The ABA-1 Allergen of the Parasitic Nematode *Ascaris suum*: Fatty Acid and Retinoid Binding Function and Structural Characterization†

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Received November 14, 1994; Revised Manuscript Received February 16, 1995⊗

ABSTRACT: We report here on the structure and function of the ABA-1 allergen protein of the parasitic nematode *Ascaris*, the first nematode allergen to be characterized in detail. Using the fluorescent fatty acid analog 11-(((5-(dimethylamino)-1-naphthalenyl)sulfonyl)amino)undecanoic acid (DAUDA), it was demonstrated that ABA-1 is a fatty acid binding protein (FABP) with a high affinity for the fluorescent analog (8.8×10^{-8} M) and for oleic acid in competition experiments (1.3×10^{-8} M), with a single binding site for ligand per monomer unit. Blue-shifting of fluorescence emission of DAUDA upon binding was unprecedented in degree among FABPs, being equivalent to that occurring in cyclohexane. A similarly blue-shifted spectrum was obtained with a probe in which the fluorophore was bound to the α carbon of a fatty acid, indicating that the carboxylate group of bound fatty acids is probably not exposed to solvent. In competition experiments and by observation of changes in their intrinsic fluorescence, retinol and retinoic acid were also found to bind in the fatty acid binding site. Circular dichroism (CD) of the ABA-1 protein revealed a high α-helix content (59%) which was consistent with the four-helix structure for the protein predicted from sequence algorithms. Fluorescence measurements showed that the single, highly conserved tryptophan residue is deeply buried in an unusually apolar environment and that this was unaffected by ligand binding. DSC studies of thermal stability indicate that unfolding of the ABA-1 dimer is cooperative and biphasic ($T_m \approx 71$ and 89 °C), suggesting a two-domain thermal unfolding process, again consistent with the predicted structure. Only the folding of the high-temperature domain is reversible on cooling. DSC confirmed the gel filtration analysis, which indicated that ABA-1 forms a dimer. Aside from being the first nematode allergen for which structure or function has been elucidated, ABA-1 provides a highly manipulable model for investigation of the interaction between hydrophobic ligands and α-helical proteins.

Parasitic nematodes cause considerable human suffering and economic losses on a global scale. Much of the pathology caused by infections with parasitic nematodes, responsible for conditions as diverse as elephantiasis and river blindness in humans, is thought to involve IgE antibody-mediated immune hypersensitivity reactions (Ogilvie & de Savigny, 1982). This, and the association between IgE antibody and protective immunity to helminth infections (Hagan, 1993), has increased the need to understand the parasite components which are selective targets of these responses, their allergens. However, neither the structure nor the biological function of any nematode allergen has been described, yet this information may be crucial to an understanding of their preferential allergenicity.

The ABA-1 protein of the nematodes *Ascaris lumbricodes* and *Ascaris suum* (parasitic in pigs and humans, respectively) is a potent allergen in the context of infection (Tomlinson et al., 1989; Christie et al., 1990); it is abundant in the somatic tissues of the organisms and is also released into the host's tissues by the invasive stages of the parasite (Kennedy & Qureshi, 1986; Kennedy et al., 1987). Recently, the amino acid sequence of ABA-1 has become available from cDNA analysis (Spence et al., 1993), making it the first nematode allergen to be so defined. ABA-1 thus represents a suitable system in which to explore the structural basis of allergenicity and other properties such as the heat resistance of its allergen activity (Christie et al., 1993) and the variability of the immune response of *Ascaris*-infected people to the protein [reviewed in Kennedy (1991)].

From both the cDNA and genomic analysis [Spence et al. (1993) and unpublished data] it is clear that the *aba-1* gene is highly unusual in that it comprises approximately 20 ABA-1-encoding units in head-to-tail array, uninterrupted by introns. The gene is expressed as a large polyprotein which is cleaved into multiple copies of the ABA-1 protein. The putative endoproteolytic cleavage sites comprise tetrads of

† This work was supported by the Wellcome Trust by Grant 031559/Z/90/1.4A to M.W.K. The biological calorimetry services at Glasgow University and the circular dichroism facility at Stirling University are funded by the Biotechnology and Biological Sciences Research Council (U.K.).

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⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1995.

0006-2960/95/0434-6700$09.00/0

arginines which, as indicated by mass spectrometry, are removed subsequent to cleavage to leave a 129 amino acid non-glycosylated protein of mass 14.6 kDa (Christie et al., 1993; Spence et al., 1993). Similar proteins occur as immunodominant antigens/allergens in other species of parasitic nematode, and the structure of the respective genes and the mode of synthesis as a polyprotein are similar (Culpepper et al., 1992; Poole et al., 1992; Tweedie et al., 1993).

We have now identified a function for ABA-1. Fluorescence studies show that it has high binding affinities for fatty acids, retinol, and retinoic acid which are likely to be functionally significant and that the binding site has unusual characteristics. Using multiple alignments of the primary sequences of all known ABA-1-like proteins, we also present a model of these as four-helix proteins and compare this prediction with experimental data on the structure and stability of ABA-1 in solution. This consensus structure is distinct from that observed for other small fatty acid and retinoid binding proteins. Besides being the first nematode allergen for which function has been ascribed, and the first product of a polyprotein precursor described with fatty acid and retinoid binding activity, ABA-1 also provides a valuable model for understanding the principles of binding of fatty acids and retinoids to α-helical proteins.

MATERIALS AND METHODS

Purification of ABA-1. Pseudocoelomic fluid from *A. suum* was prepared from adult parasites as previously described (Kennedy et al., 1987), and ABA-1 was purified from this by a combination of chromatofocussing and size-exclusion chromatography. Components of greater than 100 kDa were removed from pseudocoelomic fluid by gel filtration on Sephacryl S100HR in 0.05 M sodium phosphate buffer, pH 7.2, containing 0.5 M NaCl. Material excluded from the gel was found not to contain ABA-1 by SDS–PAGE and was discarded. Other fractions were pooled and transferred into 0.025 M Bis-Tris HCl, pH 7.1, by ultrafiltration using a 10-kDa exclusion Filtron Microsep microconcentrator (Flowgen), prior to chromatofocusing on a Mono P HR 5/20 column on a FPLC system (Pharmacia). The eluant chosen was Polybuffer 74 HCl, pH 5.0 (Pharmacia), and the resulting gradient of pH 7.0 to 5.0 was completed in a 24-mL volume, at a flow rate of 1 mL min^{-1}. The pregradient volume was 6 mL, during which a small quantity of protein, low in ABA-1, emerged before switching to the elution buffer. Eluant was monitored at 280 nm, and 0.5-mL fractions were collected. Fractions containing protein were analyzed by SDS–PAGE on a PHAST system using homogeneous 20% acrylamide gels (Pharmacia) and compared with affinity-purified ABA-1 (Tomlinson et al., 1989) and SDS Dalton Mark VII-L molecular weight standards (Sigma), to determine purity. ABA-1 was found in five main fractions, some of which also contained a 17-kDa contaminant. A peak devoid of the latter was selected and concentrated by ultrafiltration prior to removal of the Polybuffer by gel-permeation FPLC on a Superose 12 HR10/30 column using 0.05 M phosphate buffer, pH 7.2, containing 0.5 M NaCl.

The concentrations of solutions of protein were determined spectrophotometrically using an ϵ_{280} value of 10 810 M^{-1} cm^{-1}, calculated from the tyrosine and tryptophan content of the protein (Gill & von Hippel, 1989; Spence et al., 1993). This was confirmed directly by amino acid analysis on selected samples.

