Proposed lipocalin fold for apolipoprotein M based on bioinformatics and site-directed mutagenesis

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Received 6 April 2001; accepted 18 May 2001

First published online 31 May 2001

Edited by Matti Saraste

Abstract Apolipoprotein M (apoM) is a novel apolipoprotein that is predominantly present in high-density lipoprotein. Sensitive sequence searches, threading and comparative model building experiments revealed apoM to be structurally related to the lipocalin protein family. In a 3D model, characterized by an eight-stranded anti-parallel β-barrel, a segment including Asn135 could adopt a closed or open conformation. Using site-directed mutagenesis, we demonstrated Asn135 in wild-type apoM to be glycosylated, suggesting that the segment is solvent exposed. ApoM displays two strong acidic patches of potential functional importance, one around the N-terminus and the other next to the opening of the β-barrel.

Key words: Apolipoprotein M; Comparative modeling; Lipocalin; Site-directed mutagenesis

1. Introduction

Coronary artery disease (CAD) is one of the most common diseases in industrialized countries. Lipoproteins and apolipoproteins can play significant roles in the pathogenesis of CAD and structural information about these molecules will give us a better understanding of their functional relationships in both health and disease. The lipoproteins are a group of diverse particles consisting of a central core of cholesteryl esters and triglycerides and an outer coat of phospholipids, cholesterol and apolipoproteins. The apolipoproteins are responsible for the structure, function and metabolism of the lipoproteins [1,2].

The predominant apolipoprotein on the surface of high-density lipoprotein (HDL) is apolipoprotein A-I (apoA-I), which is an activator of lecithin-cholesterol acyltransferase (LCAT) and promotes reverse cholesterol flux. The other major apolipoprotein (apoA-II) is a hepatic lipase activator. Other apolipoproteins found on HDL particles are apoA-IV, apoC-I, apoC-II, apoC-III and apoD. Similar to apoA-I, apoA-IV and apoC-I are LCAT activators. ApoC-II activates lipoprotein lipase, whereas apoC-III is an inhibitor of the same enzyme [1]. ApoD is a member of the lipocalin protein family but its physiological role in HDL is still unknown [3].

Recently a new human HDL apolipoprotein of unknown function, apoM, was discovered in our laboratory [4]. This 188 amino acid residues long protein was found to be mainly associated with HDL, but could also be detected in triglyceride-rich lipoproteins and low-density lipoprotein. The expression of apoM was found to be confined predominantly to the liver and kidney. Unlike other apolipoproteins, apoM has an N-terminal signal peptide that cannot be processed by the signal peptidase and the retained signal peptide is hypothesized to anchor apoM to the single phospholipid layer of lipoproteins [4]. The apoM gene is highly conserved in several mammalian species, which is consistent with an important biological function.

Knowledge of the three-dimensional (3D) structure of a protein is of major importance in providing insights into its molecular functions, because proteins with similar folds may have similar functions. Moreover, biochemical methods to probe function without consideration of structural information may lead to the wrong conclusion and are time-consuming. Therefore, theoretical techniques allowing prediction of 3D structures followed by computer screening for potential molecular functions used prior to experimental work are very powerful research tools. At present, methods to predict protein structures can be divided in two groups, ab initio and comparative model building methods. While the complexity of the conformational space has so far precluded ab initio prediction of 3D structure of proteins, comparative (or homology) modeling is a reliable approach [5,6].

We propose that apoM is structurally related to the lipocalin protein family. This is based on sensitive sequence searches, threading and comparative model building. The X-ray structures of two lipocalins, mouse major urinary protein (MUP) [7] and human retinol binding protein (RBP) [8], were used as initial templates to build apoM. The conformation of a segment including Asn135, near the opening of the lipocalin binding pocket, was however difficult to predict (e.g. open or closed conformation). We used site-directed mutagenesis and show that Asn135 is glycosylated. Thus, this segment was built in an open conformation. We then analyze the structure of apoM and propose potential molecular functions.

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Abbreviations: ApoM, apolipoprotein M; MUP, major urinary protein; RBP, retinol binding protein; HDL, high-density lipoprotein

PII: S0014-5793(01)02544-3

2. Material and methods

2.1. Database search

The amino acid sequence of human apoM [4] (GenBank accession number AF118393) was subjected to sequence search of public databases using well established tools such as BLAST, WU-BLAST, PSI-BLAST [9] and FASTA3 [10,11]. BLOCKS [12], FingerPRINTScan (FPScan) [13] and Identify/eMotif [14] were used to search for sequence motifs. Because 3D structures are more conserved than amino acids, we also used the fold recognition approach developed by Fischer et al. [15].

