The Ov20 Protein of the Parasitic Nematode *Onchocerca volvulus*

A STRUCTURALLY NOVEL CLASS OF SMALL HELIX-RICH RETINOL-BINDING PROTEINS*

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Ov20 is a major antigen of the parasitic nematode *Onchocerca volvulus*, the causative agent of river blindness in humans, and the protein is secreted into the tissue occupied by the parasite. DNA encoding Ov20 was isolated, and the protein was expressed in *Escherichia coli*. Fluorescence-based ligand binding assays show that the protein contains a high affinity binding site for retinol, fluorescent fatty acids (11-((5-dimethylaminonaphthalene-1-sulfonyl)amino)undecanoic acid, dansyl-ΔL-α-aminoacaprylic acid, and parinaric acid) and, by competition, oleic and arachidonic acids, but not cholesterol. The fluorescence emission of dansylated fatty acids is significantly blue-shifted upon binding in comparison to similarly sized β-sheet-rich mammalian retinol- and fatty acid-binding proteins. Secondary structure prediction algorithms indicate that a α-helix predominates in Ov20, possibly in a coiled coil motif, with no evidence of β structures, and this was confirmed by circular dichroism. The protein is highly stable in solution, requiring temperatures in excess of 90 °C or high denaturant concentrations for unfolding. Ov20 therefore represents a novel class of small retinol-binding protein, which appears to be confined to nematodes. The retinol binding activity of Ov20 could possibly contribute to the eye defects associated with onchocerciasis and, because there is no counterpart in mammals, represents a strategic target for chemotherapy.

Infection with *Onchocerca volvulus* can result in perhaps the most distressing of diseases caused by parasitic nematodes, river blindness. The infection is a major cause of eye pathology in areas in which the parasite is endemic, and degenerative and disfiguring skin conditions also result (1). One of the first indications of eye damage in onchocerciasis is night blindness (1), a symptom which would be consistent with a deficiency in available retinoids. The parasites are known to accumulate retinol to a concentration an order of magnitude greater than the tissue they occupy (2, 3), and retinol-binding proteins (RBPs) from *O. volvulus* have also been reported to bind ivermectin, the drug which is the cornerstone of the program for the eradication of the infection (2, 3). RBPs are, therefore, potentially relevant to the survival of the parasite, the development of blindness in sufferers, and to therapeutic interventions against the infection.

Retinol is usually transported and protected from chemical degradation in intracellular and extracellular aqueous environments within carrier proteins. The retinol-binding proteins described to date are all β-strand-rich proteins which form β-barrel structures having a single binding site for retinol in their interiors. These proteins can be further subdivided into the predominately extracellular proteins with eight β-strands (the lipocalins), which include plasma retinol-binding protein and milk lactoglobulin (4), and the predominately intracellular 10 β-stranded FABP/P2/CRBP/CRABP family of hydrophobic ligand transporter proteins (5, 6). Members of each family bind retinoids and/or fatty acids and have been described throughout the animal phyla, ranging from vertebrates to insects and flatworms (7–9). Other proteins with retinol binding activities include interphotoreceptor retinol-binding protein, which is confined to the interphotoreceptor matrix (sub-retinal space) of the retina, and serum albumin, although retinol binding is thought to be a minor function for albumin in *vivo*, and both of these are substantially larger and more complex in structure than the β-barrel proteins.

Here we describe a new class of helix-rich retinol-binding protein, which is exemplified by the Ov20 protein of *O. volvulus*. Ov20 originally attracted attention as a glycoprotein secreted by the parasite and has been used in immunological analyses of human immune responses to the infection (10, 11). Genes encoding Ov20-like proteins have also been found in several other parasitic nematodes that are highly pathogenic in humans, and in a plant parasite.2 Ov20 is shown to possess strong retinol binding, but it is distinct from all the other known RBPs in terms of its structure and the characteristics of its binding site.