Fluorescent Probes and Competitors. The fluorescent fatty acid analogues 11-(((5-(dimethylamino)-1-naphthalenyl)sulfonyl)amino)undecanoic acid (DAUDA),[1] and 5-(octadecanoyl)aminofluorescein (OAF) were obtained from Molecular Probes, Eugene, OR. Dansyl-DL-α-aminocaprylic acid (DACA) was obtained from Sigma (Poole, Dorset, U.K.), as were *all-trans*-retinol, *all-trans*-retinoic acid, arachidonic acid, oleic acid, stearic acid, palmitic acid, squalene, cholesterol, and L-tryptophan. The small branched chain fatty acids 2-methylbutyric acid and 2-methylvaleric acid were obtained from Aldrich (Gillingham, Dorset, U.K.). The dansylated and fluoresceinated fatty acids and the retinoids were stored as stock solutions of 1 mg mL^{-1} in ethanol, in the dark at −20 °C, and freshly diluted in 171 mM phosphate-buffered saline (PBS), pH 7.2, to 1 μM for use in the fluorescence experiments. Competitors of fluorescent fatty acid binding were prepared as stock solutions in ethanol at approximately 10 mM (except for tryptophan, which was prepared in PBS) and diluted in PBS for use.

The following reference proteins were obtained from Sigma and prepared as stock solutions at 10 mg mL^{-1} in PBS: bovine serum albumin, β-lactoglobulin (BLG), ribonuclease A, ovalbumin (chicken), hemoglobin (bovine), transferrin (bovine), and hemocyanin (*Limulus polyphemus*).

Spectrofluorimetry. Fluorescence measurements were made at 20 °C in a Perkin-Elmer LS50 or a SPEX FluorMax spectrofluorimeter (Spex Industries, Edison, NJ), using 2-mL samples in a silica cuvette. Raman scattering by the solvent was corrected for where necessary using appropriate blank solutions. Quenching of protein fluorescence by succinimide was performed and analyzed as described previously (Johnson & Price, 1987), using succinimide recrystallized from ethanol before use. DAUDA, DACA, retinol, and retinoic acid were used at approximately 1 μM. All spectra are uncorrected.

Stoichiometry of Binding. To 2 mL of DAUDA at 0.825 μM were added 5-μL samples of ABA-1 at a monomer concentration of 0.12 mM, and the fluorescence was measured at 476 nm with $\lambda_{Exc} = 345$ nm. The concentration of the ethanol stock solution of DAUDA was checked by absorbance of a 1:10 dilution in methanol at 335 nm, using an extinction coefficient ϵ_{335} of 4400 M^{-1} cm^{-1}. Fluorescence data were corrected for dilution and fitted by standard nonlinear regression techniques (using Microcal ORIGIN software) to a single noncompetitive binding model to give estimates of the dissociation constant (K_d), the number of binding sites (n) per ABA-1 monomer unit, and the maximal fluorescence intensity (F_{max}). Similar nonlinear regression methods were used to analyze results of competition experiments in which oleic acid was progressively added to DAUDA/ABA-1 mixtures. Stock solutions of oleic acid were prepared in ethanol at 10 mM and freshly diluted in

[1] Abbreviations: BLG, β-lactoglobulin; BSA, bovine serum albumin; CD, circular dichroism; DACA, dansyl-DL-α-aminocaprylic acid; dansyl, (5-(dimethylamino)-1-naphthalenyl)sulfonyl; DAUDA, 11-(((5-(dimethylamino)-1-naphthalenyl)sulfonyl)amino)undecanoic acid; DMF, dimethylformamide; DSC, differential scanning calorimetry; FA, fatty acid; FABP, fatty acid binding protein; FFA, free fatty acid; A-FABP, H-FABP, I-FABP, and L-FABP, adipocyte, heart, intestinal, and liver fatty acid binding protein; GuHCl, guanidine hydrochloride; retinol and retinoic acid, *all-trans*-retinol and -retinoic acid; RBP, retinol binding protein; OAF, 5-(octadecanoyl)aminofluorescein; PBS, phosphate-buffered saline.

PBS. Increasing concentrations of oleic acid were added to a mixture containing 0.825 μM DAUDA and 0.25 μM ABA-1.

Circular Dichroism. Circular dichroism (CD) spectra were recorded at 20 °C in a JASCO J-600 spectropolarimeter using quartz cells of path length 0.02 or 0.05 cm. Protein concentrations were typically in the range 0.1−0.5 mg mL^{-1}. Molar ellipticity values were calculated using a value of 115 for the mean residue weight calculated from the amino acid sequence of the protein (Spence *et al.*, 1993). Analysis of the secondary structure of the protein was performed using the CONTIN procedure (Provencher & Glöckner, 1981) over the range 240−190 nm. Guanidinium chloride (GuHCl) (Ultrapure grade) was purchased from GIBCO-BRL, Paisley, Scotland, and the concentrations of solutions were checked by refractive index measurements (Nozaki, 1972).

Differential Scanning Calorimetry. Differential scanning calorimetry (DSC) experiments were performed by standard procedures (Cooper & Johnson, 1994) using a Microcal MC2-D instrument at a scan rate of 60 °C h^{-1} over a 20−110 °C range using sample concentrations of 0.5−1.5 mg mL^{-1}. Normalized excess heat capacity data were analyzed by standard procedures, using Microcal ORIGIN software, to give the midpoint transition temperatures (T_m) and the calorimetric (ΔH_{cal}), and van't Hoff (ΔH_{vH}) transition enthalpies.

Structural Predictions. All the known ABA-1-like proteins were compared using the ALIEN multiple alignment program (Akrigg et al., 1988). The sequences used for the multiple alignment were from the ABA-1 of *Ascaris* (EMBL/GenBank accession number L03211) and the homologues from the dog heartworm *Dirofilaria immitis* (accession number M82811), the lymphatic filarial parasites *Brugia pahangi* (of cats) and *Brugia malayi* (of humans) (gp15/400; accession number X68190), and the bovine lungworm *Dictyocaulus viviparus* (accession number U02568). The gene encoding the last of these contains several different forms of the ABA-1 homologue, and that for *D. immitis* encodes two slightly different forms. This was then analyzed to locate either positions containing conserved amino acids or positions which conserved the hydrophobic or hydrophilic nature of the amino acids. Secondary structure predictions of individual sequences were performed using the GOR (Garnier et al., 1978), Chou and Fasman (Chou & Fasman, 1974), and PHD (Rost & Sander, 1993, 1994) algorithms. The positions of secondary structure elements were located from analysis of insertions and deletions in the multiple sequence alignment (Pascarella & Argos, 1992). The ABA-1 sequence was compared to the SWISSPROT and PIR protein sequence databases using the programs SWEEP (Akrigg et al., 1988), BLAST (Altschul et al., 1990) and MPSRCH (Sturrock & Collins, 1993) to locate the best complete and local sequence similarities. The protein sequence was also compared with the sequence patterns stored in the PROSITE database (Bairoch et al., 1991) to search for potential functional domains on the basis of amino acid sequence.

A number of residue frequency matrices were constructed using the multiple alignment, and these were then used to look for similarities to other known proteins using the ADSP suite of programs (Parry-Smith & Attwood, 1992).

RESULTS

Purification of ABA-1 and Its Occurrence as a Dimer. ABA-1 was purified from the parasite as described in Material and Methods and was judged homogeneous by the criterion of a single band by SDS−PAGE or peak in gel filtration. The single gel-filtration peak eluted with an apparent molecular size of 27 400, and SDS−PAGE yielded a single band of M_r 14 400, indicating that the native protein may form a dimer (see also DSC results below). It has been established from cDNA sequencing that the parasite probably produces different forms of the ABA-1 protein (Moore et al., 1993a), but the chromatofocusing-based purification used was designated to isolate the most abundant form, as monitored by SDS−PAGE of chromofocused fractions. ABA-1 from such fractions has been amino-terminal sequenced to 68 residues (Christie et al., 1993) and found to be identical in amino acid sequence to the cDNA-derived sequence (see below).

