

Ligand Specificity of Brain Lipid-binding Protein*

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Brain lipid-binding protein (BLBP) is a member of the fatty acid-binding protein (FABP) family. Although BLBP expression in the developing central nervous system is complex, a close correlation between its expression and radial glial differentiation has been observed. Furthermore, antibodies to BLBP can block glial cell differentiation in mixed primary cell cultures. Here we describe the ligand binding properties of BLBP. The binding affinities of BLBP for oleic acid ($K_d \sim 0.44 \mu\text{M}$) and arachidonic acid ($K_d \sim 0.25 \mu\text{M}$) are similar to those reported for other FABPs, but BLBP does not bind to palmitic acid or arachidinic acid. These and other experiments establish that BLBP has a strong preference for binding long chain polyunsaturated fatty acids. A probable *in vivo* ligand for BLBP is docosahexaenoic acid (DHA), since its binding affinity ($K_d \sim 10 \text{ nm}$) is the highest yet reported for an FABP/ligand interaction, exceeding even the affinity of retinoic acid for its binding proteins. Furthermore, the requirement of DHA for nervous system development and the coincident expression of BLBP during these developmental stages suggest that the physiologic role of BLBP may involve DHA utilization. Finally, we present a structural model of BLBP/DHA interaction that provides insight into both the structural characteristics important for ligand binding and the effects of specific mutations upon BLBP/ligand interactions.

Brain lipid-binding protein (BLBP)¹ is a brain-specific member of the fatty acid-binding protein (FABP) family that is expressed at high levels in the developing central nervous system (1–3). While the function of BLBP is not clear, several lines of evidence suggest that it plays an important role in neuron/glial interactions during central nervous system development. Thus, the presence of BLBP in both the nucleus and cytoplasm of expressing cells (2) and its dynamic regulation in glial cells in response to neurons (4) both indicate that BLBP may participate in a signaling pathway that is critical for the response of glia to differentiating neurons. Furthermore, the

ability of anti-BLBP antibodies to inhibit glial process extension in response to neurons in primary cell cultures prepared during the first postnatal week is consistent with this idea (2). Given these observations, we have been interested in understanding both the regulatory pathways responsible for the dynamic pattern of BLBP expression in the developing central nervous system and its functional role in differentiating glial cells.

The high similarity between BLBP and previously characterized FABPs (2) strongly suggests that the function of BLBP in the developing nervous system involves binding to a small hydrophobic ligand, possibly a fatty acid or a fatty acid metabolite. Furthermore, the demonstration that cellular retinoic acid-binding protein (CRABP) transcription is regulated by retinoic acid (5) and the dynamic response of BLBP transcription to neurons *in vivo* and *in vitro* (4) suggests that the putative BLBP ligand might also be critical for regulation of BLBP transcription in the developing central nervous system. To understand the role of BLBP, and to investigate the potential mechanisms of its transcriptional control, we have initiated studies aimed at identifying the BLBP ligand. In this study, we present a detailed biochemical characterization of BLBP binding to a variety of potential ligands, as well as a structure/function analysis of the BLBP binding pockets to identify a physiological ligand for BLBP. We report that the binding specificity of BLBP to common fatty acids is different than that of other FABPs, since it will not bind palmitic acid or arachidinic acid. We suggest that DHA is the physiological ligand for BLBP, since the affinity of this interaction ($K_d \sim 10 \text{ nm}$) is the highest yet reported for a FABP/ligand interaction. Finally, we present a structural model for BLBP/DHA interaction that provides insight into both the chemical characteristics of the ligand that are important for BLBP binding, and some of the key amino acids in the BLBP binding pocket that we demonstrate to be critical for this interaction. These studies provide strong evidence that the role of BLBP in the developing nervous system may be closely related to the essential requirement for DHA in central nervous system development *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—Lipidex 1000 and fatty acids were from Sigma. All radioactive materials (oleic acid, palmitic acid, arachidonic acid, retinoic acid, and DHA) were from DuPont NEN.

Construction of pET Expression Plasmids for BLBP and Its Various Mutants—A 0.9-kilobase pair murine BLBP cDNA clone was cloned into the pET3a vector between the *Nde*I and *Bam*HI restriction sites (2). The mutant constructs were generated by PCR mutagenesis methods using two consecutive PCR reactions according to Ref. 6. First, the forward primer and the mutant primer were used in the first amplification reaction. These PCR products were then used as megaprimer paired with the reverse primer in a second PCR reaction. The products of this reaction were subcloned into pET3a expression plasmids using *Xba*I and *Bam*HI sites. Mutant clones were identified by DNA sequence. The authenticity of all plasmid constructs and fidelity of the entire coding sequence was determined by dideoxy chain termination sequencing with modified T7 DNA polymerase. The mutant primers were used as follows.

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¹ The abbreviations used are: BLBP, brain lipid-binding protein; FABP, fatty acid-binding protein; CRABP, cellular retinoic acid-binding protein; ALBP, adipocyte lipid-binding protein; DHA, docosahexaenoic acid; OA, oleic acid; PA, palmitic acid; AA, arachidonic acid; PCR, polymerase chain reaction; LCPUFA, long chain polyunsaturated fatty acid; M-FABP, muscle FABP.

Gly-33 → Phe: 5'-TTG GTC ACG TTg aaC ACT TGC CT-3'
 Gly-33 → Ile: 5'-TTG GTC ACG TTT atC ACT TGC CT-3'
 Phe-57 → Ala: 5'-CAA TGC ACA gcc AAG AAC ACA GAG-3'
 Arg-106 → Ala: 5'-TGT ACC gca GAA ATT AAG GAT GGC-3'
 Arg-126 → Ala: 5'-AGC CGG ATC CTA TGC CTT TTC ATA ACA ggc AAC AGC-3'

SEQUENCES 1-5

Expression and Purification of BLBP and Its Various Mutants—The expression of BLBP was accomplished using the T7 RNA polymerase based system of Studier and Moffat (7). Cells containing pET3a BLBP were induced at 30 °C for 24 h and harvested. Cell pellets were then lysed by French press. The crude extracts containing BLBP were fractionated sequentially by 30%, 60%, and 90% ammonium sulfate precipitations; the 90% pellet was dissolved in a 1-ml buffer (20 mM Tris and 2 mM EDTA, pH 8.0), and then loaded on a 100-ml G50 superfine column. BLBP elution from this column resulted in nearly homogeneous BLBP, as shown in Fig. 1. All mutant proteins (Arg-107 → Ala, Arg-126 → Ala, Gly-33 → Ile, Gly-33 → Phe, and Phe-57 → Ala) were purified to near homogeneity using the same procedure.

