

Arachidonic acid binds to apolipoprotein D: implications for the protein's function

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Abstract The lipocalin apolipoprotein D (ApoD) is associated in human plasma with lecithin-cholesterol acyl transferase. It has also been found in high concentration in the fluid of gross cystic disease of the mammary gland. Using protein fluorescence quenching, it is shown that ApoD binds arachidonic acid (K_a of $1.6 \times 10^6 \text{ M}^{-1}$) and as previously known progesterone (K_a of $2.5 \times 10^6 \text{ M}^{-1}$), but neither cholesterol nor any of the other prostanoid molecules examined had measurable affinity. This specific binding of arachidonate, also observable directly, suggests a role for ApoD in the mobilisation of arachidonic acid, and hence prostaglandin synthesis.

Key words: Apolipoprotein D; Lipocalin; Arachidonic acid

1. Introduction

Apolipoprotein D (ApoD) is a member of the lipocalin structural family. Despite a low degree of sequence homology the member proteins share a highly similar fold and an affinity for small hydrophobic molecules [1–3]. The function of this protein is unknown but it has been shown to be involved in a number of apparently unrelated biological situations. In the plasma, this protein is associated with apolipoprotein A-I and lecithin-cholesterol acyltransferase (LCAT) forming along with other components, the high density lipoproteins (HDL) [4,5]. ApoD has also been found in the fluid of cysts formed during mammary gross-cystic disease [6], where it is the major protein component. The cyst fluid is rich in many different steroid hormones at concentrations that can reach values up to 100 times higher than in plasma [7]. Whilst ApoD binds several of these steroids, it demonstrates a particular affinity for progesterone and pregnenolone but none at all for cholesterol [8,9].

The production of protein has also been detected during some growth or regeneration processes. In particular, during the regeneration of peripheral nervous tissue in the rat [10], where it was observed that the basal levels of ApoD and apolipoprotein E increase several fold. Also, during steroid modulated growth of some cancer cell lines (breast lines ZR-75, MCF-7, T37-D [11–13] and prostate LNCaP [14]) production of ApoD presents a biphasic pattern. In both situations, the ApoD level was increased when the cell multiplication stopped. Further, in normal human diploid fibroblast cell lines, ApoD is secreted when cells have reached a senescent stage [15]. The

properties and the possible functions of ApoD have recently been fully reviewed by Milne et al. [16] and Flower [17].

We report here a study of the binding of a variety of ligands to ApoD and the identification of a high affinity for arachidonic acid, leading to a proposal for the function of the protein which could relate the presence of the protein in gross cystic disease, its association with LCAT in blood plasma and in the secretions from some cancer cells.

2. Materials and methods

2.1. Protein purification

ApoD was purified from gross-cystic disease fluid (GCDF) by a modification of the published method [6]. The fluid was obtained from patients either at the Hospital de Jove, Gijón (Asturias), or from the Breast Clinic, Edinburgh Royal Infirmary. Several batches of GCDF were spun at 4°C for 1 h (30,000 rpm, Beckman rotor 50Ti) to pellet cell debris. The supernatant was concentrated 5-fold and 0.5–0.8 ml of the concentrate were loaded onto a preparative gel filtration column (Beckman TSK300SWG 21.5 × 300 mm, precolumn Beckman Sphero-gel 7.5 × 75 mm) attached to a Waters HPLC system, equilibrated and eluted at 0.2 ml·min⁻¹ with 0.1 M ammonium acetate, pH 6. A further step, based upon ApoD purification from plasma [18] was introduced to achieve higher purity. Fractions found to contain ApoD were pooled, dialysed against 1 mM K₂HPO₄, pH 8, and loaded onto a 2.6 × 70 cm hydroxyapatite column (BioRad) equilibrated with the same buffer. Protein contaminants were retained by the column and ApoD was eluted at a flow rate of 40 ml/h with the loading buffer. Analysis of the protein preparations by SDS-PAGE and by Western blotting, showed that the ApoD was effectively free from contaminating material.

2.2. Ligand-binding experiments

The ligand-binding experiments were performed at 25 ± 0.1°C with a Parker-Elmer Luminescence Spectrometer L-50 with slits set at 5 nm bandwidth, the sample excited at 280 nm and the emission recorded at 340 nm (the wavelength of maximum emission for the protein under these conditions). 50 mM KH₂PO₄ buffer, pH 7.0, was used throughout and the ethanol used for dissolving the ligands was of chromatographic grade (Rathburn). To correct for the inner filter effect [19], the titration of *N*-acetyl-tryptophanamide (Sigma) with each ligand and ethanol was measured under the same conditions as the protein with the absorbance at 280 nm adjusted to equal that of the protein.