Fluorescent Fatty Acid Binding to ABA-1. A fortuitous observation by fluorescence microscopy (M. W. Kennedy, unpublished) led us to suspect that these proteins might bind/sequester fatty acids and retinoids. Detailed investigation by spectrofluorimetry confirmed this as follows. The fatty acid binding activity of purified ABA-1 protein was examined using a range of polarity-sensitive fluorescent fatty acid probes (Table 1), all of which gave qualitatively identical results. For example, the fluorescence emission of DAUDA (11-(((5-(dimethylamino)-1-naphthalenyl)sulfonyl)amino)undecanoic acid) is known to increase substantially and exhibits a blue shift in emission wavelength when bound to fatty acid binding proteins (FABPs) (Wilkinson & Wilton, 1986). With ABA-1 (Figure 1A) we observed a shift from 543 nm (in buffer alone) to 475 nm, together with a marked increase in emission intensity. This substantial blue shift was greater than that observed with serum albumin (495 nm) or reported for rat liver FABP (500 nm; Wilkinson & Wilton, 1986). The addition of free fatty acids reversed the changes in the emission spectrum of DAUDA (see below). Control experiments with dansylamide revealed minimal binding to ABA-1, indicating that the fluorophore group is probably not contributing significantly to the binding of the dansylated fatty acid.

Blue shifting of the dansyl fluorophore emission wavelength is usually taken as a measure of the polarity of the binding site in binding proteins (Macgregor & Weber, 1986). The fluorescence spectrum of DAUDA in ethanol, dimethylformamide (DMF), and cyclohexane showed emission peaks at 506, 505, and 475 nm, respectively (Figure 1B), in order of decreasing polarity. The blue shift of DAUDA in ABA-1 is thus similar in degree to that in cyclohexane, indicating either that the environment of the protein's binding site is apolar to a remarkable degree or that there are unusual specific interactions in the binding site.

Similar blue shifts in fluorescence were found with a fluorescent fatty acid probe in which the dansyl fluorophore is attached at the α carbon (dansyl-DL-α-aminocaprylic acid; DACA), rather than to the hydrocarbon terminal, as in DAUDA (data not shown). This similarity in behavior of the two probes could indicate that the fatty acid ligand is bound entirely within the binding site and isolated from solvent.

We also investigated the binding characteristics of ABA-1 using a fluorescein-conjugated probe, OAF. OAF has a low level of fluorescence in aqueous solution, and binding to proteins can be detected by the increase in fluorescence arising from reversal of self-quenching when OAF probe molecules are transferred from aqueous micelles to a protein

Table 1: Binding of Hydrophobic Ligands by the ABA-1 Allergen of *Ascaris* and Other Representative Proteins[a]

protein	probe							refs
	DAUDA	DACA	OAF	oleic acid	oleoyl-CoA	retinol	retinoic acid	
ABA-1	+	+	+	+	+	+	+	this study
serum albumin	+	+	+	+		+	+	this study
β-lactoglobulin	−		+	+		+	+	this study; Frapin et al., 1993; Papiz et al., 1986
ovalbumin	−	−	−	−	−	−	−	this study
transferrin	−	−	−	−	−	−	−	this study
hemoglobin	−	−	−	−	−	−	−	this study
hemocyanin	−	−	−	−	−	−	−	this study
rat intestinal FABP	+			+		−	−	Sacchettini & Gordon, 1993
rat liver FABP	+			+	+	+	+	Thumser et al., 1994; Glatz & Vandervusse, 1990
P2 myelin protein				+		+	+	Cowan et al., 1993
murine adipocyte lipid-binding protein				+		−	+	Glatz & Vandervusse, 1990; Matarese & Berlohr, 1989

[a] The binding of the polarity-sensitive probes 11-(((5-(dimethylamino)-1-naphthalenyl)sulfonyl)amino)undecanoic acid (DAUDA) (Molecular Probes) and dansyl-DL-α-aminocaprylic acid (DACA) (Sigma) was followed by the changes in the intensity and wavelength of probe fluorescence. Fluoresceinated fatty acid 5-(octadecanoyl)aminofluorescein (OAF) (Molecular Probes) binding was detected by the increase in fluorescence arising from reversal of self-quenching when OAF probe molecules are transferred from aqueous micelles to a protein environment. Binding of oleic acid, retinol, retinoic acid, and oleoyl-coenzyme A was detected by competition for binding with DAUDA (see text and the caption to Figure 2). Retinol and retinoic acid binding was also detected by changes in their intrinsic fluorescence. The excitation (λ_{Exc}) and emission (λ_{Em}) wavelengths used for the probes in this study were, respectively, 345 and 476 nm for DAUDA and DACA, 480 and 517 nm for OAF, and 350 and 460 nm for the retinoids. +, binding observed; −, binding not observed; blank, not tested.

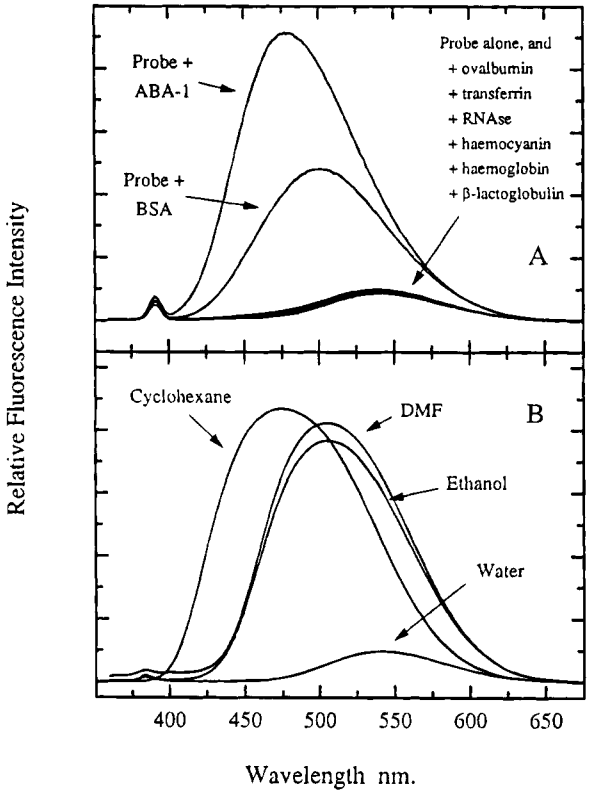

FIGURE 1: Binding of the fluorescence labeled fatty acid probe, DAUDA, to ABA-1. (A) Fluorescence emission spectra (uncorrected) (λ_{Exc} = 345 nm) of approximately 1 μM DAUDA alone or with 5 μg mL^{-1} ABA-1 or 25 μg mL^{-1} of each of the proteins indicated. (B) Comparative emission spectra of 10 μM DAUDA in different solvents.

environment (Haugland, 1992). When ABA-1 was added to this probe, a substantial increase in fluorescence was observed. This also occurred with albumin and lactoglobulin, but with none of the other reference proteins used. As before, the effect of ABA-1 was reversed in each case by addition of oleic acid.

Table 1 presents a summary of the fatty acid binding activity of ABA-1 for a range of fluorescent lipidic probes and a comparison with proteins of similar binding activity reported in the literature. Interestingly, we found that use of DAUDA and OAF differentiated the fatty acid binding activity of ABA-1 and BSA from that of BLG, in that ABA-1 and BSA bound both DAUDA and OAF, whereas BLG bound only OAF.

Stoichiometry of Binding. Titration of ABA-1 with DAUDA showed that each monomer unit of ABA-1 has a single binding site for DAUDA, with a dissociation constant (K_d) estimated to be (8.8 ± 0.5) × 10^{-8} M (Figure 2A), which is similar to that for other FABPs (Thumser et al., 1994). The binding of nonfluorescent fatty acids and other hydrophobic ligands was determined from their competitive effects on the fluorescence of the ABA-1:DAUDA complex. For example, progressive additions of oleic acid to an ABA-1:DAUDA mixture brought about a stoichiometric reversal of the fluorescence effect, presumably by displacement of the DAUDA from the protein site (Figure 2B). Analysis of such oleic acid titration curves gives an apparent K_d (oleic) of approximately 1.3 × 10^{-8} M. Such experiments can be used to map the specificity of the ABA-1 binding site (Table 1), and we found competitive displacement by arachidonic, stearic, retinoic, and palmitic acids and retinol, but not by tryptophan, cholesterol, or squalene or by the short branched chain fatty acids 2-methylbutyric acid and 2-methylvaleric acid excreted by *Ascaris* (Barrett, 1981).