Fatty Acid Binding Assays—Purified proteins were delipidated over a Lipidex 1000 column at 37 °C as reported previously (8, 9). The purified BLBP and various concentrations of oleic acid (OA) at constant ratio of hot and cold were incubated in a total volume of 500 μ l containing 50 mM potassium phosphate (pH 7.0) and 1 mM EDTA at 37 °C for 15 min. The reactions were incubated at 4 °C for an additional 15 min and loaded on 2-ml Lipidex 1000 columns. The bound oleic acid and proteins were eluted with 2 ml of 50 mM KP_i buffer (pH 7.0). The free fatty acids bound in the column were eluted with 3 ml of methanol. Both fractions were measured in liquid scintillation counter. A typical range of 0.01–10 μ M oleic acid with 10 different concentrations were used to measure the binding activity. Each binding measurement was repeated at least three times. Binding reactions containing the 100 mM Tris buffer (pH 8.0) instead of protein were used as a control. Binding of BLBP to arachidonic acid, DHA, palmitic acid, and retinoic acid was performed over Lipidex 1000 columns, except that a different range of concentrations were used as indicated in the figure legends. For each fatty acid ligand, the asymptotic maximal concentration of the bound ligand, B_{\max} , and dissociation constant, K_d , were obtained by least-squares fitting the following model to the data (program SigmaPlot was used), where F is free fatty acid concentration and B is bound fatty acid concentration.

$$B = B_{\max} \times F / (K_d + F) \quad (\text{Eq. 1})$$

The inhibition constants, K_i , were obtained from Equation 2, where K_d is the dissociation constant at zero inhibitor concentration and K_d' is the apparent dissociation constant at non-zero inhibitor concentration $[I]$, also obtained from Equation 1.

$$K_i = \frac{[I]}{K_d' / K_d - 1} \quad (\text{Eq. 2})$$

The free energy change of binding was calculated as shown by Equation 3, where R is the gas constant (8.31 J/K mol) and T is absolute temperature (310.15 K).

$$\Delta G = -RT \ln K_d^{-1} \quad (\text{Eq. 3})$$

Protein concentrations were measured by the Bradford method using bovine serum albumin as a standard.

Structural Modeling—Three-dimensional models of the wild-type BLBP, three single BLBP mutants (Gly-33 → Ile, Gly-33 → Phe, Phe-57 → Ala), and BLBP complexes with various lipid ligands (oleic acid (OA), palmitic acid (PA), arachidonic acid (AA), and docosahexaenoic acid (DHA)) were built automatically by the computer program MODELLER-3 (10, 11).² MODELLER-3 implements comparative modeling by satisfaction of spatial restraints (10). The input to MODELLER-3 was a multiple alignment of BLBP with FABPs of known three-dimensional structure. This alignment was prepared by hand because of the high sequence similarity between BLBP and other FABPs (e.g. sequence identity between BLBP and M-FABP is 62%). First, MODELLER-3 derived many distance and dihedral angle restraints on the BLBP sequence and/or ligands from their alignment with the template

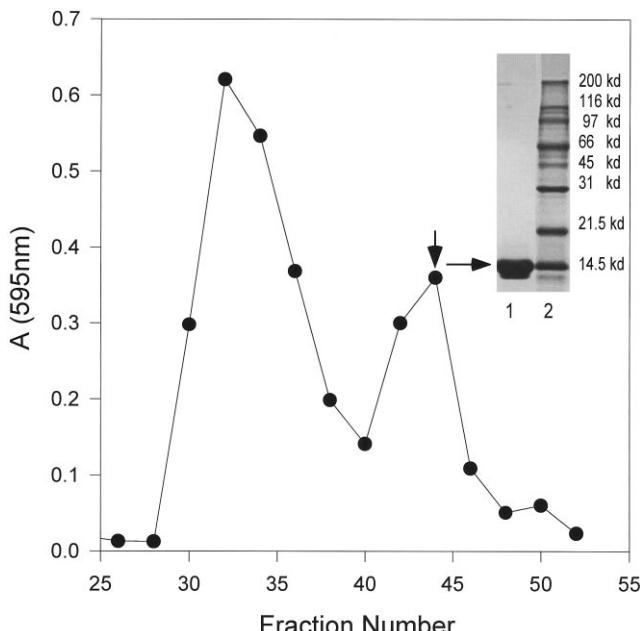


FIG. 1. Purification of recombinant BLBP. Recombinant BLBP was expressed and partially fractionated by ammonium sulfate precipitation as described under “Experimental Procedures.” The final pellets were dissolved in 1 ml of 100 mM Tris buffer (pH 8.0) and loaded on the 100-ml G50 superfine column. The elution profile of total protein from the G50 column is shown above. BLBP elutes between fractions 42 and 46 as a relatively homogeneous preparation. As shown in the inset, SDS-PAGE of fraction 44 reveals no major contaminants in the BLBP eluting from this column (lane 1). Molecular markers from top to bottom are 200, 116, 97, 66, 45, 31, 21.5, and 14.5 kDa (lane 2).

proteins and/or protein-ligand complexes. Next, these homology-derived spatial restraints and CHARMM-22 energy terms (12) enforcing proper stereochemistry were combined into an objective function. Finally, the variable target function procedure, which employs methods of conjugate gradients and molecular dynamics with simulated annealing, was used to obtain the three-dimensional models by optimizing the objective function. In each case, five slightly different three-dimensional models were calculated by varying the initial structure. The model with the lowest value of the objective function was selected as the representative model.

The template structure for BLBP and its mutants was M-FABP (13) because it has the highest sequence identity to BLBP of all the structurally defined FABPs. For the BLBP-AA and BLBP-OA complexes, the template structures were the adipocyte lipid-binding protein (ALBP)-AA (14) and M-FABP-OA complexes, respectively. ALBP-AA was used as the template because it is the only known complex with AA and has a high similarity to BLBP (56% sequence identity). The model for the BLBP-PA complex was obtained from the M-FABP-stearate complex (13) by eliminating the terminal two carbon atoms of stearate in the template structure. The BLBP-DHA complex was modeled using the ALBP-AA complex (14) as a template because DHA is closest to AA in length and the number of double bonds. DHA was built into the BLBP binding pocket mimicking the conformation of AA, followed by a relaxation of the ligand structure with 200 cycles of steepest descents and 1000 cycles of conjugate gradients energy minimization using DISCOVER (MSI, San Diego, CA); the protein non-hydrogen atoms were fixed during this minimization. All models contained water molecules found in the binding clefts of the corresponding template structures.

RESULTS

Expression and Purification of BLBP—One liter of bacteria containing the expression plasmid pET3a BLBP was induced

² MODELLER is available by anonymous ftp from guitar.rocketeer.edu and also as part of QUANTA and Insight II (MSI, San Diego, CA; E-mail: blp@MSI.com).