The abbreviations used are: RBP, retinol-binding protein; DACA, dansyl-ΔL-α-aminoacaprylic acid; dansyl, dimethylaminonaphthalene-1-sulfonyl; DAUDA, 11-((5-dimethylaminonaphthalene-1-sulfonyl)amino)undecanoic acid; FABP, fatty acid-binding protein; GC, gas chromatography; MS, mass spectrometry; GdnHCl, guanidine hydrochloride; retinol, all-trans-retinol; NPA, nematode polyprotein allergen/antigen; rOv20, recombinant Ov20 protein produced in *E. coli*; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

1 The costs of publication of this article were defrayed in part by the payment of page charges. This paper is available on line at http://www.jbc.org
2 Unpublished data base entries.
**EXPERIMENTAL PROCEDURES**

Production of Recombinant Ov20 (rOv20) Polypeptide—DNA encoding Ov20 was produced by PCR amplification and cloned into pET-15b (Novagen, Madison WI) with an intermediate cloning step into pUC-18. Oligonucleotide primers used for PCR were 5'-CTC CAT GGA AAT GGT GTT CCG TTT TC-3' and 5'-CTC GGA TTA ATG TTT TCC GCC ACC-3' designed to generate a protein starting from the predicted cleavage site for the signal peptide, Asn^{22}. The PCR product was amplified from an *O. volvulus* cDNA library made using worms obtained from Mali (10, 11). It was cloned into *Escherichia coli* host cell DH5a, in which the recombinant plasmid was maintained. For expression the plasmid was transferred into the strain BL21(DE3), and expression and purification of the protein was performed according the the manufacturer's instructions for high yield (Novagen, Madison, WI). Preliminary experiments showed that the biochemical properties of Ov20 bearing the 6xHis affinity tag and protein in which the tag had been removed with thrombin were indistinguishable. In the interests of reducing manipulation, therefore, all the assays reported here were carried out with the 6xHis tag fusion protein. Residual detergent was removed from solutions of Ov20 by passage through an Extracti-Gel D column (Pierce).

**Spectrofluorimetry and Fluorescence-based Ligand Binding—**Fluorescence emission spectra were recorded at 20 °C with a SPEX FluorMax spectrofluorimeter (Spx Industries, Edison, NJ), using 2-ml samples in a silica cuvette. Raman scattering by solvent water was corrected for where necessary using appropriate blank solutions. The fluorescence of the fatty acid analogs of dansylated fatty acids were stored as stock solutions of approximately 52,480 Mngml⁻¹, and parinaric acid were 345, 345, 350, and 319 nm, respectively. The excitation wavelengths used for DAUDA, DACA, retinol, oleic acid, and 5-[dimethylamino]naphthalene-1-sulfonamide (dansylamide) were also obtained from Sigma, and cis-[14C]-retinylamino-lactoglobulin was a kind gift of Dr. Bruce Hudson of the University of Oregon. The excitation wavelengths used for DAUDA, DACA, retinol, and dansylamide were 345, 345, 345, and 319 nm, respectively. The dansylated fatty acids were stored as stock solutions of approximately 1 mg ml⁻¹ in ethanol, in the dark at −20 °C, and freshly diluted in phosphate-buffered saline (PBS; 171 mM NaCl, 3.35 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄; pH 7.2) to 1 μM before use in the fluorescence experiments. Competitors of fluorescent fatty acid binding were prepared as stock solutions in ethanol at approximately 10 μM and diluted in PBS or ethanol for use. Free retinol is poorly soluble and unstable in aqueous solution, so was dissolved and diluted in ethanol immediately before use, and binding to proteins was tested by addition of typically 5 μl of this directly to a cuvette containing protein in PBS. The following reference proteins were obtained from Sigma and prepared as stock solutions at 10 mg ml⁻¹ in PBS: BSA, β-lactoglobulin (bovine), ribonuclease A, ovalbumin (chicken), and transferrin (bovine). The following reference proteins were obtained from Sigma and pre- pared as stock solutions at 10 mg ml⁻¹: bovine serum albumin (BSA), β-lactoglobulin (bovine), ribonuclease A, ovalbumin (chicken), and transferrin (bovine). The Ov20 sequence was compared with the SWISSPROT and PIR protein sequence data bases using the BLAST network at EPFL, Lausanne, to locate the best complete and local sequence similarities. All the protein sequences were also compared with the sequence patterns stored in the PROSITE data base (9) to search for potential functional domains on the basis of amino acid sequence. The Signap program (22) was used to predict position of any leader/signal peptides, using neural networks trained on eukaryotic and prokaryotic leader sequences. We found that this program accurately predicted the cleavage site for a nematode fatty acid-binding protein which is different from Ov20 (8). The following programs were used to analyze the sequences for predicted regions of coiledcoil: PECOIL in the GCG package, run on SEQNET, and Paircoil and Coils (23, 24), run through SwissProt. FSORT was used to predict protein localization and sorting signals (25), and was also run through SwissProt.