Retinol and Retinoic Acid Binding. The binding of retinoids to ABA-1, indicated by competition experiments (see above), was confirmed directly by the effect of the protein on their intrinsic fluorescence. Figure 3A shows the emission spectrum of retinol in which the addition of ABA-1 resulted in a substantial increase in fluorescence. Comparative spectra for retinol in BSA or BLG are also presented. The change in retinol fluorescence in ABA-1 was reversed by the addition of oleic acid, indicating that the binding site for retinol and oleic acid was competitive (Figure 3B). Similar results were found for retinoic acid. β-Lactoglobulin, which has been reported to show considerable specificity for retinoids (Papiz et al., 1986), produced a red shift in the fluorescence of both retinol and retinoic acid, but whereas

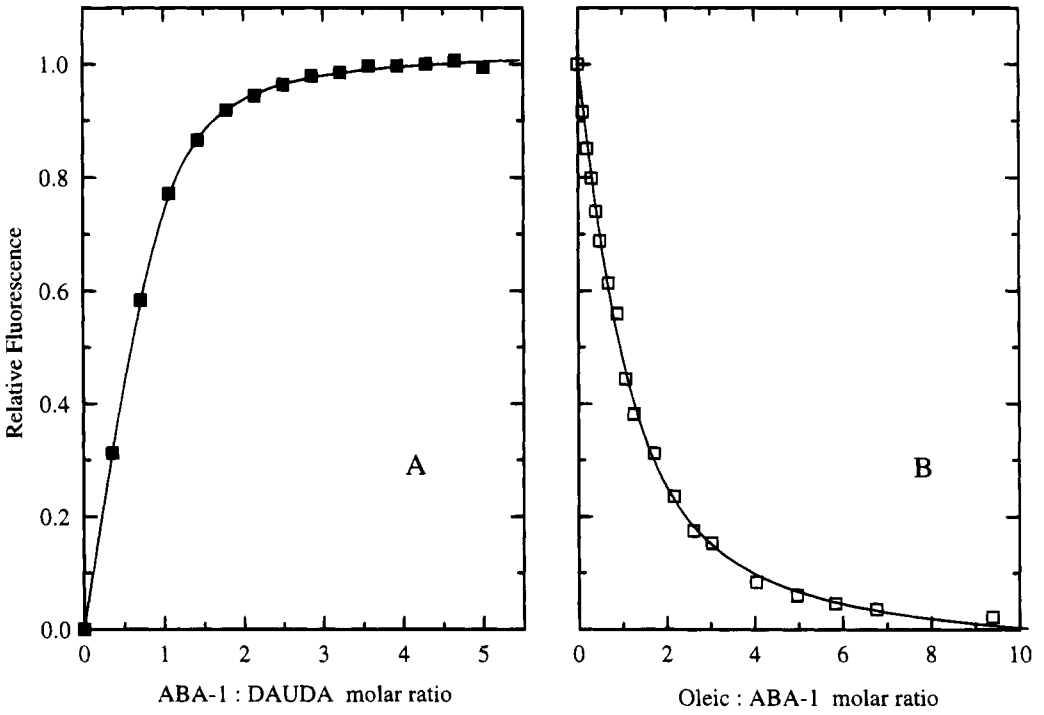

FIGURE 2: Titration curves for the binding of DAUDA and oleic acid to ABA-1. (A) Relative fluorescence intensity changes at 476 nm (corrected for dilution, $\lambda_{Exc} = 345$ nm) of 0.825 μM DAUDA in PBS, upon addition of increasing concentrations of ABA-1. The solid line is the theoretical binding curve for complex formation with a dissociation constant, $K_d = (8.8 \pm 0.5) \times 10^{-8}$ M, and binding stoichiometry $n = 1.02$ ($\pm$ 0.15) mol of DAUDA per mole of ABA-1. (B) Decrease in relative fluorescence due to displacement of DAUDA from ABA-1 by oleic acid. Increasing concentrations of oleic acid were added to a mixture containing 0.825 μM DAUDA and 0.25 μM ABA-1. The solid line is a theoretical curve for simple competitive binding of oleic acid in the DAUDA binding site of ABA-1, with apparent K_d (oleic) $\approx 1.3 \times 10^{-8}$ M.

ABA-1 and serum albumin increased the level of fluorescence, neither produced a red shift.

Tryptophan Fluorescence. Fatty acid binding proteins such as serum albumin and the family of β-barrel proteins typified by I-FABP have a tryptophan residue either within the binding cavity and involved in interaction with ligand or in close proximity to this site (He & Carter, 1992; Papiz et al., 1986; Sacchettini et al., 1989; Sacchettini & Gordon, 1993). ABA-1 and its homologues in other species of nematode have a single conserved tryptophan (Trp16), the environment of which was examined by fluorescence analysis. The fluorescence spectrum of the protein excited in the region of 290 nm showed a sharp emission peak at 307 nm (probably due to tyrosine, of which there are four in ABA-1) and a shoulder at 318 nm (Figure 4). Emission by Trp16 at this short wavelength indicates that it is highly buried within the protein (Eftink & Ghiron, 1976).

The accessibility of Trp16 to the water environment was further examined using quenching of fluorescence by succinimide at excitation and emission wavelengths of 295 and 330 nm, respectively, in order to examine the tryptophan selectively (Eftink & Ghiron, 1984). The results showed that succinimide produced minimal quenching at any concentration, confirming the highly buried position of the tryptophan; the K_{SV} is low (0.15 M^{-1}) when compared with that for tryptophan residues in other proteins, such as phosphoglycerate mutase of *Schizosacchromyces pombe* (2.2 M^{-1}) (Johnson & Price, 1987) and pig heart citrate synthase (1.68 M^{-1}) (N. C. Price, unpublished observations). Increasing concentrations of GuHCl, however, led to a red shift in the emission maximum corresponding to the increased exposure of the tryptophan with progressive denaturation of the protein (Figure 4). In 6 M GuHCl, the K_{SV} was 3.1 M^{-1}, which is of the same order of magnitude as that of *N*-acetyltryptophanamide in 6 M GuHCl (5.2 M^{-1}) (Moore et al., 1993b). The major changes in the fluorescence spectrum occurred over the range 1−3 M GuHCl, corresponding to the range in which the most radical changes occurred in the far-UV CD spectrum, suggesting that the losses in secondary and tertiary structure run largely in parallel.

Proximity of the ABA-1 Single Tryptophan Residue to the Fatty Acid Binding Site. The tryptophan fluorescence experiments showed that Trp16 of the ABA-1 monomer unit is deeply buried within the protein and therefore unlikely to be present in a binding site where some exposure to solvent would be expected in the *apo* protein. A further test of this would be to examine the fluorescence spectrum of Trp16 with and without fatty acid bound to the protein. In the case of BLG, for example, the fluorescence of its tryptophan has been shown to be increased upon binding of fatty acid (Frapin et al., 1993), presumably due to a change in the local environment of the residue brought about by proximity to the ligand or exclusion of water, or to a change in the conformation of the protein. We therefore recorded spectra of the Trp in each of BLG, BSA, and ABA-1 for comparison (Figure 5), from which it was clear that the blue shifting was the greatest for ABA-1. When oleic acid was added to each protein, the fluorescence peak of the Trp in lactoglobulin was increased (as found previously; Frapin et al., 1993), decreased in BSA, but unaltered in ABA-1.