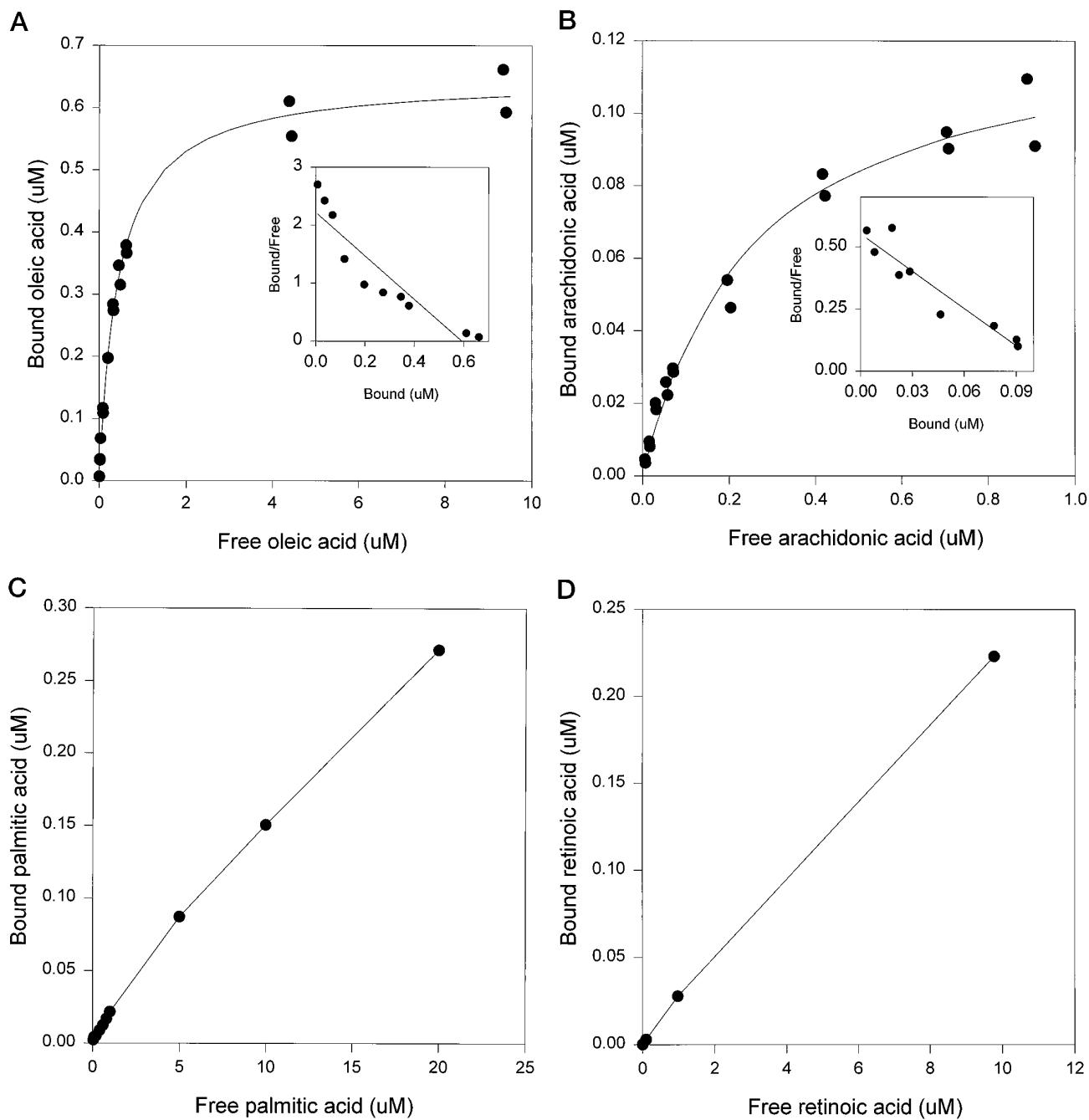


FIG. 2. Titration of fatty acid binding to recombinant BLBP. *A*, binding of oleic acid to BLBP. Ten different concentrations of oleic acid in the range of 0.01–10 μ M containing a constant proportion of 3 H-labeled oleic acid were used in binding assays with 5 μ g of recombinant BLBP as described under “Experimental Procedures.” Two independent sets of data are shown. The inset shows the Scatchard plot. *B*, binding of arachidonic acid to BLBP. Similar assays employing 10 different concentrations of arachidonic acid in the range of 0.01–10 μ M containing a constant proportion of 3 H-labeled arachidonic acid were done as described for *OA*. Two independent sets of data are shown, and the Scatchard plot is given in the inset. *C*, binding of palmitic acid to BLBP. Ten different concentrations of palmitic acid in the range of 0.01–20 μ M were used in binding assays with 5 μ g of recombinant BLBP. A representative set of data is shown, indicating no saturable binding of BLBP to PA. *D*, binding of retinoic acid to BLBP. Ten concentrations of retinoic acid in the range of 0.001–10 μ M containing a constant proportion of 3 H-labeled retinoic acid were used in binding assays with 5 μ g of recombinant BLBP. Again, no saturable binding of BLBP to retinoic acid was observed. In the top two main panels, the curves are least-squares fits of the model to the data (Equation 1).

at 30 °C overnight. Cells were harvested and lysed by French press. BLBPs were fractionated sequentially by 30%, 60%, and 90% ammonium sulfate precipitations. Pellets of 90% ammonium sulfate were dissolved in 1 ml of buffer and then were separated on a G50 superfine column. BLBP elutes in the second peak from the G50 column as expected for a monomeric 15-kDa protein. This second peak contains the nearly homogeneous BLBP, as shown in Fig. 1. That this protein is authentic BLBP was confirmed by Western blotting using rabbit anti-

BLBP antiserum (data not shown).

Ligand Binding Properties of BLBP Differ from Other Fatty Acid-binding Proteins—The binding affinities of several tissue-specific fatty acid-binding proteins for long chain fatty acids have been measured previously (8, 9, 15–17). In most cases, these proteins have been observed to bind oleic, arachidonic, and palmitic acids at approximately equal affinities with a K_d in the range of 0.2–1.0 μ M, though the adipocyte lipid-binding protein has a higher K_d of 4.4 μ M for arachidonic acid (17). To

TABLE I
Binding parameters of BLBP for several fatty acids

Ligand binding assays were performed in the presence and absence of competitors as described under "Experimental Procedures." The maximal bound concentration of the ligand, B_{\max} , dissociation constant, K_d , inhibition constant, K_i , and free energy of binding were obtained as described under "Experimental Procedures" (Equations 1–3). The means and standard deviations of three to five experiments are shown. NA, not applicable.

