Hydrophobic ligand binding properties of the human lipocalin apolipoprotein M

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Abstract **Apolipoprotein M (apoM) is a plasma protein associated mainly with HDL. ApoM is suggested to be important for the formation of preβ-HDL, but its mechanism of action is unknown. Homology modeling has suggested apoM to be a lipocalin. Lipocalins share a structurally conserved β-barrel, which in many lipocalins bind hydrophobic ligands. The aim of this study was to test the ability of apoM to bind different hydrophobic substances. ApoM was produced both in *Escherichia coli* and in HEK 293 cells. Characterization of both variants with electrophoretic and immunological methods suggested apoM from *E. coli* to be correctly folded. Intrinsic tryptophan fluorescence of both apoM variants revealed that retinol, all-*trans*-retinoic acid, and 9-*cis*-retinoic acid bound (dissociation constant = 2–3 μM), whereas other tested substances (e.g., cholesterol, vitamin K, and arachidonic acid) did not. The intrinsic fluorescence of two apoM mutants carrying single tryptophans was quenched by retinol and retinoic acid to the same extent as wild-type apoM, indicating that the environment of both tryptophans was affected by the binding. In conclusion, the binding of retinol and retinoic acid supports the hypothesis that apoM is a lipocalin. The physiological relevance of this binding has yet to be elucidated.**—Ahnström, J., K. Faber, O. Axler, and B. Dahlbäck. **Hydrophobic ligand binding properties of the human lipocalin apolipoprotein M.** ***J. Lipid Res.*** **2007.** 48: **1754–1762.**

Supplementary key words retinol • retinoic acid • intrinsic fluorescence • high density lipoprotein • signal peptide • cholesterol

Apolipoprotein M (apoM) is a 25 kDa (188 amino acid residues) protein expressed mainly in liver and kidney (1, 2). In plasma, apoM is associated mainly with HDL and to a minor extent with chylomicrons, VLDL, and LDL (1, 3). Studies of apoM in mice have suggested apoM to be important for the formation of preβ-HDL and for the efflux of cholesterol from cells to HDL, which are key events in reverse cholesterol transport. Furthermore, overexpression of apoM in LDL receptor-deficient mice challenged with a cholesterol-rich diet suggested apoM to have antiatherogenic properties (4).

A noteworthy feature of the apoM amino acid sequence is the lack of a signal peptidase cleavage site, explaining why circulating apoM contains its signal peptide (1). This unusual property is found in two other HDL-associated proteins: paraoxonase-1 and the haptoglobin-related protein (5). The retained signal peptide of apoM serves as a hydrophobic anchor, binding apoM to the phospholipid layer of the lipoproteins (O. Axler et al., unpublished data).

Structural analysis including homology modeling has predicted apoM to belong to the lipocalin protein family (6). Members of the lipocalin protein family are typically small secreted proteins, which are functionally diverse and have weak sequence similarity but high similarity at the tertiary structural level (7, 8). The majority of lipocalins are responsible for the storage and transport of compounds that have low solubility or are chemically sensitive, such as vitamins, steroids, and metabolic products (7, 8). The lipocalins share a structurally conserved β-barrel as a central motif, which consists of an eight-stranded antiparallel β-sheet closed back on itself and a short α-helix. The β-barrel encloses a hydrophobic pocket, which in many lipocalins serves as a ligand binding site (7, 8). We previously constructed an apoM model based partly on the structure of retinol binding protein (RBP) and mouse major urinary protein (**Fig. 1**) (6). RBP is a 21 kDa lipocalin present in plasma. Its main function is to transport vitamin A (all-*trans*-retinol). The three-dimensional structure of RBP has been experimentally determined, and the structural details of the retinol binding have been elucidated (9, 10).

The lipocalin family contains a highly conserved tryptophan (position 24 in RBP and position 47 in apoM) that points toward the inside of the hydrophobic pocket (9). ApoM contains a second tryptophan at position 100 (Trp100) facing the hydrophobic pocket close to its opening (Fig. 1). These tryptophans can be used for assessing the binding

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Abbreviations: 1,8-ANS, 1-anilinonaphthalene-8-sulfonic acid; apoM, apolipoprotein M; K_d, dissociation constant; RBP, retinol binding protein.

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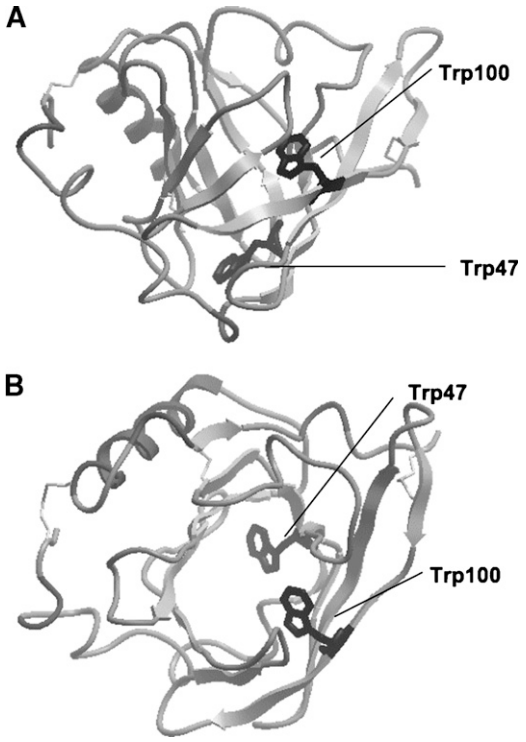

Fig. 1. The three-dimensional structure of apolipoprotein M (apoM) based on homology modeling, suggesting that apoM is a member of the lipocalin protein family. The two tryptophans (Trp), in black, at positions 47 and 100, were used for intrinsic fluorescence quenching. A: The apoM model seen from the side of the β-barrel. B: The apoM model facing the opening of the hydrophobic pocket.

of ligands in the hydrophobic pocket by intrinsic fluorescence quenching studies.

The aim of this study was to investigate the binding of several small lipophilic ligands to apoM. The predicted lipocalin domain of human apoM was produced in a prokaryotic expression system as well as in stably transfected HEK 293 cells, and intrinsic fluorescence quenching was used to test the binding of a panel of hydrophobic substances to apoM. ApoM was found to bind retinol and retinoic acid, whereas other hydrophobic vitamins, steroid hormones, and fatty acid derivatives did not associate with apoM. These results suggest that apoM is indeed a lipocalin, but the physiological importance of retinol binding remains unknown.