Circular Dichroism. The far-UV CD spectrum of ABA-1 is illustrated in Figure 6A, and shows a strong α-helix signal. Analysis with the CONTIN program provided an estimate of 59 $\pm$ 2% α-helix, 35 $\pm$ 2% β-sheet, and the remainder, 6 $\pm$ 3%. The helical content approximates closely to the

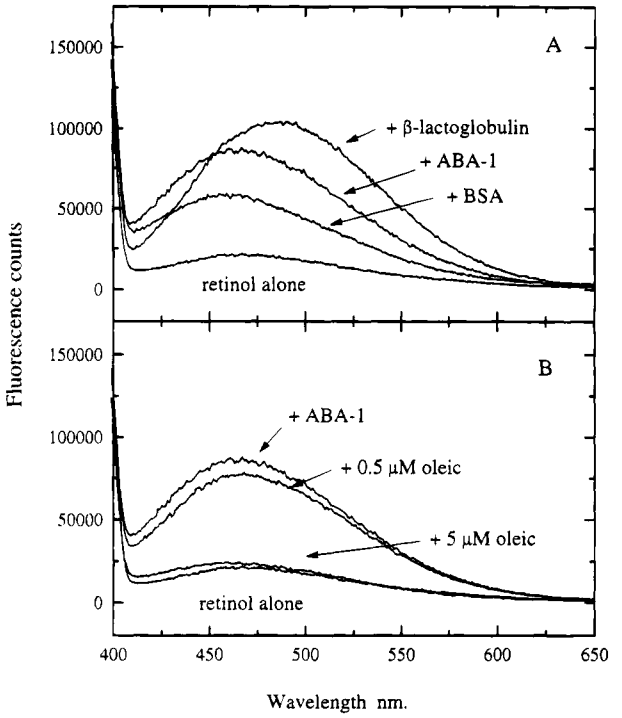

FIGURE 3: (A) Binding of retinol to ABA-1 in comparison with BSA and BLG: Fluorescence emission spectra (λ_{Exc} = 350 nm) of 1 μM retinol in the absence or presence of ABA-1, BSA, or BLG. The peak fluorescence wavelengths for retinol alone or with BSA, BLG, or ABA-1 were 467, 462, 487, and 464 nm, respectively (±2 nm). For retinoic acid, the peaks were at 464, 464, 493, and 464 nm, respectively (±2 nm) (not shown). (B) Retinol fluorescence emission curves showing competition for the ABA-1 retinoid binding site by oleic acid. The fluorescence enhancement brought about by addition of allergen ("+ ABA-1") to 1 μM retinol is reversed by subsequent addition of 0.5 or 5 μM oleic acid. Similar observations were made with retinoic acid.

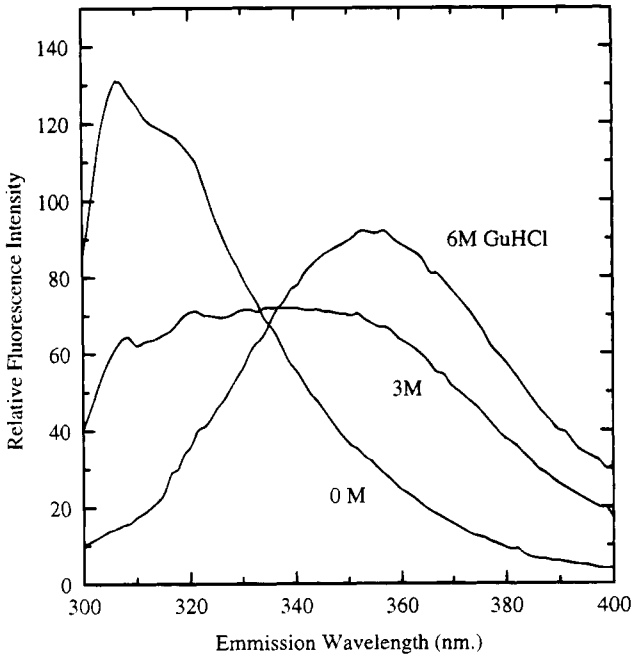

FIGURE 4: Tryptophan fluorescence spectrum of a 0.12 mg mL^{-1} solution of ABA-1 in 0, 3, or 6 M GuHCl. λ_{Exc} = 290 nm.

predicted α-helical content in the region of 53−55%, depending on where the helices are taken to begin and end (see below). Addition of increasing concentrations of GuHCl led to a progressive loss in secondary structure as detected by CD (Figure 6B), with the greater part of the unfolding occurring between 1 and 3 M GuHCl.

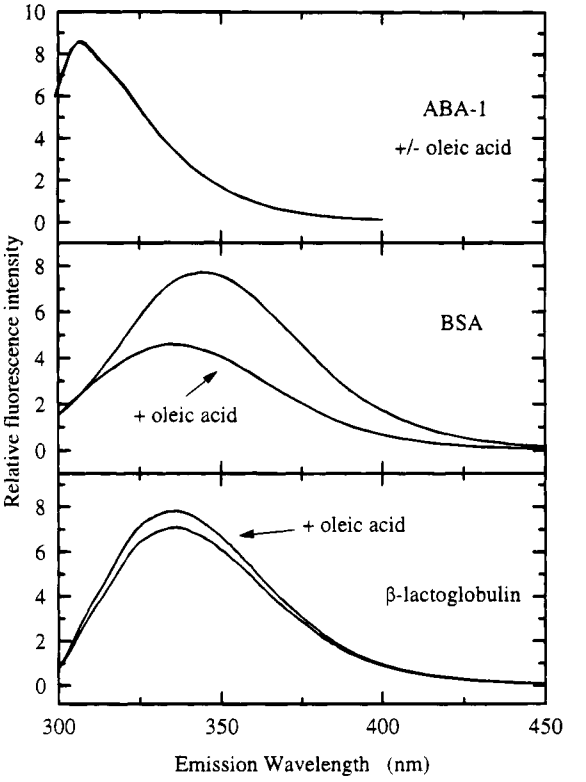

FIGURE 5: Effect of fatty acid binding on Trp16 fluorescence. Tryptophan fluorescence emission spectra (recorded at λ_{Exc} = 287 nm) for ABA-1, BSA, and β-lactoglobulin with and without oleic acid at 5.1, 1, and 1 μM, respectively. The peak of emission wavelengths for the proteins without/with oleic acid were 307/307, 344/335, and 335/336 nm for ABA-1, BSA, and β-lactoglobulin, respectively.

Differential Scanning Calorimetry. DSC experiments on purified ABA-1 in neutral buffers consistently showed two high-temperature endothermic thermal transitions at T_{m1} = 71.7 ± 0.9 °C and T_{m2} = 88.3 ± 1.6 °C (Figure 7). The biphasic nature of the transition suggests either significant heterogeneity in the protein preparation or distinct structural domains within the protein structure. Repeat DSC scans of these samples showed that the higher temperature transition is relatively reversible, even after heating to 110 °C, whereas the amplitude of the lower temperature transition decreased substantially with successive reheating. The apparent mean transition enthalpies, averaged over a series of experiments, were approximately as follows (±10%):

Transition 1: $\Delta H_{cal} \approx 110$ $\Delta H_{vH} \approx 210$ kJ mol^{-1}

Transition 2: $\Delta H_{cal} \approx 160$ $\Delta H_{vH} \approx 330$ kJ mol^{-1}

The approximate value of 2 for the ratio ΔH_{vH}:ΔH_{cal} is consistent with a dimeric cooperative unit for unfolding of the protein in each of the transitions.

Structural Predictions. An alignment of the amino acid sequence of ABA-1A with similar proteins of three other parasitic nematodes is given in Figure 8A. No significant matches to proteins other than ABA-1A homologues were detected using any of the database searching programs. All the secondary structure prediction programs predicted the sequences to be highly helical in nature. Figure 8B shows the alignment of residues 1−65 with residues 66−133 from ABA-1A, which indicates the existence of an internal repeat