Ligands	K_d or K_i μM	B_{\max} $pmol/\mu g$	ΔG $kcal/mol$	Structure
Oleic acid	0.44 ± 0.027	107 ± 3.1	-9.02	C18:1(9C)
Arachidonic acid	0.25 ± 0.034	47.3 ± 2.3	-9.37	C20:4(5,8,11,14 allC)
cis-11-Eicosanoic acid	$0.21 \pm 0.09 (K_i)$	NA	NA	C20:1(11C)
Docosahexaenoic acid	$0.030 \pm 0.015 (K_i)$	NA	NA	C22:6(4,7,10,13,16,19 allC)
Docosahexaenoic acid	0.010 ± 0.002	56 ± 4.5	-11.35	C22:6(4,7,10,13,16,19 allC)

begin to address the biochemical roles of BLBP, we first asked whether BLBP showed binding properties similar to those of other FABPs. As shown in Fig. 2A and Table I, recombinant BLBP bound oleic acid with K_d of $\sim 0.4 \mu M$ and B_{\max} of $\sim 100 pmol/\mu g$, as expected from similar studies of other fatty acid-binding proteins (8, 9, 15, 16). Also as expected, binding of arachidonic acid to BLBP was determined to have K_d ($0.25 \mu M$) and B_{\max} ($47 pmol/\mu g$) similar to previously studied FABPs (Fig. 2B and Table I). However, unlike other FABPs, BLBP does not bind palmitic acid (Fig. 2C). Since this result was entirely unanticipated, a variation of the ligand binding assay was employed to confirm it as follows. Experiments in which a constant amount of radiolabeled palmitic acid was utilized with varying concentrations of cold palmitic acid as well as experiments utilizing a constant ratio of radiolabeled and cold palmitic acid while varying total PA concentration both failed to reveal saturable binding of palmitic acid to BLBP, even with $100 mM$ palmitic acid (data not shown). These experiments were repeated with several different preparations of palmitic acid and purified BLBP. In no case was significant specific binding observed, in contrast to the reproducible specific binding of BLBP to oleic and arachidonic acids. Since the binding specificity of BLBP revealed in these experiments differs from that of other previously studied FABPs, we checked for BLBP's ability to bind retinoic acid. As shown in Fig. 2D, a nonsaturable binding curve of retinoic acid to BLBP was observed, demonstrating that BLBP did not specifically bind retinoic acid, similarly to other previously studied FABPs (15, 16).

Given these unexpected results, we established a competition assay to measure BLBP binding to a variety of potential ligands in order to understand the structural requirements for ligand binding to BLBP and to identify high affinity ligands. This assay is based upon the ability of cold ligands to compete for binding of labeled oleic acid to BLBP. As shown in Fig. 3, a standard competition curve can be obtained using oleic acid itself as a competitor. As expected from the results of the direct binding assays (Fig. 2C) described above, palmitic acid failed to compete for specific binding of oleic acid to BLBP, even at the concentration of $100 \mu M$. $100 \mu M$ cold oleic acid almost completely abolished the binding of BLBP to 3H -labeled oleic acid (Fig. 3). These results establish that the ligand binding specificity of BLBP to common long chain fatty acids is similar to but distinguishable from that of other FABPs because it does not bind palmitic acid.

Ligand Binding Specificity of BLBP—To begin to understand the chemical properties that are important for BLBP ligand binding, the affinity of a variety of compounds for BLBP was assessed using the competition assay described above. In each case, a fixed concentration of oleic acid containing trace amounts of $[^3H]$ oleic acid ($0.2 \mu M$) was included in the reaction, and competition for BLBP binding was assessed at two different concentrations of test compound ($0.2 \mu M$ and $10 \mu M$). The results were normalized to reactions containing no competitor, and expressed as the percentage of these counts remaining in

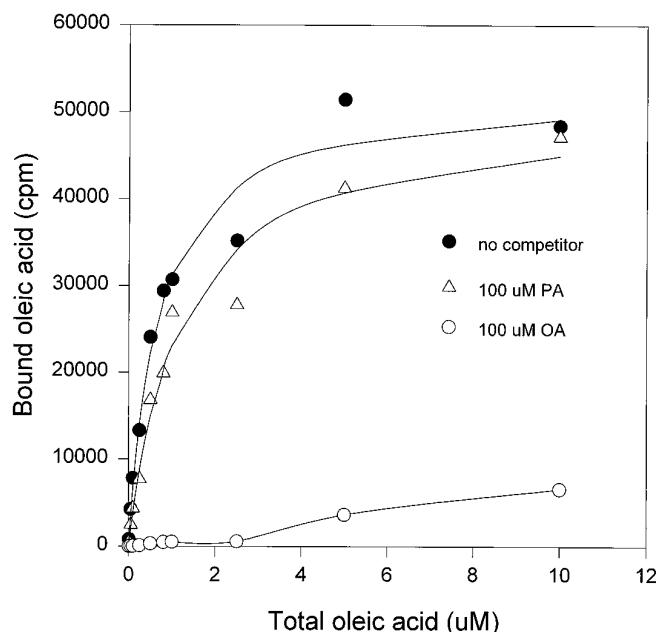


FIG. 3. Competition assays for OA and PA. Ten concentrations of oleic acid in the range of 0.01 – $10 \mu M$ containing a constant proportion of 3H -labeled oleic acid were used in binding assays with $5 \mu g$ recombinant BLBP. The points correspond to assays performed with no competitor (●), or with either $100 \mu M$ cold OA (○) or $100 \mu M$ cold PA (△) competitors. The first two curves are the least-squares fits of the model to the data (Equation 1); the model was not fitted to the third set of points because the relative error is too large.

the presence of the indicated concentration of test compound (Table II). As expected, the inclusion of cold oleic acid into the assay results in effective self-competition at both competitor concentrations. To test whether the configuration of the double bond is important for binding to BLBP, elaidic acid was used. This compound at $10 \mu M$ competed less effectively for ligand binding with 15.3% of the input counts remaining, as compared with oleic acid (7.7%). Since elaidic acid differs from oleic acid only in that its double bond is in the *trans* configuration, these results suggest that the *cis* configuration of the 9-double bond is preferred. Palmitic acid can not effectively bind BLBP, but palmitoleic acid is able to compete relatively effectively with 12.3% of the input counts/min remaining as compared to that of palmitic acid (82.7%). This suggests that an unsaturated bond is required for BLBP binding. Both arachidonic (6.4%) and 11-*cis*-eicosenoic acid (6.3%) were more effective than oleic acid (7.7%), whereas arachidinic acid (82.7%) did not effectively bind. Both α -linolenic acid and linoleic acid are good competitors. The failure of ethyl oleate to compete effectively for BLBP binding (86.2%) indicates that the negatively charged carboxyl group of fatty acids is an essential component of BLBP ligand binding. Taken together, these results demonstrate that longer chain, polyunsaturated fatty acids are preferred ligands for

TABLE II
Competition of fatty acids and their derivatives with [³H]oleic acid binding to BLBP

The ratio of the bound oleic acid in the presence and absence of a competitor is given for each competitor at 0.2 μ M (column 1) and 10 μ M (column 2) concentrations. The means and standard deviations of at least three experiments are shown. ND, not determined.