All of the titrations were performed with protein isolated from a single pool of cyst fluid (8 patients). The protein (in a weakly buffered stock solution) was diluted to $2.0 \times 10^{-6} \text{ M}$ ($\epsilon = 32,320 \text{ M}^{-1}\text{cm}^{-1}$ [20]). Ligands (100 μM in ethanol) were added to the protein solution in 2 or 4 μl aliquots to a total of 40 μl. For each ligand, three independent titrations were performed and a standard deviation estimated. A base line for the effect of the ethanol on the fluorescence of ApoD was produced by titrating the protein with ethanol, and the emission spectra were recorded at different stages of the titration. The measurements were corrected for protein concentration and averaged between assays for the same ligand before finally having the ethanol baseline (corrected and averaged as for the protein titrations) subtracted. These values were

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then further corrected if the Nacetyl-tryptophanamide titrations showed significant inner filter effect.

The ligands used were the following: progesterone (4-pregene-3,20-dione), cholesterol (5-cholesten-3 β -ol), prostaglandin E1 ((11 α ,13E,15S)-11,15-dihydroxy-9-oxoprostano-13-enoic acid), prostaglandin F2a ((5Z,9 α ,11 α ,13E,15S)-9,11,15-trihydroxy-prosta-5,15-dienoic acid), prostaglandin D2 ((5Z,9 α ,13E,15S)-9,15-dihydroxy-11-oxoprostano-5,13-dien-1-oic acid), arachidonic acid (eicosa = 5Z,8Z,11Z,14Z-tetraenoic acid), palmitic acid (hexadecanoic acid), DPPC (L- α -dipalmitoyl phosphatidyl choline) all from Sigma. 12-HETE (12-hydroxy-[S-(E,Z,Z,Z)]-5,8,10,14-eicosatetraenoic acid), 5,15-diHETE (5,15-dihydroxy-[S-[R*,R*-(E,E,Z,Z)]]-6,8,11,13-eicosatetraenoic acid), linoleic acid (octadeca-9Z,8Z,11Z,14Z-tetraenoic acid), oleic acid (octadeca-9Z-enoic acid) from Cascade Biochem, Reading. EP092 ((\pm 5)-endo-(6'-carboxyhex-2'Z-enyl)-6-exo-[1-[(N-phenylthiocarbamoyl)-hydrazono]-ethyl]bicyclo[2.2.1]heptane [21] was provided by Drs. Wilson and Jones from the Department of Pharmacology, University of Edinburgh.

2.3. Analysis of binding

A model of random binding to an unknown number of homogeneous sites was fitted using weighted nonlinear regression of F (the corrected fluorescence) versus $[L]_t$ (total ligand concentration) with F_{max} , K_d and $[P]_t$ (ligand binding site concentration) as the three parameters to be estimated. Weights were either unity or the inverse variance. The program for model fitting is written in C and will be described elsewhere. Data extracted from Figure 6 of Dille et al. [9], where progesterone binding was determined using gel filtration gave values very similar to the ones presented in that report, confirming the validity of the chosen model.

3. Results

The ethanol used to dissolve the ligands, was observed to quench the fluorescence of ApoD (data not shown). Ethanol is known to perturb the structure of another member of the lipocalin family [22] and in order to eliminate protein conformational changes as a reason for quenching, the following

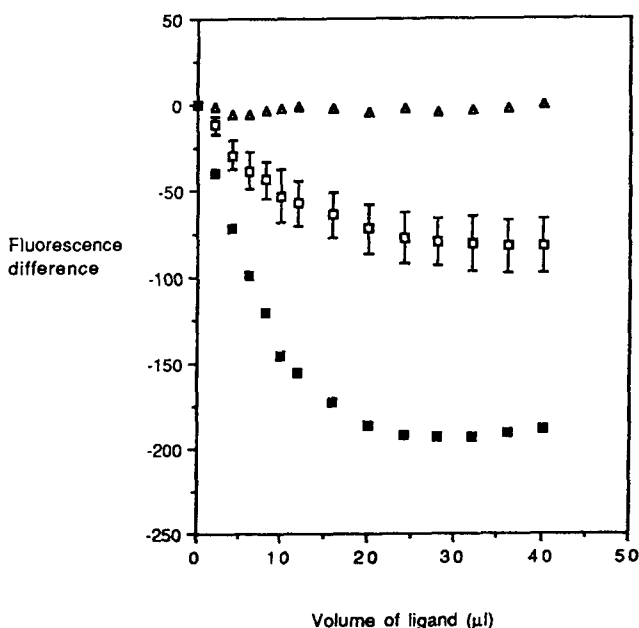


Fig. 1. Titration curves for ApoD from gross cystic disease fluid with progesterone (open squares), cholesterol (triangles) and arachidonic acid (closed squares). The ligand concentration was 100 μ M. Typical error bars for all three titration curves are shown on one of the curves only.