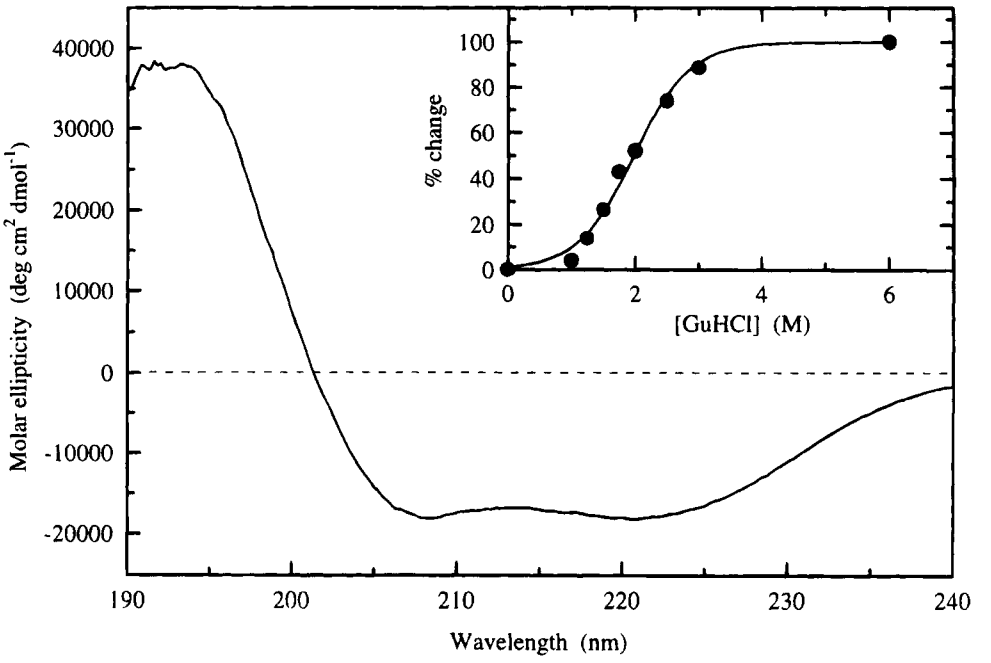

FIGURE 6: Circular dichroism (CD) spectrum recorded at 20 °C on a 0.48 mg mL^{-1} solution of ABA-1 in 8.3 mM sodium phosphate and 83.3 mM sodium chloride, path length 0.02 cm. Inset: Change in ellipticity at 225 nm with increasing concentrations of GuHCl of a 0.12 mg mL^{-1} sample of ABA-1, path length 0.05 cm. The curve is sigmoidal and merely shown for guidance.

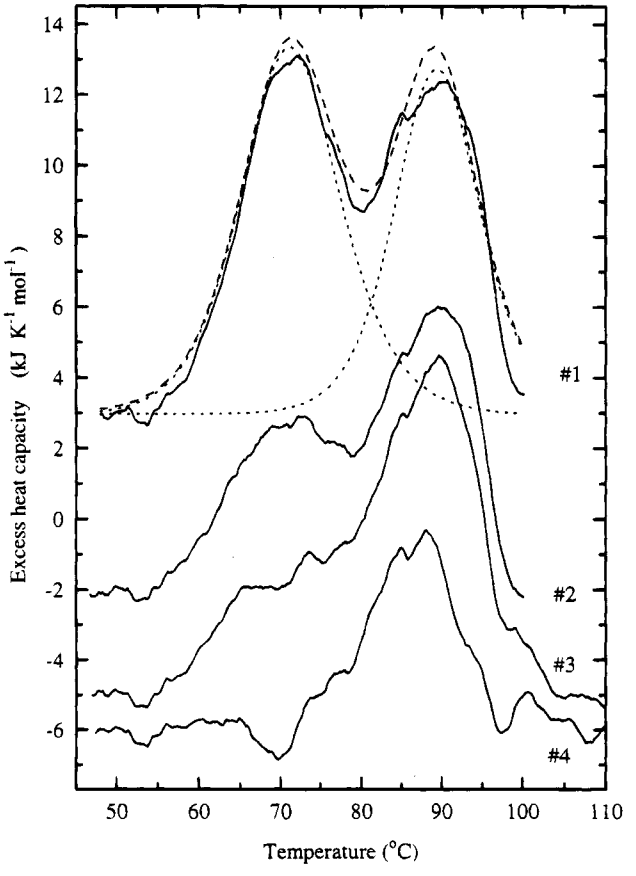

FIGURE 7: Differential scanning calorimetry (DSC) data for ABA-1 (1.56 mg mL^{-1}) in 50 mM NaP_i and 0.5 M NaCl, pH 7.2. The dashed lines (scan #1) show the theoretical curves for deconvolution into two independent unfolding transitions. Subsequent scans #2 to #4, offset for clarity, show the results of reheating this sample in the calorimeter after repeated cooling to room temperature.

in the protein sequence. The similarity between these parts of the protein is greater than given by chance ($P > 0.005$), as analyzed by the methods of Brass et al. (1992).

Parse points in the alignments (i.e., points at which there is a change in predicted secondary structure) can be determined from the places in the alignment containing either conserved glycines or prolines or positions at which insertions/deletions are indicated from the alignment. Eight potential parse points (labelled A−H) are marked on Figure 8A,B.

The region between parse points A and B shows features consistent with α-helix (see Figure 8B), which, when plotted on a helical wheel, can be seen as being primarily amphipathic, with the notable exception of the tryptophan at position 16, which appears to be isolated in a hydrophilic face. Other factors to support this region being helical come from the potential for stabilizing salt bridges within the hydrophilic surface (for example, Asp11 with Lys15 and His13 with Glu20). The region between parse points B and D is predominately hydrophilic, consistent with it being a surface loop.

The region between parse points C and D is predicted to be helical. It is difficult to know exactly where to start the helix. The start position at residue 38 was chosen primarily to match helix position 4 in the second domain (Figure 8B); however, it is possible that the helix could start two or three residues before this while still retaining an amphipathic character. Interestingly, there is a single hydrophobic residue (Leu43) within the putative hydrophilic face that is maintained as a hydrophobic residue in all ABA-1A homologues.

The predicted secondary structure in the second domain (residues 66−121) appears to be well-matched to that for the foregoing repeat domain (residues 10−65). Helix 3 is predicted to run from residue 66 to 81 with a loop (residues 82−93) connecting it to a fourth helix (residues 94−110). When these regions are arranged on helical wheels (Figure 9A), helix 4 shows a strong amphipathic nature, whereas helix 3 appears to contain mixed hydrophobic and hydrophilic regions. There is a conserved glycine near the middle of helix 3 (position 74) which could act to interrupt the secondary structure. Plotting the helix above and below this

```
                              HELIX 1                        HELIX 2
                        ____________________               ______________

Parse               A                   B  C                   D
                              10        20        30        40        50        60
ABA-1A          HHF-TLESSLDTHLKWLSQEQKDELLKMKKDGKAKKELEAKILHYYDELEGDAKKEATEHLK
gp15/400        H-EHSLDDYFRTHLSWLTDAQKDEIRKMKEEGKQKMDMQKKILDYYENLTGDGKKEAGEKLR
DvA-1B             DS-NKNFEEFIYWMTDEQKQSMNDMKTAGKSFNEIHKEGRKYFKALTIDKQSSLKEQFK
DvA-1G          E-IHSLEEAMERYLTWLSDDQKIVIKSIY-DVNDRKVLYEKIMEFFDDAIGETKQKAAKELK
DvA-1H          H-EHNLDEALEKHFTWLNEEQKSQLKTIY-ESEDREALHKKVWEFFEAGAG-----------
DvA-1J          H-EHNLEEALEKYLTWLNEEQKSQMKTIY-ESEDREALYKKVLEFFEAATGEVKEKAAVELK
DvA-1K          H-EHNLEEAMGKYLSWMSDEQQAQVKKIY-GTGDRLATYNKVMELFESVPSDEKEKATSQLK
DvA-1L          Y-EIDVDEAISKYLTWLNEEQKAEIKQLK-EKDEKQTIGKKIMEFFELTSGDDKEKAREQLK
Di1             RNDHNLESYFQTYLSWLTDAQKDEIKKMKEEGKSKMDIQKKIFDYFESLTGDKKKKAAEELQ
Di2             -NDHNLESYFQTYLSWLTDAQKDEIKKMKEEGKSKMDIQKKIFDYFESLTGDKKKKAAEELQ
Di3             -NDHNLESYFQTYLSWLTDAQKDEIKK-----------------------------------
PHD                   HHHHHHHHHHHHHHHHHHHHHHH...HHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
                    +•++ •++ • ••++ ++   • +• +   + +   •   +••+••+  •   + +++•  +•+

                            HELIX 3                     HELIX 4
                       ________________             ________________

Parse              E                F       G                     H
                          70        80        90       100       110       120
ABA-1A          GGCREILKHVVGEEKAAELKNLKDSGASKEELKAKVEEALHAVTDEEKKQYIADFGPACKKI
gp15/400        GGCRELLRQIVGDEKMAELKQMKESGLGQQELRAKVDEMLEHVTDEAKKQKIHEYGPACRKI
DvA-1B          DKCKKYFMQIANSDEVEKIKSL-----NDDEIRHVVKNAVARLNGEDKEFAV-KMETLCEDV
DvA-1G          DACKHYVKDLIGEENGNLLREMKENGASNEAIATKVEEMIEAITDETKRAQAMRASTSCRKV
DvA-1H          -----------------------------------------------------LRASNASKKI
DvA-1J          SACRHYIKDYIGDEKAEKIKEMKESGVSTEEISKKVDEFIAMITDDEKKAKALRASNACKKI
DvA-1K          AACRHYIKDFIGKDNLAVIKEMKESGATNEAIGEKIDEFIAGLHDEQKKAQAQRAASACKKI
DvA-1L          AACKHYVKMYVGEEKAAELKKLKDSGISLEEMSKKVTETIETIEDEAVRAKARR--------
Di1             QGCLMALSEIIGNEKMLMLKEIKDSGADPEQIRMKVEDMLKLVVDKEKKKRIDEYAPVCRKI
Di2             EGCRMALREIVGEEKWTVLRQMKDSGATPKELSMKVEEMFKDVVDKDKKEKIDEYAPVCRKI
Di3
PHD             ...HHHHHHHHHHHHHHHHH....HHHHHHHHHHHHHHHHHHHHHHHHHHHHH.....E
                  •   ••   •• +++   •++•+++ •+ + •    •++••  • ++   +  • +•  ••++•

                      130
ABA-1A          YGV--HTSRRRR
gp15/400        YED--RH-KRNN
DvA-1B          LAF--KARKN
DvA-1G          YGVVQRFRRDHH
DvA-1H          YGVAKRFRR
DvA-1J          YGVAKRLRRDHH
DvA-1K          YGVKSRKRREH-
DvA-1L          ------------
Di1             YAA--MNERRKR
Di2             FAV--IHERRKR
Di3
PHD             EEEE........
                •      +++++
```

```
                 Helix 1                           Helix 2
Parse     A                      B    C                       D              E
          |                      |    |                       |              |
       HHFTLESSLDTHLKWLSQEQKDELLKMKKDGKAKKELEAKILHYYDELEGDAKKEATEHLKGGC 65
Consensus        ~    ~ ~~~E  EL  ~K  G ~K EL AK~         ~  ~ KK~      ~~C
           66 REILKHVVGEEKAAELKNLKDSGASKEELKAKVEEALHAVTDEEKKQYIADFGPACKKIYGVHTSRRRR
                              |       |                       |
Parse                         F       G                       H
                 Helix 3                           Helix 4
```