METHODS

Reagents

Arachidonic acid, β-estradiol, bilirubin, cholesterol, linoleic acid, oleic acid, palmitic acid, platelet-activating factor, progesterone, prostaglandin E1, vitamin A (all-*trans*-retinol), all-*trans*-retinoic acid, 9-*cis*-retinoic acid, and vitamin E (DL-α-tocopherol) were purchased from MP Biomedicals. 1-Anilinonaphthalene-8-sulfonic acid (1,8-ANS) was from Fluka. Acetyl-CoA, vitamin D_3 (cholecalciferol), and 3-hydroxy butyric acid were from Sigma-Aldrich. Vitamin K was from Roche. Testosterone was kindly provided by Dr. Pirkko Härkönen (University Hospital Malmö). Human purified RBP was a kind gift from Dr. Bo Åkerström (Lund University).

Polyclonal and monoclonal antibodies were generated in house using recombinant human apoM as antigen. Polyclonal antibodies were raised in rabbits and monoclonal antibodies (mab23, mab42, and mab58) were raised in mice using standard techniques (11).

Vector construction for expression of the human apoM lipocalin domain in *Escherichia coli*

The cDNA encoding the lipocalin domain of apoM (amino acid residues 22–188) was introduced into a pET15b bacterial expression vector (Novagen) using the *Bam*HI and *Nde*I cleavage sites. The pET15b vector contains the coding sequence for an N-terminal histidine tag followed by a thrombin cleavage site. After the thrombin cleavage site, the vector construct encodes five amino acids, GSHMN, before the start of the apoM sequence. The thrombin cleavage site was thus placed a few amino acids before the start of the apoM sequence, the reason being that we had experienced poor cleavage in other constructs in which the apoM sequence followed directly upon the cleavage site. This is presumably attributable to steric hindrance, as the cysteine at position 23 is involved in a disulfide bridge with cysteine 167 (6). The five extra amino acid residues did not seem to affect the folding, as demonstrated in Results. The BigDye Sequencing Kit (Perkin-Elmer) was used to confirm that the inserted apoM sequence was correct.

Mutagenesis of apoM

ApoM (accession number NM 019101) contains two tryptophans, Trp47 and Trp100, which is why we denote the wild-type variant $apoM^{WW}$ (Fig. 1). Three mutants, Trp47Phe ($apoM^{FW}$), Trp100Phe ($apoM^{WF}$), and Trp47Phe, Trp100Phe ($apoM^{FF}$), were constructed using the Quickchange Site-Directed Mutagenesis Kit (Stratagene). Two complementary oligonucleotides for each mutation were used for this purpose. The sequences for the sense oligonucleotides were 5′-GAGGTCCACTTGGGCCAGTTC-TACTTTATCGCAGGG-3′ (FW and FF) and 5′-CTCTGTGTG-CCCCGGAAATTCATCTACCACCTGACTG-3′ (WF and FF). Mutated nucleotides are highlighted by underlined letters. The oligonucleotides were ordered from MWG Biotech. The mutations were verified by DNA sequencing using a BigDye Sequencing Kit (Perkin-Elmer).

Expression and purification of recombinant apoM in *E. coli*

The pET15b vector containing the apoM sequence was transfected into *E. coli* strain BL21 (DE3). Cultures were grown under agitation at 37°C in 2 liters of Luria-Bertani medium containing 100 μg/ml ampicillin. Gene expression was induced when the absorbance at 600 nm was 0.6–0.8 by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM. Expression was allowed for 4 h, and then the cells were harvested by centrifugation and resuspended in ice-cold 20 mM Tris-HCl and 0.15 M NaCl, pH 7.4. Lysozyme (Amersham Biosciences; 100 μg/ml final concentration) and benzamidine (0.5 M) were added to the bacteria, followed by incubation for 45 min at room temperature. The bacteria were sonicated in intermittent 10 s bursts for 30 min until the solution was clear and nonviscous and then centrifuged again. The pellet containing the inclusion bodies was suspended in 30 ml of 6 M guanidine-HCl and 20 mM Tris-HCl, pH 8.0, containing 10 mM reduced glutathione overnight at room temperature and then centrifuged again. The supernatant was collected and applied to a nickel column (20 ml) (nickel

resin Superflow; Qiagen) equilibrated with the same buffer. After washing with the equilibration buffer, the apoM was eluted with a linear 0–0.5 M imidazole gradient (260 ml). Fractions containing recombinant apoM were identified by dot-blot analysis using a polyclonal anti-human apoM antiserum.

The fractions with apoM were pooled and refolded by dialysis at 4°C. The pool (diluted to 100 μg/ml in the dialysis buffer) was first dialyzed against 3 M guanidine HCl, 20 mM Tris-HCl (pH 8.0), 4 mM reduced glutathione, 0.4 mM oxidized glutathione, and 10% glycerol for 2–3 days and then against 20 mM Tris-HCl (pH 8.0), 4 mM reduced glutathione, 0.4 mM oxidized glutathione, and 10% glycerol for 1 day. Iodacetamide (5 mM) was added to block free sulfhydryl groups before final dialysis against 20 mM Tris-HCl, pH 8.0, containing 10% glycerol for 1 day. The recombinant apoM was further purified through anion-exchange chromatography on a Q-Sepharose column (Amersham Biosciences). The column (100 ml) was equilibrated in 20 mM Tris-HCl (pH 8.0) and 10% glycerol, and after application of the refolded apoM and washing with the same buffer, the column was eluted with a 500 ml linear NaCl gradient (0–0.3 M). Fractions were analyzed by unreduced 15% PAGE in the presence of SDS, and those containing monomeric apoM were pooled and dialyzed against 20 mM Tris-HCl (pH 8.0) and 10% glycerol overnight at 4°C. The histidine tag was successfully cleaved off by thrombin. After thrombin cleavage, the apoM was repurified on another Q-Sepharose column (25 ml; 400 ml 0–0.3 M NaCl gradient). Finally, the pooled fractions from the second Q-Sepharose purification were applied on a cation-exchange chromatography column (SP-Sepharose; Amersham Biosciences) (2 ml) equilibrated with 20 mM Tris-HCl (pH 8.0) and 10% glycerol to remove the remaining thrombin; thrombin bound to the column, whereas apoM passed through. The apoM concentration was determined by absorption at 280 nm using an extinction coefficient (E1%, 1 cm) of 13.7, which was determined as described (12). The purified apoM was analyzed by 15% SDS-PAGE and by nondenaturing gel electrophoresis (13).