**RESULTS AND DISCUSSION**

The cDNA encoding Ov20 of *O. volvulus* predicts a 20580.6-Da protein with a hydrophobic leader which is presumably removed posttranslationally (11). The Signap program, trained on eukaryotic sequences, predicts that this cleavage should occur between Ala^{15} and Asn^{17} to give a 18781.2-Da polypeptide, assuming no further modifications. DNA encoding Ov20 was amplified by PCR from cDNA using primers designed to exclude the hydrophobic leader, and recombinant protein with a 6xHis affinity tag plus 20 vector-encoded amino acids, was produced in bacteria and purified as detailed under “Experimental Procedures.” The recombinant polypeptide (rOv20; molecular mass 20944.6) was judged to be homogeneous on the basis of a single band on SDS-polyacrylamide gel electrophoresis. Data base searching revealed no proteins of known function similar to Ov20, but a short N-terminal amino acid sequence of a retinol-binding protein from the dog heart worm *Dirofilaria*
immitis showed similarities to the Ov20 sequence. The beginning of this sequence aligns exactly at the predicted cleavage site for the removal of the hydrophobic leader in Ov20.

Ligand Binding—The retinol binding activity of rOv20 was investigated using changes in retinoid fluorescence that occur upon interaction with binding proteins (26). The fluorescence emission of retinol was minimal in buffer alone, but was dramatically enhanced when added to a solution of Ov20 (Fig. 1). Similar changes were observed with bovine milk lactoglobulin, human serum retinol-binding protein, and recombinant ABA-1 allergen of Ascaris lumbricoides (data not shown), all of which have well described retinol binding activities (26). Transferrin, ovalbumin, or RNase had no effect on retinol fluorescence (data not shown). The fluorescence enhancement brought about by Ov20 was reversed upon addition of oleic acid, presumably through displacement of retinol from a protein-binding site into solvent. This therefore indicated that Ov20, in common with many retinol-binding proteins, will also bind fatty acids and that the binding sites for retinol and fatty acids are coincident or interact competitively.

The dissociation constant of retinol:Ov20 binding was estimated by fluorescence titration in which increasing quantities of retinol were added to an rOv20 solution in the cuvette. Fig. 2 shows the dissociation curve, corrected for free retinol, consistent with a single retinol-binding site per molecule of rOv20 (assuming monomeric dispersion), which provided a consistent with a single retinol-binding site per molecule of rOv20 (assuming monomeric dispersion), which provided a dissociation constant, $K_d = 8.5 \times 10^{-8}$ M. The line for free retinol was obtained with increasing concentrations of retinol in PBS in the absence of protein, and the best fit line is shown. This was used to correct for free retinol to produce the corrected curve. The line drawn for the corrected data is the theoretical binding curve for complex formation with a dissociation constant, $K_d = 8.5 \times 10^{-8}$ M, and apparent stoichiometry $n = 0.8$ per monomer unit.