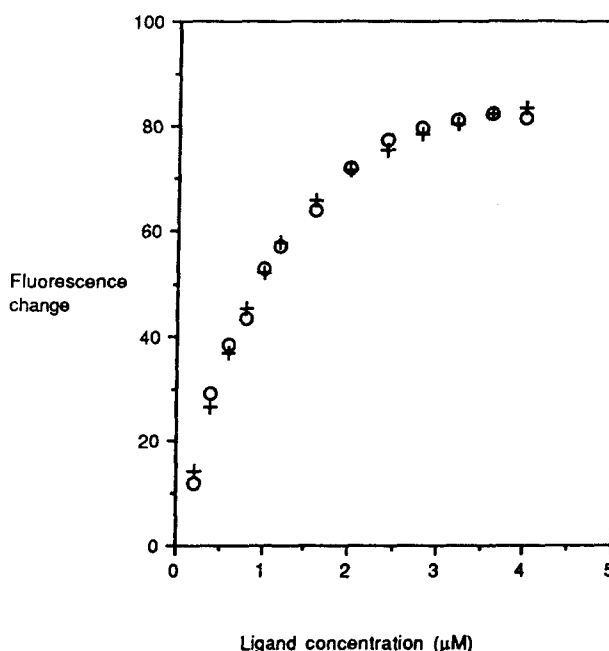


Fig. 2. Fit of the corrected data for ApoD from GDCF with progesterone (○) and that calculated from the model assuming an unknown number of ligand binding sites of equal affinity (+).

controls were made. The emission spectra at different ethanol concentrations were measured and no wavelength shift of the maximum emission was observed with increasing ethanol concentration, indicating that there was no major conformation change affecting the solvent-inaccessible fluorescent groups (data not shown). The absorbance spectra of ApoD (240–350 nm), recorded in the presence of ethanol within the concentration range 0–4% (v/v), did not reveal any changes besides a rise in the overall base line. The CD spectrum of ApoD in the presence of 4% ethanol was recorded between 190–260 nm and compared to the native spectrum. The spectra were superimposable except for the 190–200 nm range, where the noise was clearly greater. Thus, the small increase in derived α -helix content in the presence of ethanol as determined by the CONTIN procedure [23] was almost certainly due to the noise increase in the far UV range. Taken together, these facts indicate that no major conformational change occurs in the range of ethanol concentrations to which ApoD was exposed. A correction for the small ethanol effect was applied to the ligand titration curves by subtraction of the ethanol quenching from the overall ethanol/ligand quenching.

The corrected titration curves for progesterone and cholesterol are shown in Fig. 1. These two molecules served to test the method because it was known that ApoD binds progesterone [8,9] tightly but not cholesterol [8]. The marked difference between the titration curves, showing that progesterone-quenched protein fluorescence reaches saturation while cholesterol did not affect the fluorescence at all, demonstrated that the method was capable of detecting binding. However, the error bars plotted for progesterone were typical and demonstrate that the errors in this experimental system were quite large. Analysis of the weighted binding data gave a good fit to

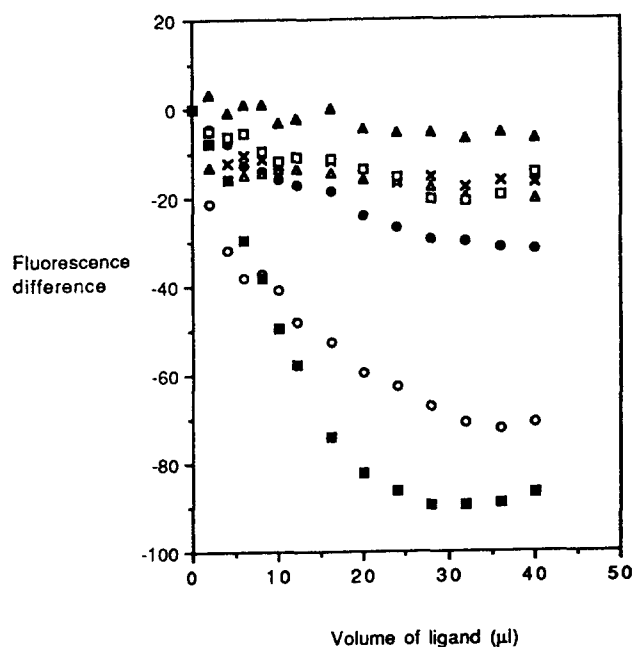


Fig. 3. Titration curves for the ApoD from GCDF with a variety of ligands. Arachidonic acid (filled squares), prostaglandin D2 (filled circles), 5,15-diHETE (filled triangles), 12-HETE (open squares), EP092 (open circles), prostaglandin E1 (open triangles), prostaglandin F2a (crosses). The ligand concentration was 100 μ M.

the model (Fig. 2) and produced an association constant of $2.53 (\pm 0.31) \times 10^6 \text{ M}^{-1}$ similar to the reported values [8,9]. In addition, we found that ApoD purified from plasma had a similar affinity for progesterone.