Vector construction for the expression of the human apoM lipocalin domain in a eukaryotic expression system

To express soluble apoM, a signal peptidase cleavage site was generated by mutagenesis of glutamine 22 to alanine (apoM^{Q22A}) in full-length apoM cDNA in pcDNA3 (Invitrogen) (6) using the Quikchange Site-Directed Mutagenesis Kit (Stratagene). The sequence for the sense oligonucleotide (MWG Biotech) was 5′-CCTTAACTCCATCTACGCGTGCCCTGAGCACAGTC-3′, with the mutated oligonucleotides being underlined. The presence of the mutation was verified by DNA sequencing using the BigDye Sequencing Kit (Perkin-Elmer).

Expression and purification of apoM produced in HEK 293 cells

The apoM^{Q22A} cDNA was used to transfect HEK 293 cells using Lipofectin (Invitrogen), and stable transfectants were selected using G-418 and tested with the apoM ELISA. Clones secreting high levels of apoM were expanded, and serum-free Optimem medium was collected and stored at −20°C. To purify apoM, ammonium sulfate (70%) was added to the medium (1 liter) and the precipitate was collected by centrifugation at 5,000 rpm for 25 min. The pellet was dissolved in 20 mM Tris, pH 8.0, dialyzed against the same buffer overnight at 4°C, and loaded onto a 5 ml mab23 (9 mg/ml) HiTrap column (Amersham Biosciences) (3). The column was washed with TBS, pH 7.4, and apoM was eluted with 0.1 M glycine, pH 2.2, the pH being immediately neutralized with unbuffered Tris. The purity of the apoM was assessed by 15% SDS-PAGE.

ApoM sandwich ELISA

A sandwich ELISA for apoM based on two monoclonal antibodies, mab58 and mab42, was used to quantify apoM, as described previously (3, 11). In brief, a 96-well Costar plate (Corning, Inc.) was coated with mab58 and quenched according to standard techniques. Serial dilutions of wild-type apoM (apoM^{WW} and apoM^{Q22A}) and apoM mutants and Li-heparin plasma were made in TBS (50 mM Tris-HCl and 0.15 M NaCl, pH 7.4) containing 1% BSA (Sigma-Aldrich) and 1% Triton X-100 (MP Biomedicals) and added to the wells. ApoM bound to mab58 was detected by biotinylated mab42, streptavidin-avidin-horseradish peroxidase (DAKOCytomation A/S), and 1,2-phenylenediamine dihydrocloride tablets (DAKOCytomation A/S), according to the manufacturer's instructions.

Gel filtration of apoM

Recombinant apoM (0.5 ml of 20 μg/ml) in TBS containing 1% BSA (Sigma-Aldrich) was applied to a gel filtration column, Superose 12 10/300 GL (Amersham Biosciences), and equilibrated in TBS. Fractions of 0.27 ml were collected and analyzed using the apoM ELISA. The total protein concentration in the fractions was measured with the Bio-Rad Protein Assay according to the manufacturer's instructions.

Intrinsic fluorescence quenching

The ligand binding experiments were performed at 25 ± 0.1°C with a Fluoromax-3 spectrofluorometer (Jabin Yvon Horiba Group) with slits set at 5 nm bandwidth. The binding studies were performed using excitation at 295 nm. In initial experiments, increasing concentrations of the proteins (in TBS) were tested until the signals for intrinsic fluorescence were high enough. The emission maximum for each protein was determined from the emission spectra received during these titrations. Ligands were added stepwise to a 1 ml apoM protein solution in 0.5–5 μl aliquots from a 1 mM stock solution of the ligand, the maximum volume of ligand added not exceeding 20 μl. The slight volume increase in the course of the titration was neglected. After each added aliquot, the protein sample was mixed and incubated in the dark for 1 min before the fluorescence measurement. In each of the measurements, a blank consisting of the ligand in TBS was subtracted from the actual fluorescence. For each ligand, three to five independent titrations were performed. Binding was evaluated by following the quenching of protein fluorescence attributable to energy transfer. The ligands tested were the following: bilirubin, cholesterol, β-estradiol, 3-hydroxy butyric acid, oleic acid, palmitic acid, platelet-activating factor, progesterone, prostaglandin E1, all-*trans*-retinol, all-*trans*-retinoic acid, 9-*cis*-retinoic acid, vitamin D_3 that had been dissolved in DMSO, arachidonic acid, testosterone, vitamin K that had been dissolved in 99.5% ethanol, vitamin E that had been dissolved in acetone, and acetyl-CoA that had been dissolved in distilled water.

Data analysis of binding

The binding affinity between apoM and the different ligands was calculated by fitting to two different equations, 1 and 2, according to the theory of bimolecular complex formation.

$$I = I_P\left(1 - \frac{[L]}{[L] + K_d}\right) + I_{PL}\left(\frac{[L]}{[L] + K_d}\right) \qquad \textit{(Eq. 1)}$$

$$[\mathrm{L}] = \frac{[\mathrm{L}]_t - K_d - [\mathrm{P}]_t}{2} + \sqrt{\left(\frac{[\mathrm{L}]_t - K_d - [\mathrm{P}]_t}{2}\right)^2 + K_d[\mathrm{L}]_t} \qquad \textit{(Eq. 2)}$$

$[P]_t$ and $[L]_t$ represent the total concentration of protein and ligand, respectively, and [L] represents the concentration of free ligand. I indicates the corrected fluorescence. I_P and I_{PL} represent the fluorescence of free protein without added ligand and the protein-ligand complex (at saturating concentrations of ligand), respectively.

K_d is the apparent dissociation constant for the binding between apoM and the ligand. K_d and I_{PL} were fitted as free parameters. See the supplementary material for the derivation of the equation.