2.2. Model construction

The atomic coordinates of mouse MUP (PDB entry 1mup) [7] and RBP (PDB entry 1rbp) [8] were downloaded from Protein Data Bank [16]. The sequence alignment of MUP, RBP and apoM was obtained after manual adjustment of the sequence alignment output obtained from the fold recognition experiment, multiple sequence alignments of several lipocalins and structural alignment of MUP and RBP. The MUP 3D structure was then used to predict apoM but for two regions (see below). After analysis of the experimental templates, it was observed that that the N-terminal segments of MUP and RBP are structurally very different. The superposition of the two proteins is possible only around residues Asn14 (in RBP) and Asn13 (in MUP). The segment N-terminal to these Asn residues adopts a very different conformation. In RBP, Cys4 is disulfide bonded to Cys160, and the N-terminal segment points in a very different direction as compared to MUP. Because of the location of the Cys residues in the apoM sequence and previous experiments [4] suggesting that all apoM Cys are involved in S–S bonds, it is very likely that a disulfide bond involving apoM Cys23–Cys167 mimics the RBP Cys4–Cys160 bridge (see Fig. 1). Therefore, we built the first residues of apoM following the overall structure of RBP. The segment including apoM Asn135 was also difficult to build because this residue is part of a consensus sequence for *N*-glycosylation. The corresponding residue in MUP is essentially buried in the structure and because we show below that apoM Asn135 is glycosylated, the MUP structure could not be used to build this region of apoM. The equivalent segment in RBP is essentially solvent exposed and was thus used to build apoM (see Fig. 1). The predicted transmembrane helix within the N-terminal region of apoM was not built.

The apoM model was constructed with the Modeller package [17] running on DEC Alpha workstations. This program uses spatial restraints from the X-ray templates as input and the model to build has to satisfy as much as possible the set of restraints. Extra constraints were also applied on secondary structure elements at the beginning of the modeling process. Ten potential structures were generated and the one with the fewest restraint violations was chosen for further geometric optimization. Energy refinement was carried out with CHARMM along with the all atoms CHARMM22 parameter set [18,19]. Hydrogen atoms were added and partial charges assigned. Backbone constraints were added at the beginning of the calculations but were gradually decreased during the minimization. This structure was then subjected to a simulated annealing protocol with all secondary structures fixed. The aim here was to allow the loop structures to relax. Temperature was slowly tuned down from 500 K to 100 K (20 K every 10 ps after 20 ps equilibration) and at 100 K the simulation was continued for another 50 ps. The final structure was then briefly energy minimized.

2.3. Electrostatic calculations

Electrostatic properties are important for macromolecular recognition and assembly [20,21]. Thus, we decided to compute some of the electrostatic properties of apoM. Computations were carried out using GRASP [22] or DelPhi [23,24]. The inner and outer dielectric constants were set to 4 and 80, respectively and formal charges were assigned to the main protein titratable groups (Arg, Lys, Asp and Glu).

2.4. Site-directed mutagenesis

Two Asn residues, Asn135 and Asn148, could possibly be glycosylated in apoM. To investigate which of these two were glycosylated, two apoM mutants, Asn135Gln (N135Q) and Asn148Gln (N148Q), were constructed using cloning vector pcDNA3 (Invitrogen) and Quikchange Site-Directed Mutagenesis kit (Stratagene). Two complementary oligonucleotides for each mutant were employed. The sequences of the sense oligonucleotides were: 5-GGTGGAATCATGCTGCAGGAGACAGGCCAGGG (N135Q), 5-CGCTTTCTCCTCTACCAGCGCTCACCACATCCTCC (N148Q). The mutated nucleotides are highlighted by underlined letters. The oligonucleotides were from DNA Technologies A/S. Mutations were verified by DNA sequencing using the BigDye Sequencing kit (Perkin Elmer).

2.5. In vitro expression

The mutants and the wild-type apoM were expressed in TNT Couple Reticulocyte Lysate Systems (Promega) with microsomes (canine pancreatic microsomal membranes, Promega) according to the manufacturer's instructions. [^{35}S]Methionine molecules (Amersham Pharmacia) were incorporated during translation. The expressed products (5 μl) were treated with 5 μl purified PNGase (0.25 μg/μl) at 20°C overnight. The PNGase-treated and untreated expression products were analyzed by 12% reducing SDS–PAGE [25] and visualized using a PhosphorImager (Molecular Dynamics).