Competitor	0.2 μ M	10 μ M	Structure
None	100 \pm 4.5	100 \pm 4.5	
Oleic acid	78.4 \pm 2.4	7.7 \pm 0.2	C18:1(9C)
Elaidic acid	74.6 \pm 10	15.3 \pm 1.6	C18:1(9T)
Palmitolic acid	99.3 \pm 1.1	12.3 \pm 1.8	C16:1(9C)
Linoleic acid	63.0 \pm 5.1	7.8 \pm 0.6	C18:2(9C,12C)
α -Linolenic acid	64.6 \pm 4.9	7.5 \pm 0.4	C18:3(9C,12C,15C)
Arachidonic acid	62.6 \pm 1.0	6.4 \pm 0.4	C20:4(5C,8C,11C,14C)
cis-11-Eicosenoic acid	74.0 \pm 6.5	6.3 \pm 0.7	C20:1(11C)
Ethyl oleate	92.3 \pm 9.3	86.2 \pm 5.3	C18:1(9C)
Palmitic acid	103 \pm 2.2	82.7 \pm 5.3	C16:0
Arachidinic acid	83.5 \pm 0.5	68.9 \pm 3.9	C20:0
Retinoic acid	98.5 \pm 5.1	77.2 \pm 6.2	
Prostaglandin E ₂	98.9 \pm 7.3	ND	
Leukotriene B ₄	87.0 \pm 2.5	ND	
Leukotriene E ₄	113 \pm 9.4	ND	
Lipoxin B ₄	87.0 \pm 7.9	ND	
5-OH HETE	108 \pm 0.5	ND	
12-OH HETE	111 \pm 6.1	ND	
15-OH HETE	112 \pm 10	ND	
HETE standard mixture 2 (equal amounts 8-OH, 9-OH, and 11-OH)	110 \pm 15	ND	

BLBP binding.

The importance of arachidonic acid and its metabolic derivatives as signaling molecules prompted us to test for BLBP binding to several additional members of this family of compounds including prostaglandin E₂, leukotriene B₄, leukotriene E₄, lipoxin B₄, 5-OH HETE, 12-OH HETE, 15-OH HETE, and even a mixture of 8,9,11-OH HETE. In no case was significant competition with oleic acid binding observed. The combination of the following two factors prompted us to search for a ligand that binds BLBP with significantly higher affinity than OA or AA: 1) the proposed role of BLBP as a component of a signaling pathway similar to the retinoic acid pathway and 2) the fact that the affinity of retinoic acid for CRABP (K_d \sim 20 nm) is considerably stronger than that of oleic acid or arachidonic acid for BLBP (K_d \sim 300 nm).

Identification of DHA as a High Affinity Ligand for BLBP—Based on the structural characteristics required for fatty acid binding to BLBP, the abundance of DHA in the nervous system (18, 19), and the demonstration that DHA is required *in vivo* for timely development of the human nervous system (20), we chose to measure the binding of DHA to BLBP. As shown in Fig. 4A, DHA is a very effective competitor with oleic acid for binding to BLBP, demonstrating substantial competition at concentrations as low as 0.1 μ M and complete competition at 0.2 μ M. The K_i for DHA (0.03 μ M) is approximately 10-fold higher than either the K_m of oleic acid or arachidonic acid (see Table I). To confirm this very high affinity binding of DHA to BLBP, direct binding assays were performed as shown in Fig. 4B; the results of these assays demonstrate that the K_d for DHA binding to BLBP is \sim 10 nm with a B_{max} of \sim 56 pmol/ μ g. This is the highest affinity interaction yet observed between a fatty acid and its ligand-binding protein, exceeding even the affinities for retinoic acid binding to its carrier proteins (20 nm) (21).

Structural Characteristics of BLBP Required for Ligand Binding—The unique ligand specificity of BLBP and the abundance of structural information available for other members of the fatty acid-binding protein family suggested to us that further insight into BLBP/ligand interaction could be obtained from site-directed mutagenesis experiments combined with protein structure modeling. Both Arg-106 and Arg-126 are highly conserved among FABP family and CRABP family (22). Previous studies have shown that Arg-126 is important for binding of retinoic acid to CRABP II (21), while both Arg-126

and Arg-106 are important for binding of retinoic acid to CRABP I (22). Crystallographic studies of muscle fatty acid-binding protein (13) have shown that Arg-126 and Arg-106 contact the carboxylate group of fatty acid. Since our ligand competition studies with ethyl oleate (Table II) demonstrated that the carboxylate group is required for BLBP/ligand interaction, and since these two Arg residues are conserved in BLBP, we sought to confirm their contact with the carboxylate of the ligand. Thus, BLBP single point mutants in which each of these Arg residues was replaced with Ala were prepared (Arg-106 \rightarrow Ala, Arg-126 \rightarrow Ala) and tested for interaction with oleic acid. No detectable binding of Arg-106 \rightarrow Ala to oleic acid was observed in any assay, whereas Arg-126 \rightarrow Ala bound oleic acid with significantly reduced B_{max} of about 33-fold less than that of wild type (K_d \sim 1.2 μ M; B_{max} \sim 3.3 pmol/ μ g) (Table III and Fig. 5). These results are different from those for ALBP where mutation of Arg-126 to Gln did not change the K_d for 12-(9-anthroyloxy)-oleate and *cis*-parinaric acid (17). Our binding results are consistent with the three-dimensional model of the BLBP-ligand complex (Fig. 7), which shows the two highly conserved Arg residues critical for ligand binding due to their interaction with the carboxylate moiety on the terminus of the fatty acid as has been observed in crystallographically determined structures and molecular dynamics simulations of FABP-ligand complexes (13, 14, 23, 24).

It has also been suggested that the Phe-57 side chain may make important contacts with one of the carbon atoms of the fatty acid backbone (25). To test this idea, a third mutant BLBP was prepared (Phe-57 \rightarrow Ala) and its interaction with oleic acid measured. In this case, only a 2-fold reduction in B_{max} and a 2-fold increase in K_d were observed (K_d \sim 0.94 μ M; B_{max} \sim 39 pmol/ μ g), indicating that this residue is not a major contributor to the BLBP/ligand interaction.

Finally, we wished to prepare BLBP mutants for use in biological experiments in which the binding pocket was occluded, preventing BLBP/ligand interactions. To achieve this, a model of BLBP structure was calculated (see below). It was noted that Gly-33 is adjacent to the opening of the BLBP binding pocket. This was then used to predict that mutation of Gly-33 into a bulkier amino acid could preclude ligand binding by preventing access of the ligand to the binding pocket and by occupying part of the volume normally occupied by the ligand (Fig. 7). Thus, Gly-33 was replaced with either Phe or Ile