FIGURE 8: (A) Alignment of the amino acid sequences deduced from cDNA of the ABA-1 allergen of *Ascaris suum* (EMBL/GenBank accession number L03211) with homologues for which information is available from three other genera of parasitic nematode: gp15/400 is from the filarial nematode *Brugia malayi* (X68190); Di1 and Di2 are derived from *Dirofilaria immitis* (M82811); and DvA-1B, G, H, J, K, and L are from *Dictyocaulus viviparus* (U02568). The line labeled PHD gives the predicted secondary structure from the program PHD (Rost & Sander, 1993, 1994) marked with α-helix (H), loop (.), and β-sheet (E). Residues which are conserved throughout are highlighted in bold, and where the nature of each residue is conserved they are indicated as follows: conserved hydrophobic (•), conserved hydrophilic (+). Also marked are the parse points and the predicted helices (see text). (B) Sequence of ABA-1A showing the similarity between the two putative domains comprising helix 1 + 2 and helix 3 + 4. Conserved residues are indicated, and those with conserved biochemical properties are indicated (~). Parse points (see text) are also indicated.

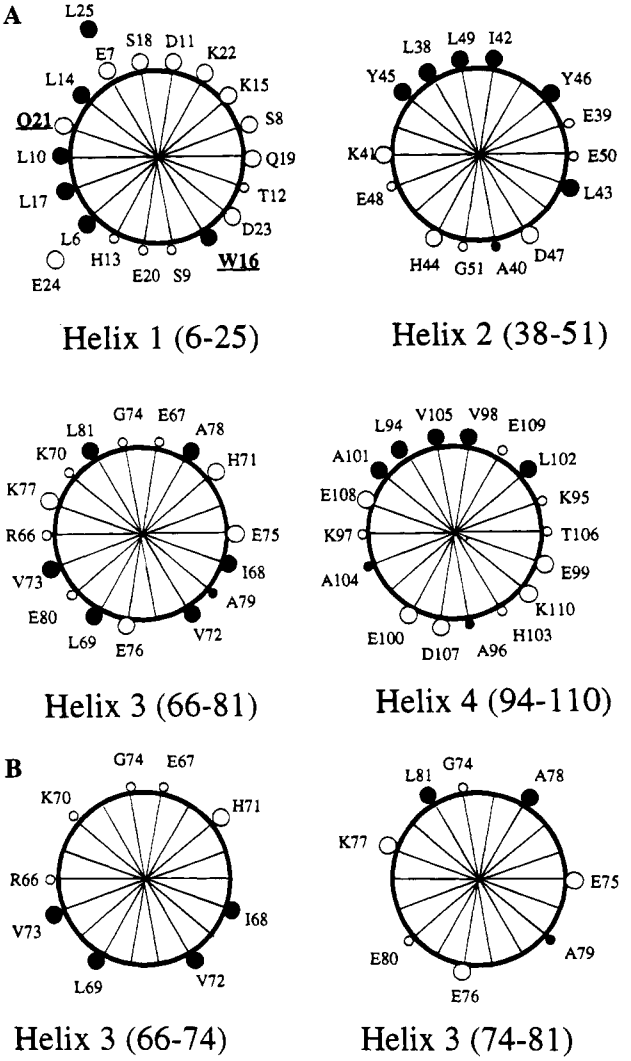

FIGURE 9: (A) Helical wheels for the four predicted helices of ABA-1A. Hydrophobic residues are indicated by filled circles, and hydrophilic residues, by open circles. Large circles mean that the property of the residue (hydrophilic or hydrophobic) is maintained across the complete alignment in Figure 8A. The two totally conserved residues (W16 and Q21) are marked in bold and underlined. (B) Helical wheels for the regions above and below Gly74 in helix 3.

glycine (Figure 9B) revealed that the helix may have two hydrophobic faces, separated by the glycine. Helix 2 also has a conserved glycine (position 51), but this occurs toward the COOH-terminal end of the helix and so would not cause a major disruption of the helix's structure.

The proposed four-helix structure for ABA-1 is conserved across the whole family of proteins aligned in Figure 8A. The residues that have conserved hydrophilic or hydrophobic natures across the entire alignment are also indicated in Figure 8. It can be seen from this that the hydrophobic/hydrophilic nature of the residues seen in ABA-1 is well conserved across all the nematode ABA-1-like proteins, strongly suggesting that this pattern is structurally or functionally significant. It is also interesting to note from this that the majority of the conserved residues are found at the hydrophobic faces as identified in Figure 9A.

DISCUSSION

We predict that the ABA-1 family of proteins are fatty acid and retinoid binding proteins sharing a common structural motif based on a four-helix structure. There is an intriguing possibility that ABA-1 monomer may be a four-helix bundle protein, such as myohemerythrin of invertebrates and cytochrome b_{562}, but set apart from other binding proteins of its size and ligand specificity (Sheriff et al., 1987; Lederer et al., 1981; Harris et al., 1994). Acyl-CoA binding protein also has a four-helix structure, but it cannot bind free fatty acids (Kragelund et al., 1993).

