the adult nematodes as final products of the carbohydrate metabolism are proposed to serve as an energy source for developing embryos in *Ascaris suum* [1] but were not found to bind to the AgFABP. Similar results were obtained for DvA-1 from *Dictyocaulus viviparus* [11] which suggest that AgFABP is not involved in the transport of these fatty acids. Nevertheless, the observations that FABP from *O. volvulus* is localized within

the cells of developing parasite embryos, As-p18 (a FABP from *Ascaris suum*) is in the perivitelline fluid [41], and ABA-1 is abundantly expressed in reproductive tissue of *Ascaris suum* [18] support the idea that an important function of FABPs is the supply of fatty acids as an energy source to the developing stages of the worms. In other parasite nematodes it has been discussed that FABPs are involved in sequestration and distribution of hydrophobic ligands which are taken up via the cuticle. This suggestion is supported by the predominant localization of FABPs in the cuticle [30] and directly beneath it in the underlying hypodermis of these parasites [5,29]. Another possible function of these proteins is their capacity to bind and to sequester hydrophobic toxic compounds [42]. Further, most of the nematode FABPs are not only present at a high abundance inside the parasite, but are also secreted by the parasites [5,30]. This observation implies that these proteins might be involved in the host-parasite relationship possibly by interacting with the host's immune response [8].

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