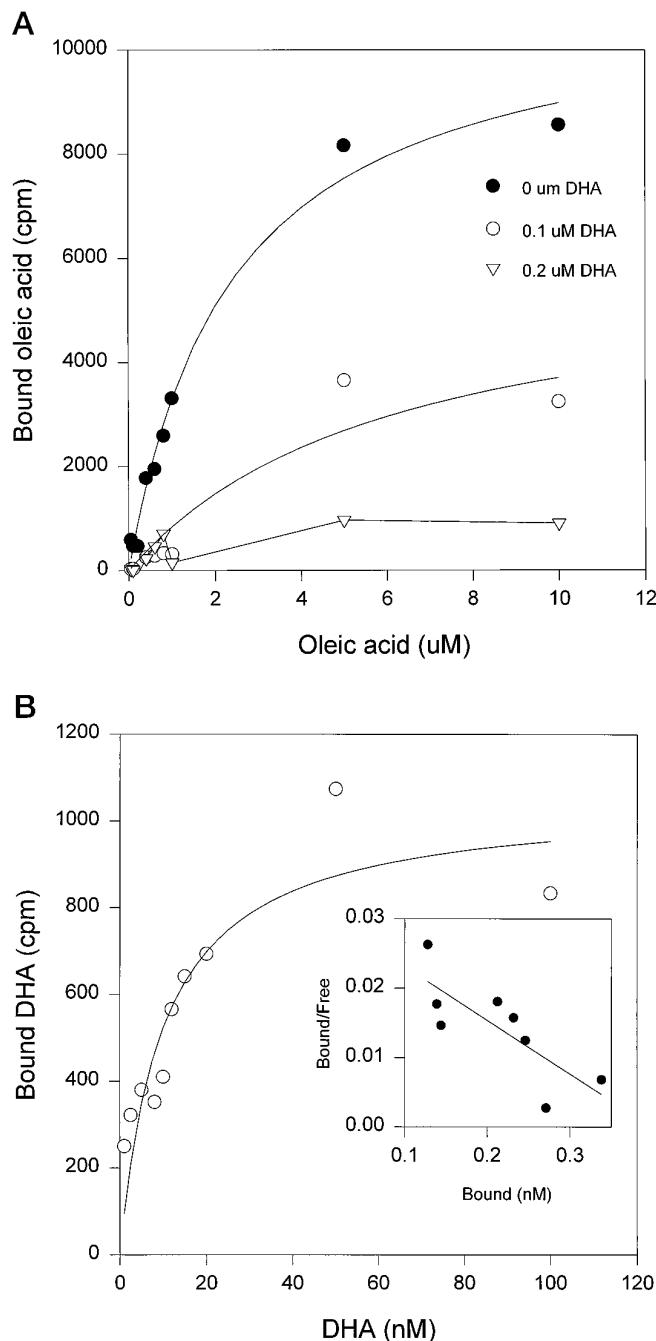


FIG. 4. Binding of DHA to recombinant BLBP. A, inhibition of oleic acid binding to BLBP by DHA. Ten different concentrations of oleic acid in the range of 0.01–10 μ M containing a constant proportion of 3 H-labeled oleic acid were used in binding assays with 5 μ g recombinant BLBP in the presence of 0 μ M (●), 0.1 μ M DHA (○), and 0.2 μ M DHA (▽) as described under “Experimental Procedures.” B, direct binding of DHA to BLBP. Ten concentrations of DHA in the range of 1–100 nM containing a constant proportion of 3 H-labeled DHA were used in binding assays with 5 μ g recombinant BLBP as described under “Experimental Procedures.” A representative set of data is shown. The Scatchard plot is shown in the inset. The curves in the main panels are least-squares fits of the model to the data (Equation 1), except for the curve describing the 0.2 μ M DHA data in panel A, which is not a least-squares fit because the relative error is too large.

(Gly-33 → Phe, Gly-33 → Ile), and the two mutants were tested for ligand binding. As expected, the Gly-33 → Ile and Gly-33 → Phe mutations decreased B_{\max} approximately 30- and 6-fold, respectively, and increased K_d for oleic acid for almost 20- and 10-fold, respectively.

A Structural Model for BLBP/Ligand Interaction—To gain

TABLE III
Effect of single mutations on binding of oleic acid to BLBP

Binding assays were performed, and B_{\max} , K_d , and ΔG were calculated as described under “Experimental Procedures” (Equations 1 and 3). The means and standard deviations of three to five experiments are shown. K_d/K_d' is the ratio of mutant to wild type K_d , similarly, B_{\max}/B_{\max}' is the ratio of mutant to wild type B_{\max} . ND, not detectable.

Mutants	K_d	Relative K_d/K_d'	B_{\max}	Relative B_{\max}/B_{\max}'	ΔG			
						μ M	pmol/ μ g	kcal/mol
Wild type	0.44 \pm 0.027	1	107 \pm 3.1	1	-9.02			
Phe-57 → Ala	0.94 \pm 0.09	2.13	39 \pm 1.3	0.36	-8.55			
Gly-33 → Phe	3.83 \pm 0.97	8.70	16.6 \pm 2.6	0.16	-7.69			
Gly-33 → Ile	7.75 \pm 4.33	17.6	3.9 \pm 1.2	0.036	-7.25			
Arg-126 → Ala	1.17 \pm 0.2	2.66	3.3 \pm 0.2	0.031	-8.42			
Arg-106 → Ala	ND	ND	ND	ND	ND			

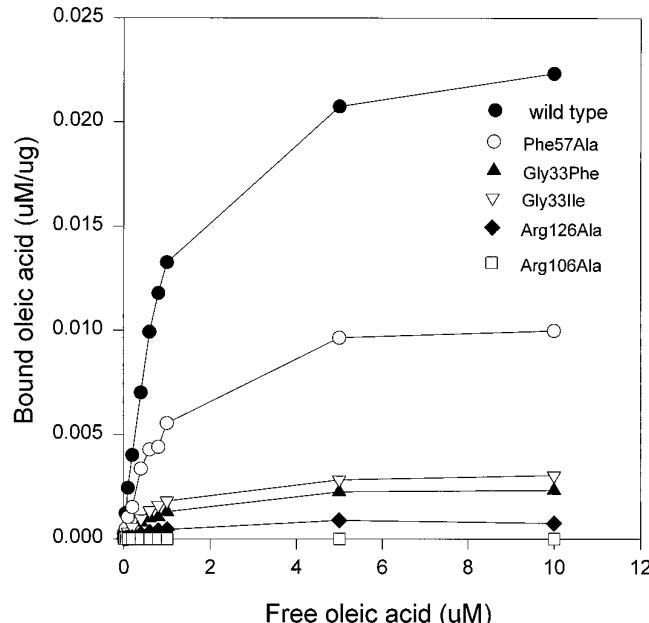


FIG. 5. Binding of OA to wild type and mutant BLBP proteins. Ten concentrations of oleic acid in the range of 0.01–10 μ M containing a constant proportion of 3 H-labeled oleic acid were used in binding assays with 20 μ g of recombinant BLBP as described under “Experimental Procedures.” Representative sets of data for each of the mutant proteins are shown: wild type (●); Phe-57 → Ala (○); Gly-33 → Phe (▲), Gly-33 → Ile (▽); Arg-12 → 6Ala (◆); Arg-106 → Ala (□).

additional insight into the BLBP/ligand interaction, structural models of BLBP and various BLBP-ligand complexes were prepared by homology modeling relying on the M-FABP and ALBP high resolution crystallographic structures. These proteins were chosen because of the high similarity between M-FABP, ALBP, and BLBP (Fig. 6). All FABPs, including BLBP, consist of a single domain composed of interacting α -helices packed at the edge of two orthogonal, four and six stranded antiparallel β -sheets (Fig. 7). The backbone structures of the BLBP models are virtually indistinguishable from those of the M-FABP or ALBP complexes (Fig. 7). The models passed all stereochemical criteria implemented in PROCHECK (23) as well as the protein structure evaluation test with program ProsaII (26). Generally, the errors in a homology-derived model of a target sequence are similar to the structural differences between two proteins that have the same sequence similarity as the template structure and the target sequence (11). Accordingly, this indicates that most of the main-chain atoms in the BLBP model have a root-mean-square error of about 1 \AA , corresponding to the 62% sequence identity between BLBP and M-FABP (27).