1,8-ANS competition assay

1,8-ANS is a hydrophobic probe that is known to interact with hydrophobic regions in proteins (14–17). The affinity of other ligands for the 1,8-ANS binding site can be assessed by observing the decrease of 1,8-ANS-apoM fluorescence in the presence of increasing concentrations of competitor ligand (16). 1,8-ANS also shows a reversible shift of its own fluorescence emission maximum from 512 to 470 nm when excited at 370 nm after binding to hydrophobic surfaces. 1,8-ANS-bound fluorescence measurements were performed with the ligands binding to apoM in the intrinsic fluorescence measurements. A precomplex was formed by adding 2 μl of a 5 mM stock solution of 1,8-ANS in DMSO to 1 ml of 1.6 μM $apoM^{WW}$. Fluorescence intensity was measured at 500 nm after excitation at 370 nm. Just as in the intrinsic fluorescence experiments, ligands were added to the protein in 0.5–5 μl aliquots of 1 mM stock solutions to a total of 20 μl. After each added aliquot of ligand, the protein sample was mixed and incubated in the dark for 1 min before the fluorescence measurement. Slit widths of 5 nm were used for both excitation and emission. For each ligand, five independent titrations were performed.

RESULTS

Expression and purification of recombinant apoM variants

The lipocalin domain of human apoM was expressed both in prokaryotic (*E. coli*) and eukaryotic (HEK 293) cells. The hydrophobic signal peptide (residues 1–21) was excluded from the apoM constructs, as we aimed to obtain a soluble apoM for ligand binding studies. In the *E. coli* system, in addition to $apoM^{WW}$, three tryptophan mutants, Trp47Phe ($apoM^{FW}$), Trp100Phe ($apoM^{WF}$), and Trp47Phe, Trp100Phe ($apoM^{FF}$), were produced. The recombinant proteins expressed in *E. coli* were recovered from inclusion bodies using reducing and denaturing conditions. The N-terminal histidine tag allowed purification of the proteins using chromatography on a nickel column. The apoM protein was refolded through dialysis, a protocol including oxidized and reduced glutathione. Pure apoM was obtained after ion-exchange chromatography on Q-Sepharose. The N-terminal histidine tag was cleaved off with thrombin, the apoM was again purified on a Q-Sepharose column, and the remaining thrombin was removed on a small SP-Sepharose column. The purified apoM variants migrated as single bands on SDS-PAGE under both reducing and nonreducing conditions, the apparent molecular masses being just below 20 kDa (**Fig. 2**).

Even on heavily overloaded gels (data not shown), no additional bands were seen and the apoM variants were judged to be >95% pure. The yield of refolded $apoM^{WW}$ per liter of bacterial culture varied between 50 and 75 mg. The yield of $apoM^{WF}$ was similar to that of $apoM^{WW}$, whereas the yields of $apoM^{FW}$ and $apoM^{FF}$ were much lower (i.e., 10–30% of the yield of $apoM^{WW}$). The low yield of these two mutants was attributable mainly to poor recovery during the refolding process. On nondenaturing agarose gel electrophoresis, apoM migrated as a homogenous band to the α_2 region (data not shown). On gel filtration chromatography, the elution volume of $apoM^{WW}$ was similar to that of RBP, suggesting apoM to be monomeric. $ApoM^{WW}$ was stable upon storage in a refrigerator for weeks. It was highly soluble and could have been concentrated to 30 mg/ml without any visible precipitation.

$ApoM^{Q22A}$ was expressed in HEK 293 cells and used to compare with the prokaryotically expressed apoM. The Q22A mutation created a signal peptidase cleavage site, and stably transfected HEK 293 cells expressed soluble

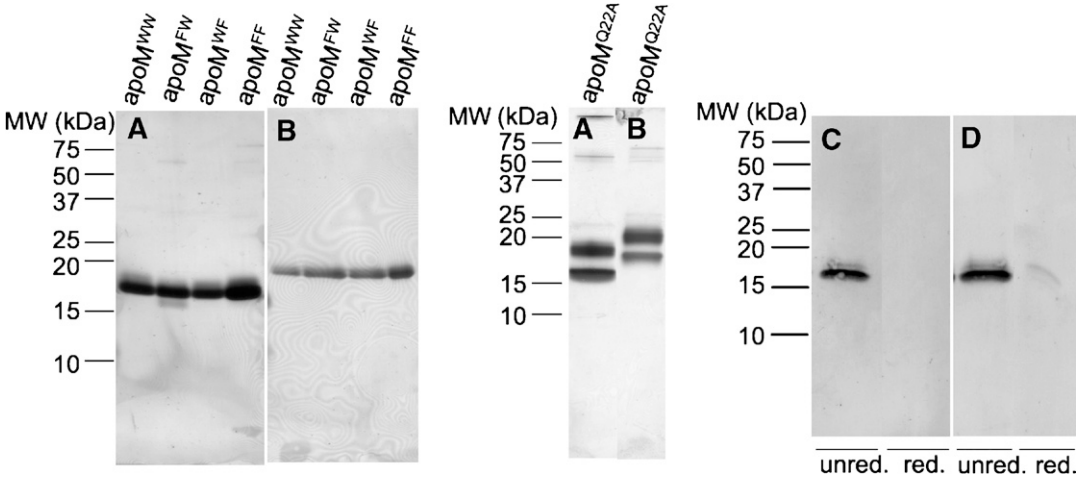

Fig. 2. Electrophoresis of recombinant apoM. A, B: $ApoM^{WW}$, $apoM^{Q22A}$, and apoM mutants as indicated were subjected to a 15% SDS-PAGE under nonreducing (A) and reducing (B) conditions. To each lane, 2 μg was added. C, D: Western blotting of $apoM^{WW}$ under nonreducing (unred.) and reducing (red.) conditions using mab42 (C) and mab58 (D). A total of 20 ng of $apoM^{WW}$ was added to each lane. MW, molecular mass.

apoM (residues 22–188) lacking the signal peptide at a concentration of ~3 mg/l. The yield of purified apoM^{Q22A} was 200 μg/l Optimem medium. The purified apoM^{Q22A} migrated as two bands on SDS-PAGE under both reducing and nonreducing conditions, which corresponded to glycosylated (20 kDa) and nonglycosylated (15 kDa) apoM (Fig. 2A, B). On gel filtration chromatography, the elution volume of apoM^{Q22A} was similar to that of apoM^{WW}.