![Fig. 1](image1.png)  
**FIG. 1.** Retinol binding activity of rOv20 and competitive displacement by oleic acid. Fluorescence emission spectra (uncorrected) of 0.35 $\mu$M retinol in PBS or when added to 3.55 $\mu$M rOv20. The effect of addition of 5.7 $\mu$M oleic acid to the retinol:Ov20 complex is also shown. $\lambda_{exc} = 350$ nm.

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The fatty acid binding activity of purified Ov20 was examined using solvent polarity-sensitive fluorescent lipid analogues. The fluorescence emission intensity of DAUDA is increased and shifted to shorter wavelengths when bound to fatty acid-binding proteins (FABPs) such as rat liver FABP (27). In buffer alone, the peak emission of DAUDA occurred at 541 nm, but moved to 485 nm upon addition of rOv20 (Fig. 3), together with a marked increase in emission intensity. Blue shifts of this magnitude are taken as indicative of entry of the fluorophore into a highly apolar environment and removal from collisional contact with water (28). This blue shift was greater than that reported for serum albumin (495 nm) (26) and rat liver FABP (500 nm) (27), and approached that reported for DAUDA binding to the ABA-1 fatty acid-binding protein of

![Fig. 2](image2.png)  
**FIG. 2.** Titration curve for the binding of retinol to rOv20. Change in relative fluorescence intensity at 470 nm of 1.06 $\mu$M rOv20 monomer on addition of increasing concentrations of retinol. $\lambda_{exc} = 350$ nm. The line for free retinol was obtained with increasing concentrations of retinol in PBS in the absence of protein, and the best fit line is shown. This was used to correct for free retinol to produce the corrected curve. The line drawn for the corrected data is the theoretical binding curve for complex formation with a dissociation constant, $K_d = 8.5 \times 10^{-8}$ M, and apparent stoichiometry $n = 0.8$ per monomer unit.

![Fig. 3](image3.png)  
**FIG. 3.** Binding of DAUDA to recombinant rOv20 and competition with fatty acid. Fluorescence emission spectra ($\lambda_{exc} = 345$ nm) of 0.64 $\mu$M DAUDA alone or upon addition of 1.84 $\mu$M rOv20. Also shown is the reversal of changes in DAUDA emission by competition with oleic acid (5.7 $\mu$M) added to the rOv20-DAUDA complex. The wavelengths of peak emission by DAUDA when in PBS or when bound to the protein are as indicated.

Ascaris (475 nm) (26). Control experiments with dansylamide revealed minimal binding to rOv20, indicating that the fluorophore group itself does not contribute to the binding of the dansylated fatty acid.

Similar blue-shifts in fluorescence emission (from 543 to 483 nm) upon interaction with Ov20 were found with a fluorescent fatty acid probe in which the dansyl fluorophore is attached to the $\alpha$ carbon (dansyl-OL-$\alpha$-aminocaproic acid), rather than to the hydrocarbon ($\omega$) terminal, as in DAUDA (data not shown). Assuming that the binding of both the dansylated fatty acids is representative of natural ligands, this similarity in behavior of the two probes indicates that the ligand is held entirely within the binding site of rOvA-1L and isolated from polar solvent. X-ray crystallographic studies of the $\beta$-barrel retinol- and fatty acid-binding proteins show similar isolation of ligand from

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3 T. V. Rajan, personal communication.
solvent (4, 6) (with exception of liver fatty acid-binding protein, which holds one of two ligand molecules close to the surface of the protein) (29), which would serve to protect oxidation-sensitive ligands, such as retinol and other hydrophobic ligands during transport within an organism or cell.