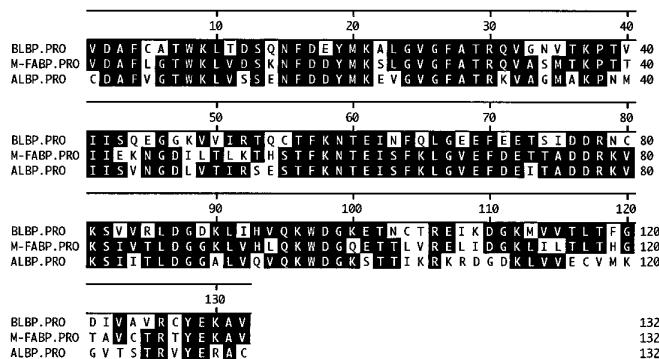


FIG. 6. Sequence alignment of BLBP, ALBP, and MFABP. Amino acid sequences of mouse brain lipid-binding protein (*blbp.pro*), mouse adipocyte lipid-binding protein (*ALBP.pro*), and muscle fatty acid-binding protein (*M-FABP.pro*) are shown. Residues matching an ideal consensus derived from these three proteins using the Megalign program (DNAStar) are shaded.

DISCUSSION

Previous studies of BLBP expression in the developing nervous system (1–3), and BLBP function in primary mixed neuronal/glial cell cultures (2), have suggested that BLBP may play an important role in glial cell differentiation. BLBP is expressed in radial glia during neuronal migration, and inhibition of BLBP function can result in failure of primary glia to elaborate long processes. Furthermore, promoter sequences necessary for BLBP regulation in radial cells *in vivo* also dictate the transcription of BLBP in response to neurons *in vitro* (4). These observations have led to a model for BLBP function and regulation, which is based on similar studies of cellular retinoic acid-binding proteins (5). As is the case for CRABP, we expect that regulation of BLBP transcription occurs through a nuclear receptor activated by the same ligand that is bound by BLBP. If this is the case, discovery of the BLBP ligand is a critical step in understanding the signaling system in which BLBP participates, since this ligand can be used to identify a putative nuclear receptor regulating the transcription of BLBP and other genes involved in glial differentiation.

In this study, we report three major conclusions regarding the ligand binding properties of BLBP. First, we have established that BLBP is a fatty acid-binding protein with ligand binding properties that are similar to but different from those of previously characterized members of this family. BLBP binds oleic acid and arachidonic acid with similar affinities as the heart or liver fatty acid-binding proteins (15, 16), but unlike these family members it does not bind saturated fatty acids such as palmitic or arachidinic acid. The strong preference of BLBP for long chain, polyunsaturated fatty acids may be a distinguishing feature of BLBP. Second, BLBP binds DHA with a very high affinity ($K_d \sim 10$ nM). This is approximately 20-fold higher affinity than that of any previously reported FABP/fatty acid interactions and is approximately equal to the affinity of CRABP/retinoic acid interaction ($K_d \sim 20$ nM). The very high affinity BLBP/DHA interaction, the enrichment of DHA in the brain (18, 28), and the fact that DHA is essential for timely development of the human central nervous system (18–20), strongly suggest that DHA is the natural ligand for BLBP. Third, we report a model for BLBP interactions with lipid ligands, which explains the results of our ligand binding and mutagenesis studies. In particular, this model suggested specific explanations for the selective binding of BLBP to various fatty acids and allowed construction of BLBP mutants with designed ligand binding properties.

The identification of DHA as the putative BLBP ligand is of significant biological interest. DHA is the most abundant long

chain polyunsaturated fatty acid (LCPUFA) in the nervous system. It is taken up by both the central nervous system and neural retina from the liver through the blood (28). A great deal of evidence that DHA is an essential nutrient for nervous system development has accumulated in studies of both experimental animals (18) and human infants (19, 20). For example, it has recently been shown that the well documented difference in neural maturation between breastfed infants and formula-fed infants (29) can be completely overcome if the formula is supplemented with DHA (20). This is consistent with the facts that infant formula is fortified only with precursors to LCPUFA such as linolenic acid (19) and that infants are unable to metabolize their full requirement of LCPUFA from these precursors (19). While the role of BLBP in DHA metabolism is not clear, the very high affinity of BLBP for DHA ($K_d \sim 10$ nM) as well as the correlation between the timing of BLBP expression throughout the developing nervous system (2) and the requirement for exogenous DHA (20, 29) strongly suggest that BLBP plays some role in the DHA function. Whether this role reflects the requirement for BLBP in glial cell differentiation in culture remains to be determined.

Three-dimensional models for a number of BLBP-ligand complexes were calculated in an attempt to explain some of the observed affinity differences in the binding of fatty acids to BLBP *versus* other FABPs. We first asked whether the structural models for BLBP could provide insight into the failure of BLBP to bind palmitic acid. The strong conservation of the amino acid residues in the binding pockets of BLBP and other FABPs with known structure are consistent with their similar affinities for OA and AA. The largest difference between the BLBP and M-FABP binding pockets is that the BLBP binding pocket has Ile at position 75, while the other FABPs have a residue with a small side chain, such as Gly or Ala; the other 9 residues defining the pocket in M-FABP (13) are identical. It is conceivable that this single side-chain change results in a steric clash between PA and BLBP, thus providing an explanation for the weak binding of PA to BLBP. While this is the most reasonable explanation we can infer from the BLBP model, it is certainly not the only possibility. Confirmation will require, for example, that the Ile-75 \rightarrow Gly mutation in BLBP results in an increase in affinity of BLBP for palmitic acid.

A second and more important issue we wished to address using the model is the approximately 20-fold increase in affinity of BLBP for DHA relative to OA or AA. The free energy of DHA binding to BLBP is approximately 2 kcal/mol greater than that for the AA binding (Table I). The BLBP-DHA model shows that the lipid binding cavity in BLBP is probably large enough to accommodate the two additional carbon atoms of DHA (Fig. 7). Thus, the tail of DHA does not have to protrude out of the binding cleft. As a consequence, the formation of the BLBP-DHA complex may result in approximately 80 \AA^2 of additional buried hydrophobic surface area compared to the BLBP-AA complex. Since the hydrophobic effect is worth about 24 cal/mol \AA^2 (30), this can account for the observed increase in the binding free energy ($24 \text{ cal/mol \AA}^2 \times 80 \text{ \AA}^2 = 2 \text{ kcal/mol}$). The model also shows that the DHA aliphatic chain can be extended by at most one carbon atom and still fit in the cavity. This indicates that a longer ligand with a higher affinity than that of DHA is not likely to exist.