Reactivity of apoM variants in a monoclonal antibody-based sandwich ELISA

A sandwich ELISA for apoM based on two monoclonal antibodies (mab42 and mab58) was used to compare the immunoreactivities of the recombinant apoM variants with that of apoM in plasma. On Western blotting, the two monoclonal antibodies only reacted with unreduced apoM (Fig. 2C, D), showing that their epitopes were conformation-dependent. Similar dose-response curves were obtained with the five recombinant apoM variants as with apoM from plasma (**Fig. 3**). The ELISA was used to determine the concentration of the mutants, using the apoM^{WW} values to create the standard curve. The reason for this approach was that the 280 nm absorbance is dependent on the number of tryptophans in the molecule. A semiquantitative Western blot using polyclonal antibodies against apoM confirmed that the concentration determination made with the ELISA was correct (data not shown).

Intrinsic fluorescence of recombinant apoM variants

Tryptophan is the dominant amino acid contributor to the fluorescence of a protein. The quantum yield at the maximum wavelength associated with intrinsic fluorescence is very sensitive to the polarity of the environment (18, 19) and therefore is used to monitor interactions. When investigating the emission spectra of apoM after excitation at 295 nm, emission maxima for the different proteins were found at 335–350 nm (**Fig. 4**). ApoM^{WW} expressed in *E. coli* had a similar spectrum as apoM^{Q22A} expressed in HEK 293 cells (Fig. 4). The apoM mutant without any tryptophans (apoM^{FF}) did not give any intrinsic fluorescence and served as a negative control, showing that the intrinsic fluorescence was highly specific for tryptophans (Fig. 4). The emission spectra for apoM^{WW}, apoM^{FW}, and apoM^{WF} had different emission maxima. The sensitivity to the polarity of the immediate environment and the fact that the two mutants apoM^{FW} and apoM^{WF} have only one tryptophan were sufficient to alter the emission maximum of the protein (20).

Ligand binding studies using quenching of intrinsic fluorescence

A panel of small hydrophobic substances was tested in the ligand binding assay. All-*trans*-retinol and its derivatives all-*trans*-retinoic acid and 9-*cis*-retinoic acid quenched the intrinsic fluorescence of apoM in a dose-dependent manner, suggesting binding of these molecules (**Figs. 5**, **6**). The emission maxima of all apoM variants shifted slightly toward red with increasing concentrations of all-*trans*-retinol, possibly as a result of a conformational change in apoM upon the binding of retinol. The quenching data fitted well to equations 1 and 2 (see Methods). The estimated K_d values are presented in **Table 1**. There was no decrease or only a slight decrease in the intrinsic fluorescence from the other substances (e.g., cholesterol, arachidonic acid, and vitamin K or D_3) (**Fig. 7**). Some of these substances might interact with the apoM molecule, but because the quenching was not more pronounced we considered the binding too weak to investigate further. Vitamin E and testosterone were difficult to assess with this technique, because they gave in themselves a fluorescence signal after excitation at 295 nm. Human RBP was used as a

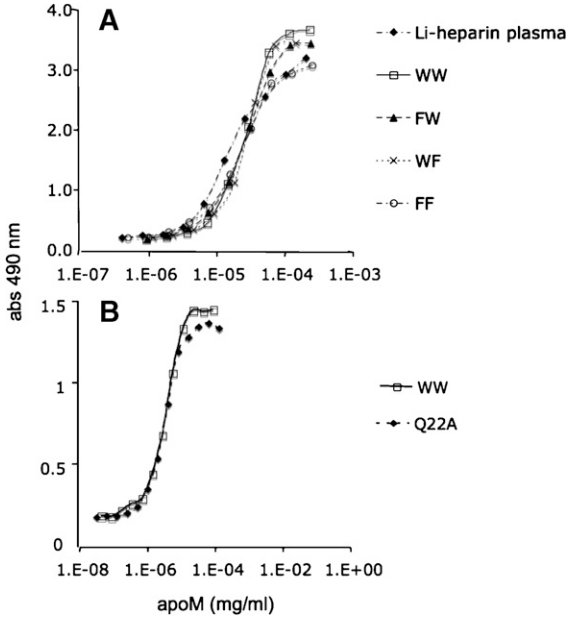

Fig. 3. Dose response of apoM variants in a monoclonal antibody-based ELISA. Serial dilutions of Li-heparin plasma, apoM^{WW} and apoM mutants (A), and apoM^{WW} and apoM^{Q22A} (B) were run on a sandwich ELISA that used the two conformation-specific monoclonal antibodies mab42 and mab58. The wells were coated with mab58, and the bound apoM was detected with biotinylated mab42.

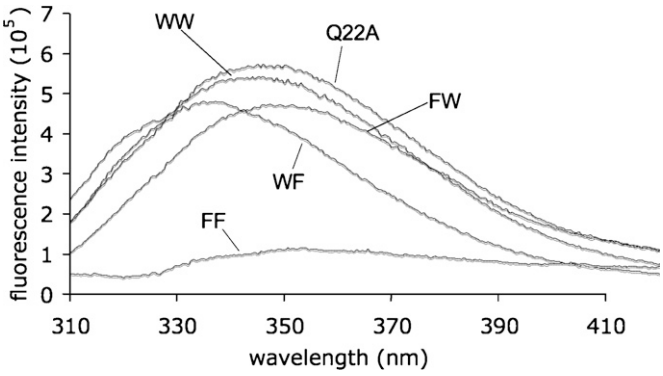

Fig. 4. Intrinsic fluorescence of apoM when excited at 295 nm. Emission spectra for apoM^{WW}, apoM^{Q22A}, and the three mutants. The emission maxima after excitation at 295 nm were 345, 350, and 335 nm for apoM^{WW} and apoM^{Q22A}, apoM^{FW}, and apoM^{WF}, respectively.