Fluorimetric titration of rOv20 with DAUDA (Fig. 4A) gave a progressive increase in relative fluorescence intensity, with an apparent dissociation constant ($K_d$) of $9.7 \times 10^{-7}$ M. This value approximates that obtained for other lipid-binding proteins (30), and a similar value was obtained for progressive addition of a natural, nonfluorescent fatty acid (oleic acid) to a rOv20:DAUDA mixture ($K_f$ apparent = $3.6 \times 10^{-7}$ M; Fig. 4B). A titration with the naturally fluorescent fatty acid parinaric acid gave a value of $8.6 \times 10^{-7}$ M, which is of the same order of magnitude to the values for DAUDA and oleic acid. Competition was also used to test for binding with other natural ligands, which showed that arachidonic acid, but not cholesterol, binds to rOv20 (data not shown).

To examine the hydrophobic ligands that bind to Ov20 in a biological context, a sample of Ov20 which had been affinity-purified as described under “Experimental Procedures,” but with no further treatment, was subjected to extraction with ethyl acetate, and the extracted material was subjected to GC-MS. The mass spectra revealed single ions consistent with the presence of palmitoleic, palmitic, heptenoic, oleic, and stearic acids. No evidence for significant amounts of retinol was found, which would be consistent with the fact that E. coli does not synthesize retinoids.

**Structural Analysis and Stability**—The far UV CD spectrum of Ov20 is illustrated in Fig. 5A, and shows a strong $\alpha$-helix signal. Analysis of the data over the range 190 to 240 nm by the CONTIN procedure yielded the following estimates of secondary structure: $60 \pm 1.3\%$ $\alpha$-helix, $32 \pm 1.6\%$ $\beta$-sheet, and the remainder $8 \pm 2.7\%$. In our experience, the application of the CONTIN procedure to proteins with a significant content of $\alpha$-helix can overestimate the $\beta$-sheet content considerably (31), but the estimate of the $\alpha$-helix is reliable. Addition of increasing concentrations of GdnHCl led to a progressive loss in secondary structure as detected by CD (Fig. 5B), with the greater part of the unfolding occurring between 3 and 4 M GdnHCl. Ov20 therefore appears to be more robust to this means of denaturation than are many proteins, including the ABA-1-type proteins (26, 32), although detailed quantification of the stability of Ov20 and its reversibility remains to be carried out.

This conformational stability was confirmed in the absence of denaturants by differential scanning calorimetry experiments which indicated an endothermic unfolding transition $T_m$ in excess of 90 °C, but accompanied by exothermic irreversible aggregation for Ov20 in PBS, which precludes any detailed thermodynamic analysis (data not shown). This would indicate that the stability of the protein to chemical denaturation is mirrored by extreme thermal stability, which is also in excess of that exhibited by the ABA-1-type proteins (26, 32).

**Structural Predictions**—An alignment of the amino acid sequence of Ov20 with similar proteins of other nematodes (animal-parasitic, plant-parasitic, and free-living) is given in Fig. 6. No significant matches to other proteins were found from data base searching. Secondary structure prediction algorithms applied to the whole alignment predicted a highly helical confor-
A Structurally Novel Retinol-binding Protein from Nematodes

**Fig. 6. Structural predictions for Ov20 and its homologs.**

| Ov20 | 1 | myhglilma...ligvima | NUVFVSMN1 | PEEKKGFLPE | EVKMPFVNT1 |
| Sm20 | 2 | myhrrilla...lyvttma | NVFVSN21 | PEEYKGFIPF | EVRKFVYDF1 |
| PCN | 3 | mgrllictgy asfvlf1ga | SFPPFFSI1 | SEQYRLKF | EVIDPFTNT1 |
| C1e | 4 | miratlilaa vaalafspAVP | PEFPVPD11 | PEPYKLYLA | EYVEHSLST1 |
| C2e | 5 | miraflylva asvafspFI | PEPYFQPD11 | PEPYKLYLA | EYVEHSLST1 |
| Consensus | M...r...i...la.... | a...s..l...v...a... | . . . | . . . | . . . |
| PHD | LHHHHHHHH | HHHHHH | ...L.L.L.H | HHHHHHHHH | HHHHHHHHH |

**Table 1.**

| Ov20 | 1 | 50 |
| Sm20 | 2 | 101 |
| PCN | 3 | 150 |
| C1e | 4 | 151 |
| C2e | 5 | 190 |