Finally, models for several mutant forms of the BLBP-OA complex were built to find mutations likely to disrupt ligand binding to BLBP. As shown in Fig. 7, Gly-33 on the C-terminal helix is situated at the entrance to the binding pocket. Therefore, a mutation of this side chain into a larger side chain was expected to interfere with the entrance and binding of the ligand in the cavity. The large side chains of Ile and Phe in

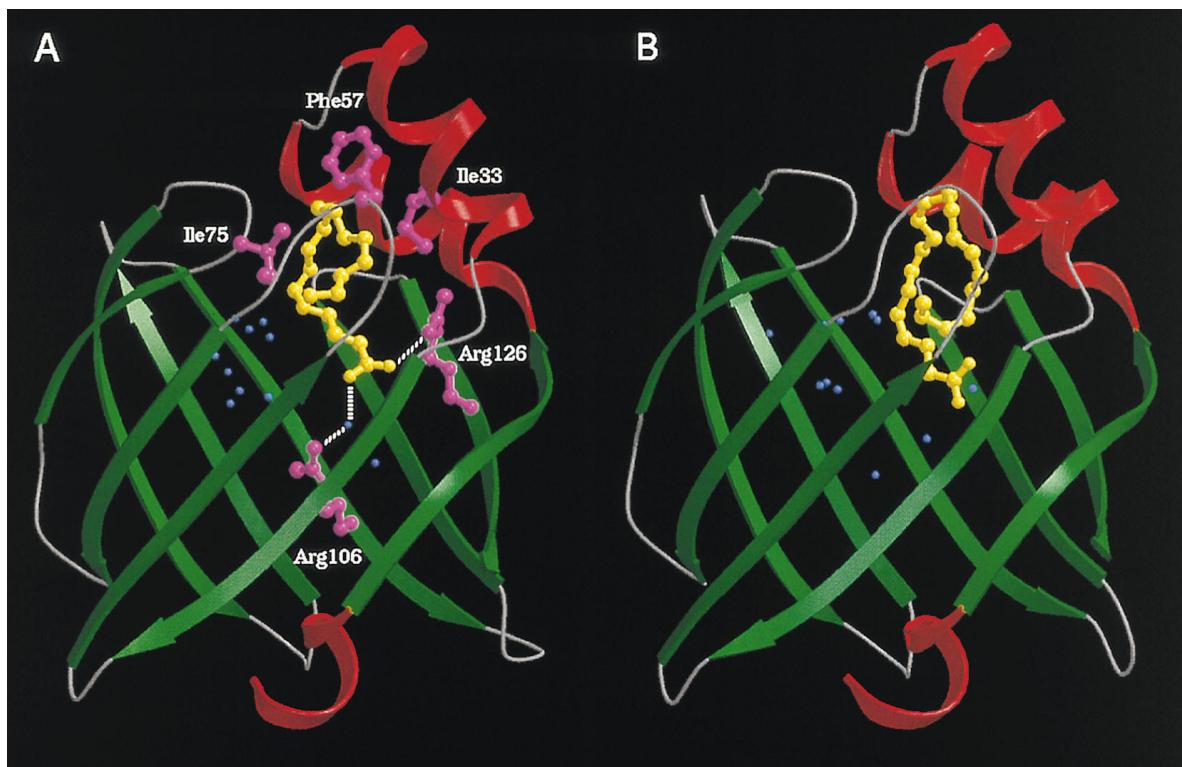


FIG. 7. Ribbon diagrams of models of the Gly-33 → Ile BLBP-oleic acid (A) and BLBP-DHA complexes (B). Lipid ligands, yellow; α -helices, red; β -strands, green; side chains of selected residues, magenta; water molecules, blue; hydrogen bonds, white dashed lines. The models were calculated with MODELLER (10, 11). The figure was prepared with programs Molscript (35) and Raster3D (36).

particular were selected because they are likely to preserve the local helical conformation (31). As predicted, the two mutants had the OA affinity for a factor 10-fold smaller than that of the wild type (Table III and Fig. 5). However, their affinities still were significant ($K_d \sim 4 \mu\text{M}$). This is consistent with the fact that the Gly-33 → Ile and Gly-33 → Phe mutations can be accommodated in the models by local conformational changes without significant violations of the model tests implemented in PROCHECK (23) and ProsaII (26).

In the case of the mouse adipocyte FABP, structural studies have demonstrated that FABP ligands have tails protruding out of the binding pocket and that tails interact with the Phe-57 side chain (25). Phe-57 is located on the surface of BLBP near the entrance to the binding pocket as shown in Fig. 7. The structural models of BLBP-ligand complexes predict that mutations of Phe-57 should have little effect on ligand binding because the bound ligand is buried in the interior of the protein and does not make any contact with the Phe-57 side chain. This is consistent with the binding studies of the Phe-57 → Ala mutant, which demonstrate that this change has almost no effect (0.4 kcal/mol) on the binding free energy of OA (Table III). These data indicate that the BLBP ligands are indeed buried within the binding pocket, as are the ligands in M-FABP. On the other hand, some ALBP ligands (OA, PA, but not AA) protrude from the pocket and make contacts with the surface of the protein that are important in ligand binding (14, 25). Phe-57 mutations, therefore, might be a useful indicator in distinguishing between these two modes of FABP/ligand interaction.

Structural modeling of BLBP/ligand interactions may help to design mutant BLBP proteins that are likely to facilitate testing of the physiological relationship between BLBP and DHA. For example, while the space-filling model of the BLBP-DHA complex predicts that there is sufficient space in the BLBP binding pocket to allow DHA binding, and that the additional

contacts between DHA and BLBP within the pocket may account for the increase of binding free energy of DHA relative to AA, it also predicts that fatty acids with significantly longer chains cannot be bound to BLBP in this mode (data not shown). Although it remains possible that such long chain fatty acids could bind BLBP in some other manner, we interpret this model as additional evidence that DHA could be the physiologic ligand for BLBP. To test this idea, we are presently trying to design BLBP mutants that specifically interfere with DHA binding to BLBP without affecting OA or AA binding. If such mutant proteins can be obtained, then definitive biological tests of DHA as the physiological ligand for BLBP function can be designed.

Finally, the similarities between the regulation of CRABP transcription by retinoic acid receptors (5) and the dynamic regulation of BLBP transcription in response to neurons (4) suggest that a DHA nuclear receptor may be involved in transcription of the BLBP gene. The recent demonstration that peroxisome proliferator-activated receptors can be activated by fatty acids and their metabolites (32–34), and that there are several members of this family that do not have known ligands, suggest these receptors as obvious candidates for transcriptional regulators involved in BLBP expression. Our current efforts are aimed at identifying this putative receptor.

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