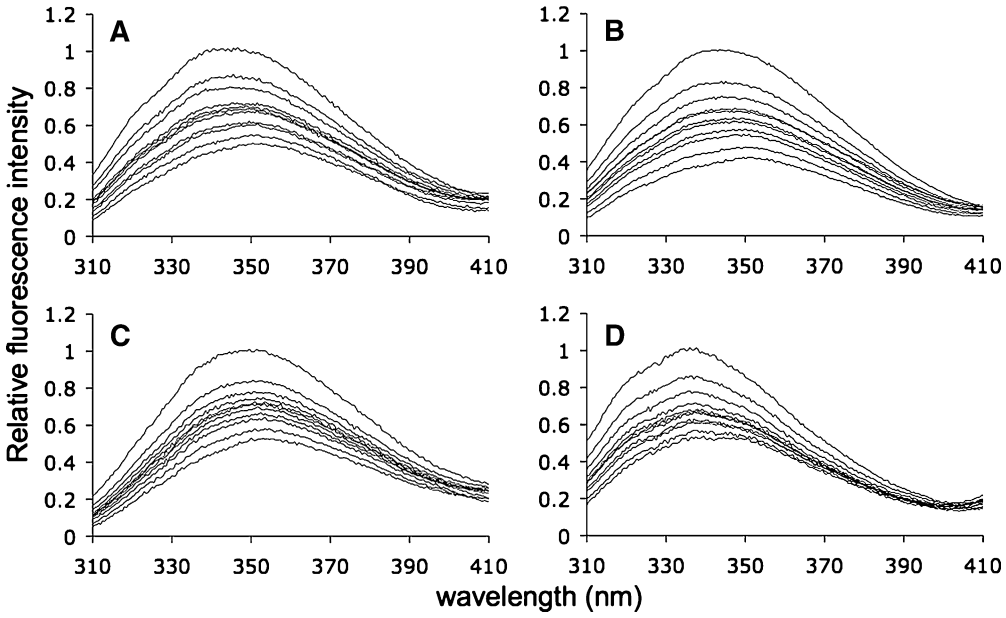

Fig. 5. Emission spectra of apoM^{WW} (A), apoM^{Q22A} (B), apoM^{FW} (C), and apoM^{WF} (D) excited at 295 nm in the absence and presence of increasing concentrations of all-*trans*-retinol. In each panel, the most intense spectrum is that of the protein alone, and the remaining spectra with decreasing intensities correspond to those recorded in the presence of increasing concentrations of all-*trans*-retinol, the final concentrations being 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 15, and 20 μM.

positive control, and all-*trans*-retinol and all-*trans*-retinoic acid were found to bind to RBP with K_d of ~0.1 μM, which corresponds well with previously reported results (21, 22). ApoM^{WW} expressed in *E. coli* bound all-*trans*-retinol and its two metabolites in the same manner as apoM^{Q22A} expressed in HEK 293 cells (Fig. 6, Table 1). Also, the two apoM mutants apoM^{FW} and apoM^{WF} bound all-*trans*-retinol and its two metabolites with the same K_d values as did apoM^{WW} (Table 1). The large variance observed in the experiment using all-*trans*-retinoic acid was caused by high background fluorescence from the substance itself. This also explains the negative values that were seen in some experiments at high concentrations of all-*trans*-retinoic acid after subtraction of the background fluorescence from the fluorescence of the apoM-retinoic acid complex (Fig. 6B).

1,8-ANS competition assay

In an attempt to determine the specificity and the site of ligand binding to apoM, a 1,8-ANS competition assay was performed. In fluorescence quenching, the mean ± SD K_d of ANS binding to apoM was determined to be 22.3 ± 7.3 μM (i.e., much higher than the K_d values measured for retinol and retinoic acid). In the competition assay, apoM^{WW} was allowed to interact with 1,8-ANS and then aliquots of the investigated ligands were added stepwise. When mixing apoM^{WW} with 1,8-ANS, the fluorescence of 1,8-ANS increased dramatically and there was a shift of the emission maximum from 512 to 470 nm. Addition of all-*trans*-retinoic acid and 9-*cis*-retinoic acid resulted in partial displacement of 1,8-ANS, detected as quenching of the ANS fluorescence (**Fig. 8**) and a reversed shift of the emission maximum toward longer wavelengths. All-*trans*-retinol has in itself fluorescence that interfered with the 1,8-ANS fluorescence and therefore could not be investigated in this type of assay.

DISCUSSION

ApoM is associated mainly with HDL particles in plasma but also to a minor extent with chylomicrons, VLDL, and LDL (1). An unusual feature of apoM is that the mature protein retains its signal peptide due to the lack of a signal peptidase cleavage site. This signal peptide binds to the single-layer phospholipids of lipoproteins, thus anchoring apoM to the lipoproteins (O. Axler et al., unpublished data). To date, no free apoM has been found in plasma and the hydrophobic nature of the signal peptide precludes the isolation of the native protein without the use of detergents. As apoM is predicted to be a lipocalin, with a characteristic hydrophobic pocket, it is of interest to investigate whether apoM has the ability to bind small hydrophobic substances (6). However, this cannot be done in the presence of detergents; therefore, to be able to investigate the binding properties of apoM, we developed methods to produce recombinant apoM lacking the signal peptide (i.e., the lipocalin domain of apoM) in both prokaryotic and eukaryotic systems. ApoM^{Q22A} from HEK 293 cells served as a control for the folding and functional properties of the refolded apoM from the inclusion bodies of the *E. coli* system. The apoM sequence contains a site for *N*-linked glycosylation, and apoM in plasma is partially glycosylated. Similarly, the apoM^{Q22A} preparation was also found to be partially glycosylated. ApoM^{WW} and apoM^{Q22A}