3. Results and discussion

ApoM is a new apolipoprotein with unknown function. In order to facilitate the elucidation of its physiological function, we decided to predict and analyze its 3D structure. A fold relationship to an already characterized protein often allows information to be derived about a possible function of a protein. Protein functions can be divided into phenotypic, cellular and molecular types depending on whether the known functions are at the level of the whole organism, at the cellular, or

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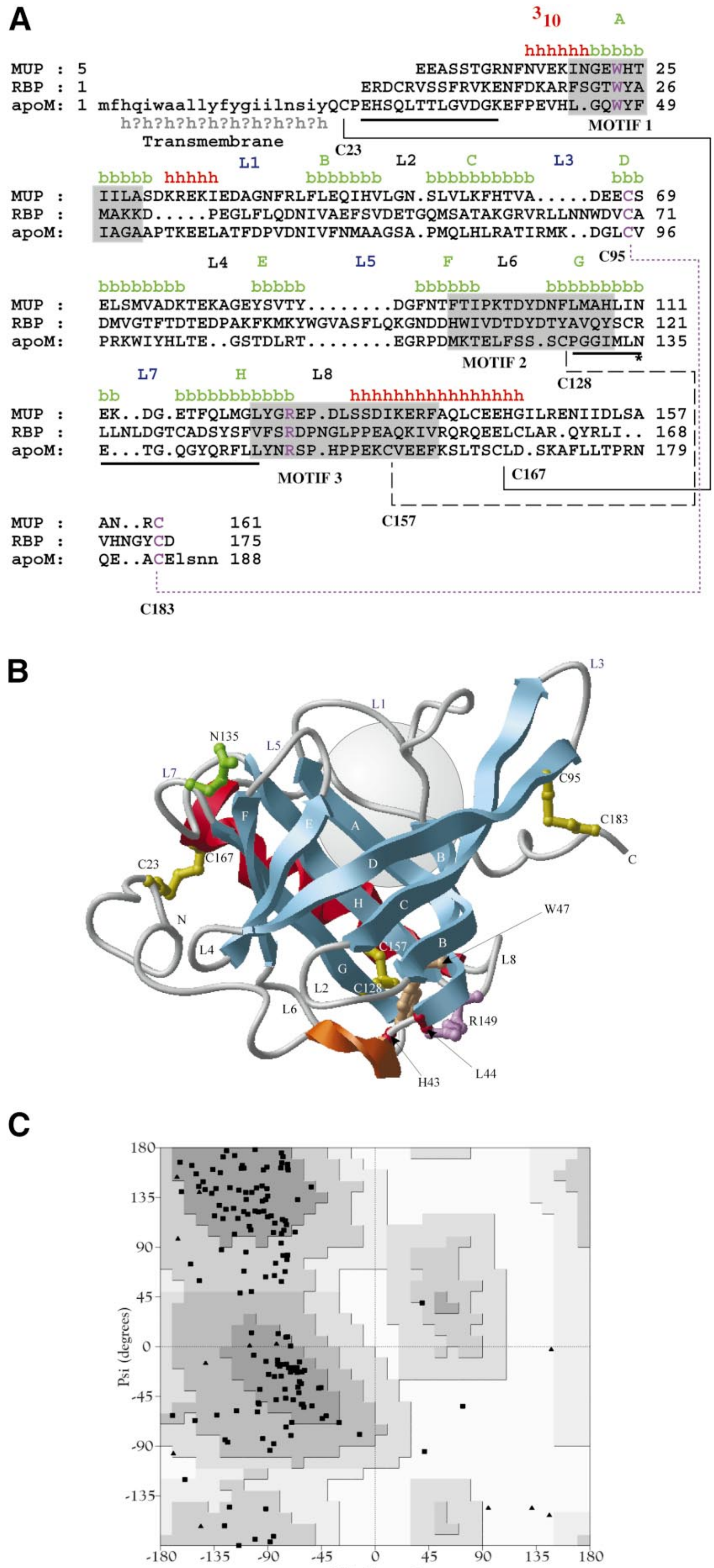
Fig. 1. A: Structural alignment. Structure-based sequence alignment of MUP, RBP and apoM. Because of the extreme sequence divergence in the lipocalin family and some structural deviations, slight variations in the alignments are possible. The numbering follows the amino acid sequence for apoM and was taken from the PDB files for MUP and RBP. The shadowed areas represent the conserved sequence motifs that characterize the kernel lipocalins. Only motifs 1 and 2 are present in apoM. A star marks the experimentally identified glycosylated Asn (residue 135). The MUP X-ray structure was used as template to build apoM but for the two underlined regions that come essentially from the RBP structure. The lower case letters in the apoM sequence indicate regions that were not predicted (the present apoM model runs from residues 22 to 184). The hydrophobic N-terminal segment of apoM is not a cleavable signal peptide but rather a transmembrane α-helical signal anchor. The proposed disulfide (S–S) bonds for apoM are shown (dashed lines). A conserved contact in the lipocalins involves packing of an Arg (apoM Arg149) against a Trp (apoM Trp47) together with hydrogen bonds between the guanido group of this Arg with main chain carbonyl groups of the N-terminal 3_{10} helix (apoM His43 and Leu44). Secondary structure elements are taken from the MUP structure (hhhh symbol defines helices and bbbb defines strands as part of the eight-stranded anti-parallel β-barrel surrounding the ligand binding site, labeled A–H). The loops are labeled L1–L8. These segments are essentially conserved in apoM but for the α-helix between strands A and B, only present in MUP. B: Ribbon drawing of apoM. Predicted secondary structure elements and loops are labeled as in A. Loops L1, L3, L5 and L7 are located near the opening of the binding pocket. The other loops (L2, L4, L6 and L8) are on the opposite end. The regions where important structural changes are observed between MUP and RBP involve the N-terminal segment, the L1, L2, L3, L5 and L7 loops. The glycosylated Asn135 residue is shown. The predicted S–S bonds are displayed in yellow. A conserved contact in the lipocalin family that corresponds to apoM residues Arg149, Trp47, His43 and Leu44 is illustrated. A sphere highlights the lipocalin binding cavity. C: Ramachandran plot for apoM. The plot was generated with Procheck. The Gly residues are shown as triangles. Residues are essentially located in the energetically allowed regions.