**Table 2.**

| Ov20 | 1 | 50 |
| Sm20 | 2 | 101 |
| PCN | 3 | 150 |
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**Fig. 6.** Structural predictions for Ov20 and its homologs. A, alignment of Ov20 (GenBank™ accession no. L276866) with putative homologs from the human lymphatic filariasis parasite *B. malayi* (Bm20, GenBank accession no. U69169), the potato cyst nematode *Globodera pallida* (PCN, GenBank™ accession no. Y09293), and two sequences from *C. elegans* (Ce1 and Ce2, sequences F02A9.2 and F02A9.3, clone CEL F02A9, GenBank™ accession no. Z19555). The amino acids of the presumptive hydrophobic leader/signal peptide are shown in lowercase letters. The alignment was generated using the MultiAlin program through SwissProt set for the Dayhoff comparison matrix. In the consensus line, uppercase letters refer to conservation of amino acid position across the entire array, and lowercase letters refer to cases where that amino acid occurs in that position more than half of the sequences. Other symbols are as follows: % is for either F or Y, and # is for anyone of NDQEBZ. The PHD line shows the secondary structure prediction from submission of the multiple alignment to the PHD secondary structure prediction program; H = helix, L = loop, * = no prediction; no extended/b struct was predicted), and the final line indicates conserved hydrophobic (+) and hydrophobic (●) positions. Consensus N-glycosylation sites are underscored and the consensus casein kinase phosphorylation site is in boldface type.

**Fig. 7.** When plotted on helical wheels, many of the predicted helical stretches can be seen to be strongly amphipathic. This applies both for the hydrophobic residues occurring in Ov20, and, even more dramatically, for hydrophobic sites conserved across the entire array. This is exemplified by the regions of highest probability helix, from Pro27 to Thr62, and from Asp78 to Arg117, and from Met 126 to Ala153.

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stage of the nematode *Ascaris suum*, and a DNA sequence encoding similar proteins has arisen from the *C. elegans* genome project (8). Why it is that nematodes produce highly helical lipid-binding proteins such as Ov20 and the ABA-1-like helical protein (42), might be descended from proteins similar to the nematodes and vertebrates, and that the helix-rich proteins have been lost in the latter, although it remains conceivable that serum albumin, which is helix-rich and that the helical RBPs/FABPs were produced by common ancestors to the nematodes and vertebrates, and that the helix-rich proteins have been lost in the latter, although it remains conceivable that serum albumin, which is helix-rich and thought to have arisen through multiplication of an original helical protein (42), might be descended from proteins similar to Ov20 or the NPAs.

The function and true in vivo binding propensities of Ov20 remain to be established, although its secretion into the tissues of the infected host could be important to survival of the parasite. It is possible, for example, that it is released to sequester retinol, and the *holo* protein might then be reabsorbed by the parasite to contribute to the considerable retinol store accumulated by *O. volvulus*; there is evidence that retinol is essential for the metabolism, development, and reproduction of the parasite (2, 3). Moreover, reduction in available retinol could also have immunomodulatory effects on the host; retinol-deficient animals are known to have an impaired immune response to parasitic nematodes (44); and vitamin A deficiency is thought to contribute to the pathogenesis of the infection (1). Local inflammatory and immune mediators such as prostaglandins and leukotrienes operate at sufficiently low concentrations for a binding protein to influence their function (45, 46). With respect to this, we found that Ov20 binds arachidonic acid, which is itself an inflammatory mediator in addition to being the precursor for prostaglandins, leukotrienes, and thromboxanes (45, 46). The fact that Ov20 appears to have no counterpart in the mammalian host is encouraging in that it could provide a novel therapeutic target for onchocerciasis therapy. It may be relevant here that an approximately 19-kDa retinol-binding protein from *O. volvulus* is reported to bind ivermectin, currently the cornerstone drug for the treatment of onchocerciasis (2, 3, 47).

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