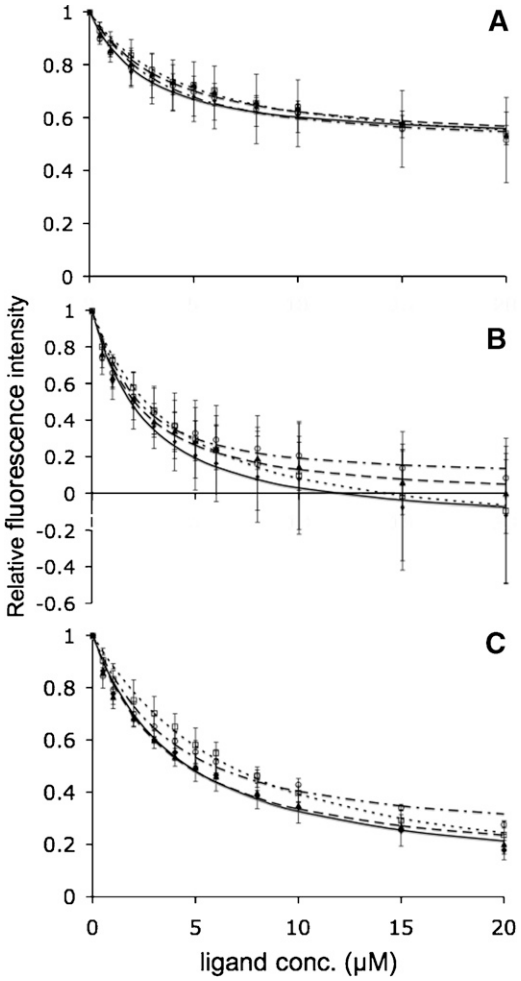

Fig. 6. Intrinsic fluorescence quenching studies. The quenching of intrinsic fluorescence of apoM^{WW} (closed diamonds), apoM^{FW} (closed triangles), apoM^{WF} (open squares), and apoM^{Q22A} (open circles) by all-*trans*-retinol (A) and its two derivatives, all-*trans*-retinoic acid (B) and 9-*cis*-retinoic acid (C), was investigated. The relative fluorescence intensities at 345, 350, and 335 nm for apoM^{WW} and apoM^{Q22A}, apoM^{FW}, and apoM^{WF}, respectively, were plotted against the total concentration of the ligand added to the system. The lines demonstrate the fitting of the data to equations 1 and 2 in Methods: apoM^{WW} (continuous line), apoM^{FW} (dashed line), apoM^{WF} (dotted line), and apoM^{Q22A} (dashed and dotted line). Points represent means ± SD.

TABLE 1. K_d values for the binding of various ligands to recombinant apoM

Protein	Ligand	K_d	SD	n
		μM		
ApoM^{WW}	All-*trans*-retinol	2.2	1.5	5
	All-*trans*-retinoic acid	1.8	1.2	5
	9-*cis*-retinoic acid	3.3	1.2	5
ApoM^{FW}	All-*trans*-retinol	3.2	0.7	5
	All-*trans*-retinoic acid	1.9	0.5	3
	9-*cis*-retinoic acid	3.3	0.6	3
ApoM^{WF}	All-*trans*-retinol	3.3	2.1	5
	All-*trans*-retinoic acid	2.2	0.9	3
	9-*cis*-retinoic acid	5.3	2.1	3
ApoM^{Q22A}	All-*trans*-retinol	2.5	0.4	3
	All-*trans*-retinoic acid	1.6	1.2	3
	9-*cis*-retinoic acid	3.2	2.0	3

ApoM, apolipoprotein M; K_d, dissociation constant. Results are given as means and SD. The differences between the different substances and the apoM mutants were not significant. The concentrations (in μM) of recombinant apoM used during the intrinsic fluorescence were 1.6, 0.7, 2.5, and 1.2 for apoM^{WW}, apoM^{FW}, apoM^{WF}, and apoM^{Q22A}, respectively.

demonstrated the same immunoreactivity in a monoclonal antibody-based ELISA as did plasma-derived apoM. In this context, it is noteworthy that both monoclonal antibodies that were used in the ELISA reacted with conformation-dependent epitopes. Together, our results suggest that the prokaryotically expressed apoM was correctly folded and functionally similar to apoM from the eukaryotic system and that the highly efficient prokaryotic system is suitable for the production of large amounts of soluble apoM.

Among the lipocalins, a tryptophan located at the bottom of the hydrophobic pocket is highly conserved (9, 23). According to homology modeling, the tryptophan at position 47 in apoM corresponds to the conserved tryptophan. In addition, apoM has a tryptophan at position 100 (i.e., at the top of the binding pocket) (Fig. 1) (6). These two tryptophans made it possible to use intrinsic fluorescence quenching to investigate the binding of hydrophobic ligands. Intrinsic fluorescence quenching studies have been used successfully for ligand binding studies for RBP, apoD, and several other lipocalins (21, 22, 24, 25). In this study, RBP was used as a positive control. All-*trans*-retinol and all-*trans*-retinoic acid bound to RBP with a K_d of ~0.1 μM, which agrees well with previously reported results (21, 22).

Of the many different hydrophobic substances tested, only vitamin A (all-*trans*-retinol) and its two derivatives, all-*trans*-retinoic acid and 9-*cis*-retinoic acid, showed significant binding to apoM, with K_d values of ~2–3 μM using this method. The three substances bound to apoM^{Q22A}, apoM^{WW}, and the two apoM mutants, apoM^{FW} and apoM^{WF}, with similar K_d values (Table 1). Together with the observation that the mutations did not affect binding to the monoclonal antibodies, this indicates that the apoM mutants were correctly folded. The binding of the three substances quenched the intrinsic fluorescence of apoM^{FW} and apoM^{WF} to the same extent as was observed for apoM^{WW}. This suggests that both tryptophans are involved in the quenching of the fluorescence in apoM and that the polarity of the environment around both tryptophans is affected by the binding of retinol and retinoic acid. In vivo, apoM is linked to the lipoproteins with the retained signal peptide, and at present we cannot exclude the possibility that the signal peptide affects the ligand binding properties of apoM, even though the lipocalin domain of apoM is an independently folded unit.

In an attempt to evaluate whether retinol and retinoic acid bound in the hydrophobic pocket of apoM, an ANS competition assay was used. 1,8-ANS is a much used "hydrophobic probe" for proteins (e.g., apoD), membranes, etc. It is noteworthy that ANS bound to apoM with much lower affinity than did retinol and retinoic acid. Moreover, in the competition assay, both retinoic acids were only able to partially displace bound 1,8-ANS from apoM. The displacement of 1,8-ANS could be the result of a confor-