The predicted high degree of α-helix was confirmed directly by CD analysis of purified ABA-1. The prediction of 53−55% of the amino acids involved in helices is close to the observed estimate of 59 ± 2%. Each of the predicted helices of ABA-1 has relatively well-defined hydrophobic faces, and the simplest hypothesis is that they all orient toward the hydrophobic core of the protein. The helix for which amphipathicity was the least clear is helix 3, but this has a glycine (position 74) which would act to interrupt the secondary structure at this point. If the residues above and below Gly74 are plotted on a helical wheel (Figure 9B), then all the hydrophobic residues are similarly oriented, and the amphipathic character is thereby retained. Moreover, salt bridging between Glu67 and His71, and Lys77 and Glu80 would stabilize a helical conformation above and below Gly74, respectively. The conservation of this glycine throughout the ABA-1-like proteins indicates that the distortion of the helix at this point is probably an important structural feature.

Helix 1 is also notable in that the protein's single tryptophan (Trp16) residue appears to be located within the hydrophilic external face of the helix. Paradoxically, the fluorescence emission maximum at 318 nm indicated that Trp16 is not exposed to a hydrophilic environment, and the value of the Stern−Volmer constant from succinimide quenching experiments showed that this residue is deeply buried within the structure. Experiments to unfold the protein with GuHCl showed that Trp16 became progressively more exposed to a water environment between 1 and 3 M GuHCl, at which concentrations CD showed progressive loss of secondary structure.

DSC analysis revealed two major transitions at approximately 71 and 89 °C, which is consistent with the existence of two distinct structural domains within the ABA-1 protein. A value of approximately 2 for the ratio ΔH_{vH}:ΔH_{cal} at both transitions indicates a dimeric cooperative unit, which is consistent with our estimate of the M_r of the native form of ABA-1 by gel-exclusion chromatography. Taking the DSC data together with our other findings and predictions, therefore, the results could be tentatively explained as follows. Each unit of ABA-1 comprises two domains A and B, in which domain A interacts with domain A of a partner molecule in the dimer. An increase in temperature results first in the unfolding of domain B. A further increase in temperature causes the unfolding of domain A, which occurs simultaneously with the separation of the dimer partners. The unfolding of domain A and separation of the dimer is probably causally linked in that such dimerization interactions can stabilize interacting helical domains (Steif et al., 1993), which in turn may explain the high transition temperature in this case. A further speculation would be that the helices involved in dimerization are helices 1 and 2, because only these have hydrophobic amino acids on opposite faces of the helices which would be exposed on the surface of the protein to interact with a complementary region on the dimer partner. The existence of two discrete

domains in a four-helix protein has also been inferred for the ROP protein of *Escherichia coli* (Steif et al., 1993).

The other reason for considering the existence of two discrete domains is the apparent internal duplication within the amino acid sequence of a single monomer unit of ABA-1. There has, however, been a substantial degree of divergence since the putative duplication event which gave rise to the modern ABA-1 monomer. It is likely that this occurred early in the evolution of the nematodes given that cDNAs encoding ABA-1-like proteins are also encoded in the genome of the free-living species *Caenorhabditis elegans* (Moore et al., 1993c; unpublished observations). The duplication event may therefore have preceded the advent of parasitism in this group of organisms.

Our experiments with fluorescent fatty acid analogues or retinoids showed that the ABA-1 allergen is a fatty acid and retinoid binding protein. We have also found that homologues of ABA-1 which occur as important antigens in a wide range of parasitic nematodes have similar binding characteristics (Kennedy et al., 1995; unpublished observations). If the structural predictions for ABA-1 are correct, then it is distinct from the family of small lipid binding proteins which includes intestinal fatty acid binding protein (I-FABP), heart FABP (H-FABP), cellular retinoic acid binding protein, cellular retinol binding protein, bovine lactoglobulin (BLG), plasma retinol binding protein, and myelin P2 protein, all of which are β-barrels with only minor content of α-helix (Sacchettini & Gordon, 1993; Thumser et al., 1994). Serum albumin is, however, an exception to this in that it binds fatty acids and is predominately helical, but it is substantially larger than ABA-1, its structure is more complex, and it has multiple binding specificities (He & Carter, 1992).

The fluorescent fatty acid analogue used here, DAUDA, displayed a substantial increase and blue shifting in its fluorescence upon binding to ABA-1 (from 541 to 475 nm). The degree to which this occurred was indicative of the probe having entered an environment which was sufficiently apolar to drive the blue shift close to its limit, as defined by that observed for DAUDA in cyclohexane. The shift upon binding to BSA was found to be considerably less (495 nm), which is similar to that reported for I-FABP (500 nm; Wilkinson & Wilton, 1986). Therefore, either the environment of the ABA-1 binding site is hydrophobic to an unusual degree or unexpected interactions occur within it.

Titration experiments provided an estimate of the K_d of DAUDA for ABA-1, the value of 8.8×10^{-8} M representing an affinity comparable with those reported for other FABPs and RBPs (Lowe et al., 1987; Richieri et al., 1992; Frapin et al., 1993; Thumser et al., 1994). Competition experiments showed that oleic acid binds in the DAUDA site with a greater affinity, estimated at 1.3×10^{-8} M. The experiment also indicated that there was one binding site for DAUDA or oleic acid per 14.6-kDa subunit of ABA-1. Whether this means that each monomer unit has a single independent binding site or dimerization leads to the formation of a binding site with capacity for two ligand molecules remains to be determined.

The carboxylate group of bound fatty acid in ABA-1 may be held deep within the binding site, and not exposed to solvent, as appears to be the case for I-FABP (Sacchettini et al., 1989; Sacchettini & Gordon, 1993). This was inferred from the use of a fatty acid analogue in which the ((dimethylamino)-1-naphthalenyl)sulfonyl fluorophore was attached to the α carbon of the fatty acid (DACA), rather than to the terminal methyl group of the acyl chain as in DAUDA. The blue shift in the fluorescence emission of the α-conjugated DACA on binding to ABA-1 was similar to that for DAUDA, indicating that the carboxyl group was similarly inaccessible to solvent.

Many FABPs have been found also to bind retinoids (Sacchettini & Gordon, 1993), and the same appears to be the case for ABA-1. Moreover, the ligand competition experiments indicated that the binding site for FA is the same as that for retinol and retinoic acid. In our current experiments, a red shift in retinol was observed with BLG, perhaps reflecting its greater specialization toward retinoid binding, but with neither ABA-1 nor BSA. This may indicate that the conformation of retinol when held in the binding site, and the mechanism of its binding, in the latter two proteins is similar, but distinct from that of BLG. Whether this is a reflection of a dichotomy in binding mechanism between the α-helical and β-barrel lipid binding proteins is worthy of attention.

In conclusion, ABA-1 is quite unlike other fatty acid binding proteins of its size. It might, therefore, provide a valuable model with which to study interactions of fatty acids and retinoids with α-helical proteins which may be different in principle from β-barrel proteins. Moreover, ABA-1 is a far simpler and more compact protein than serum albumin for genetic manipulation to examine ligand binding specificities such as has been carried out with its β-barrel equivalents (Sacchettini & Gordon, 1993). To this end, we have already produced recombinant ABA-1 in *E. coli* which has identical ligand binding characteristics to the parasite-derived protein used here, opening the way for site-directed mutagenesis studies of its ligand specificity, structure, and heat stability.

Infection with *Ascaris* can result in a biomass of parasites which is unsurpassed in nematode infections. This, combined with the fact that ABA-1 is the most abundant protein present in the internal fluid of *Ascaris*, means that the potential for the parasite to sequester dietary retinoids would be substantial and may therefore relate to vitamin A deficiency ascribed to ascariasis in humans (Nesheim, 1989). Moreover, *Onchocerca volvulus*, the causative agent of river blindness, is noted for its accumulation of large quantities of retinoids (Sani et al., 1985), and retinoid binding proteins of the parasite are reported to bind ivermectin, the most potent anthelmintic currently in clinical use (Sani & Vaid, 1988). The binding of the drug to ABA-1-type binding proteins may therefore be crucial to its distribution and activity within the respective parasites.

ACKNOWLEDGMENT

We are indebted to Ian Davidson of the Protein Sequencing facility at the University of Aberdeen for carrying out the protein hydrolysis of ABA-1 and to Isla Kennedy for technical help.

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BI942649K