A
3 10 A
hhhhhhbbbbb
MUP : 5 EEASSTGRNFNVEKINGEWHT 25
RBP : 1 ERDCRVSSFRVKENFDKARFSGTWYA 26
apoM: 1 mfhqiwaallyfygiilnsiyQCPEHSQLTTLGVDGKEFPEVHL.GQWYF 49
h?h?h?h?h?h?h?h?h
Transmembrane
C23
MOTIF 1
L1 B L2 C L3 D
bbbbb hhhhh bbbbbbb bbbbbbbbbb bbb
MUP : IILASDKREKIEDAGNFRLFLEQIHVLGN.SLVLKFHTVA.....DEECS 69
RBP : MAKKD.....PEGLFLQDNIVAEFSVDETGQMSATAKGRVRLLNNWDVCA 71
apoM: IAGAAPTKEELATFDPVDNIVFNMAAGSA.PMQLHLRATIRMK..DGLCV 96
C95
L4 E L5 F L6 G
bbbbbbbb bbbbb bbbbbb bbbbbbbbb
MUP : ELSMVADKTEKAGEYSVTY........DGFNTFTIPKTDYDNFLMAHLIN 111
RBP : DMVGTFTDTEDPAKFKMKYWGVASFLQKGNDDHWIVDTDYDTYAVQYSCR 121
apoM: PRKWIYHLTE..GSTDLRT........EGRPDMKTELFSS.SCPGGIMLN 135
MOTIF 2
C128
L7 H L8
bb bbbbbbbbbbb hhhhhhhhhhhhhhhh
MUP : EK..DG.ETFQLMGLYGREP.DLSSDIKERFAQLCEEHGILRENIIDLSA 157
RBP : LLNLDGTCADSYSFVFSRDPNGLPPEAQKIVRQRQEELCLAR.QYRLI.. 168
apoM: E...TG.QGYQRFLLYNRSP.HPPEKCVEEFKSLTSCLD.SKAFLLTPRN 179
MOTIF 3
C167
C157
MUP : AN..RC 161
RBP : VHNGYCD 175
apoM: QE..ACElsnn 188
C183
B
L3
L1
N135
L5
L7
C95
C183
F
E
A
D
B
C
C23
C167
H
C
N
W47
B
L4
C157
L8
G
C128
L2
L6
R149
H43
L44
C
180
135
90
45
0
-45
-90
-135
-180
-135
-90
-45
0
45
90
135
180
Psi (degrees)
Phi (degrees)

at the molecular level [26]. Several lines of evidence outlined below suggest that apoM adopts the lipocalin fold, which will form the basis for future experimental approaches aiming at the investigation for apoM of known lipocalin functions. Furthermore, the molecular surface of the apoM model was screened in search of regions of potential functional importance such as putative binding sites.