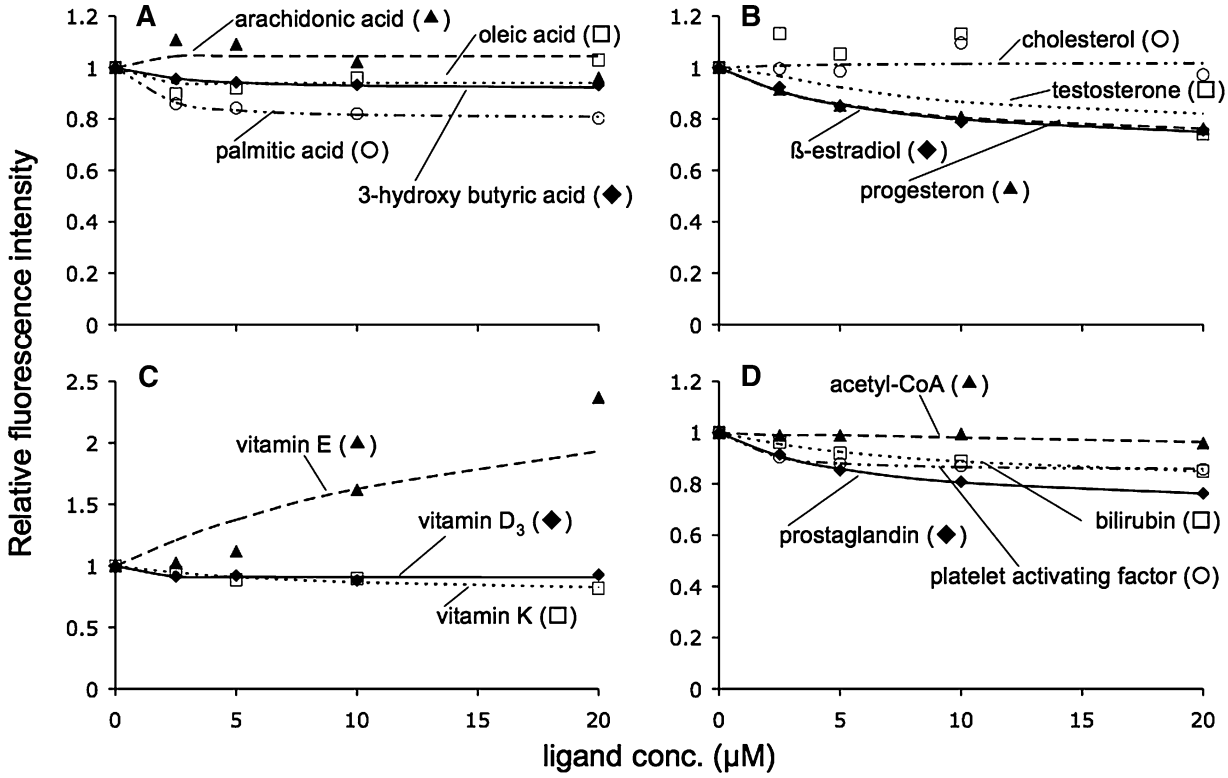

Fig. 7. Intrinsic fluorescence quenching studies of the ligands that did not affect, or only slightly affected, the intrinsic fluorescence of apoM^{WW}. A: Steroids. B: Fatty acids. C: Vitamins. D: Other substances. The relative fluorescence intensities were plotted against the total concentration of the ligand added to the system. The lines demonstrate the fitting of the data to equations 1 and 2 in Methods.

mational change in apoM upon binding of retinoic acid or direct competition between the substances. 1,8-ANS is conventionally considered to bind to hydrophobic surfaces through its nonpolar anilinonaphthalene group. However, Matulis et al. (17) showed that 1,8-ANS binding to a number of proteins can occur through electrostatic forces, making it possible for several 1,8-ANS molecules to bind to the same apoM molecule. In conclusion, the experiments using ANS suggested that ANS and the retinoids possibly bind to the same site, the affinity for the retinoids being higher than that for ANS. It is possible that the relatively bulky ANS molecule did not fit in the hydrophobic pocket of apoM, whereas the smaller retinoids could fit better.

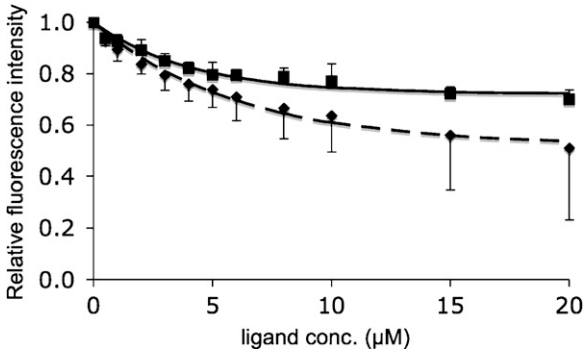

Fig. 8. Quenching of the extrinsic fluorescence in the 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS) competition assay. ApoM was precomplexed with 1,8-ANS. Increasing concentrations of retinoic acid were added. The relative extrinsic fluorescence was plotted against the total concentration of the ligand added to the system. All-*trans*-retinoic acid (closed diamonds, dashed line), 9-*cis*-retinoic acid (closed squares, continuous line). Points represent means ± SD.

Retinoids (vitamin A and its derivatives) exhibit multiple and diverse biological functions and are important for vision, the regulation of gene expression affecting reproduction, hematopoiesis, and growth (10, 26, 27). All-*trans*-retinol represents the biological nonactive transport form and serves as the precursor of active intracellular retinoids as all-*trans*-retinoic acid and 9-*cis*-retinoic acid (28). With the exception of vision, the actions of retinoids are mediated by these two retinoic acid isomers. Several human lipocalins have been shown to bind retinoids (21). ApoD binds all-*trans*-retinol tightly but also many other substances, including cholesterol, fatty acids, and pheromones (21, 24, 25, 29). RBP is the main transporter of retinol in blood, but there are alternative ways to transport retinoids (30). Dietary retinoids are incorporated into chylomicrons as retinyl esters along with the other dietary lipids in the intestine. The majority of dietary retinoids are cleared by and stored within the liver. The liver secretes retinol into the circulation bound to RBP to meet the vitamin A requirements of tissues. Compared with other tissues, the eye displays a strong preference for retinol uptake when retinol is delivered bound to RBP (28, 31, 32).

RBP knockout mice are viable and fertile even though they have a phenotype with several different features. The RBP knockout mice are able to use an alternative pathway to acquire retinol to the eye (28, 30, 31). Whether or not apoM is involved in the transport of retinol as a backup system for RBP remains to be determined.

In conclusion, the binding of retinol and retinoic acid to recombinant apoM supports the hypothesis that apoM is a lipocalin, although the physiological relevance of this binding has yet to be elucidated.

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ERRATUM

The authors of "Hydrophobic ligand-binding properties of the human lipocalin apolipoprotein M" (*J. Lipid Res.* 2007. 48: 1754–1762) have advised the *Journal* that Equation 1 on page 1756 is incorrect.

The incorrect Equation 1 as originally published:

$$I = I_P\left(1-\frac{[L]}{[L]K_d}\right)+I_{PL}\left(\frac{[L]}{[L]K_d}\right)$$

Equation 1 should read:

$$I = I_P\left(1-\frac{[L]}{[L]+K_d}\right)+I_{PL}\left(\frac{[L]}{[L]+K_d}\right)$$

The erroneous equation appeared initially online but has since been corrected.

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