The titration curves of several molecules tested for affinity for ApoD (EP092, prostaglandins D2, E1 and F2a, arachidonic acid, 12-HETE, 5,15-diHETE) are presented in Fig. 3. Of these, only EP092 (a thromboxane antagonist [21]) and arachidonic acid demonstrated significant affinity as judged by the quenching of ApoD fluorescence. The binding of arachidonate was confirmed by Lipidex-based methods [24] and size-exclusion HPLC of ApoD incubated with radioactive arachidonic acid. The amount of arachidonate observed to bind directly was comparable to that calculated from the fluorescence data, and higher than those observed for other putative ligands for ApoD, including progesterone and progestagen derivatives (data not shown).

Finally, of the small series of fatty acids (arachidonic (C20:4), linoleic (C18:2), oleic (C18:1) and palmitic (C16:0)) and one phospholipid (dipalmitoyl phosphatidyl choline) tested, only arachidonic acid showed any affinity for ApoD. The association constant determined for arachidonic acid was $1.62 (\pm 1.06) \times 10^8 \text{ M}^{-1}$ with the observed weights applied.

4. Discussion

Fluorescence spectroscopy was chosen for this study because it is a technique which has already been used for ligand-binding studies with other lipocalins [25,26]. We have shown that arachidonic acid has the highest affinity for ApoD so far re-

ported, with an association some 20-fold higher than that of progesterone. Interestingly, none of the metabolites of arachidonic acid that were tried showed any affinity for the protein, which may well be because they are all generally more hydrophilic. The same, highly specific binding was found among the small series of fatty acids tried: only arachidonic acid showed significant binding. These factors make the relevance of this ligand in vivo all the more intriguing.

It is tempting to speculate that the affinity of ApoD for arachidonic acid is the unifying factor for all of the biological situations where the protein is present. The eicosanoids and prostaglandins are formed from arachidonic acid and are involved in a wide variety of biological phenomena [27]. The understanding of their mechanisms of action is incomplete and suffers because of their multiple, complex and sometimes contradictory, actions. However, it is known that these compounds are produced locally throughout the organism in response to different stimuli [28], the major step-dependent factor for their production being the presence of the precursors, arachidonic acid, eicosapentanoic acid and homo- γ -linolenic acid. Arachidonic acid is stored in inactive form as phospholipids (in cellular membranes and in lipoproteins), triglycerides (only in the renal medulla) and as cholesteryl-ester (in lipoproteins). The release of arachidonic acid from these inactive forms depends mainly upon the regulation of the activity of phospholipases A2 and C on the membrane phospholipids and of LCAT in HDL, although this latter role in the metabolism of the acid is still unclear.

Through its association with LCAT, ApoD may therefore play a role in regulating metabolism of arachidonic acid in lipoproteins. LCAT is known to act both as a phospholipase and as an esterase [29,30] with the combination of these activities resulting in the synthesis of fatty acid cholesteryl-ester from cholesterol and phosphatidyl-choline. ApoD could scavenge arachidonic acid thus preventing its further conversion to cholesterol ester.

Another aspect of the affinity shown between ApoD and arachidonic acid, is related to their involvement in growth and regeneration processes which foresees a possible role for the protein in these processes. It is known that arachidonic acid metabolism is directly connected to cyclic-AMP formation [31] in, for example, sensitization to pain, the activity of nervous cells and cell proliferation. ApoD, on the other hand, is over-expressing during regeneration of peripheral rat nerve with maximum expression being attained 2–3 weeks after injury when active myelination is occurring [10]. Further, over-expression is seen during the non-growth stages of several, particularly cancerous, cell lines [11–14]. Thus, ApoD could be acting as a carrier of arachidonic acid, removing this active precursor from the cell when division or growth is slowing down.

In conclusion, we propose that the strong specificity of ApoD for arachidonic acid implies a role for this protein in the control of levels of this important metabolite and hence in cell regulation [17]. The understanding of its exact role requires further work.

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