The lipocalins form a family of proteins with very limited amino acid sequence identity (often below 20%) but with a common tertiary structure. In this family, an eight-stranded anti-parallel β-barrel surrounds a central cavity generally lined with hydrophobic/aromatic residues that enable binding of small hydrophobic molecules [27–30]. Lipocalins fulfill a wide variety of functions such as pheromone activity [7], enzymatic [31], cell regulation [32], and immune regulation [33]. They can be involved in the pathogenesis of diseases [34–37]. The lipocalin scaffold has been used in biotechnology and medicine [38]. The kernel group of lipocalin shares three conserved sequence motifs which correspond to conserved structural elements, while a more diverse group (outlier lipocalins) of the family shares no more than two of these motifs. The disulfide bonding pattern is not always conserved and for instance RBP seems to be the only lipocalin containing three S–S bridges [27]. Some lipocalins have a free cysteine responsible for homo- and/or heterodimerization. Only three lipocalins (apoD, Blc and lazarillo) are known to be attached to biological membranes, either via a hydrophobic loop or via a glycosylphosphatidylinositol anchor, and they all appear to play a role in membrane biogenesis and repair [39].

3.1. Proposed lipocalin fold for ApoM

Database sequence searches (BLAST, WU-BLAST, PSI-BLAST and FASTA3) were not able to match the apoM sequence with any protein family, while protein motif searches (BLOCKS, FPScan and eMotif) suggested the amino acid sequence of apoM to be related to the lipocalin family. ApoM has two lipocalin sequence motifs and can be classified into the more divergent group, the outlier lipocalins. The fold recognition method indicated that the apoM sequence could be accommodated unambiguously only into the lipocalin fold. The most closely related experimental structure identified during this search was MUP (PDB entry file 1mup, z-score = 6.29) followed by apoD (theoretical model PDB entry 2apd [40]). Because the z-score is above the confidence threshold of the method and since the protein motif searches suggested that apoM adopts a lipocalin fold, we initially decided to predict the 3D structure of apoM using the structural framework of MUP.

3.2. Rationale of the modeling process and evaluation of the apoM structure

The structure-based sequence alignment that was used to build apoM is presented in Fig. 1A. Secondary structures for the model were defined according to Kabsch and Sanders [41] (Fig. 1B). The stereochemistry of the apoM model was evaluated with Procheck [42] (Fig. 1C). The root mean square deviation for the C_α trace between MUP and apoM is 1.37 Å (121 residues selected). Charged residues are essentially solvent exposed or appropriately counterbalanced via formation of salt bridges.

Earlier experimental results showed that apoM does not form inter-molecular covalent bonds with other macromolecules [4], suggesting that all cysteines are involved in intramolecular disulfide bonds. It was possible to build the apoM Cys23–Cys167 disulfide bond when the RBP structure was used as initial template. The Cys95–Cys183 bond of apoM is conserved in MUP (Cys68–Cys161) and RBP (Cys70–Cys174). We assumed that apoM Cys128–Cys157 forms a disulfide bond because these two Cys are located close in space in the 3D model. We thus suggest that apoM, like RBP, has three disulfide bridges. The overall apoM model appeared reasonable at this stage with regard to the disulfide bridges but one key structural problem remained that involved the region of Asn135. This residue is part of a consensus sequence for *N*-glycosylation and is near the opening of the hydrophobic binding pocket, which is characteristic of lipocalins. When this segment of apoM was built using MUP as structural template, Asn135 was essentially buried. We concluded that this conformation is most likely incorrect, because Asn135 is glycosylated and it is clear that a glycosylated residue has to be essentially solvent exposed. There are two potential glycosylation sites in apoM, one at Asn135 and the second at Asn148, although the latter position has a proline downstream of the Asn-Arg-Ser sequence and is therefore likely not glycosylated. SDS–PAGE analysis of apoM, derived either from in vitro translation experiments or from plasma, demonstrated that there are two bands, one at 26 kDa representing the glycosylated protein and another less pronounced, at approximately 23 kDa representing non-glycosylated apoM [4]. Thus, it seems that a small quantity of non-glycosylated apoM coexists with glycosylated apoM in vivo. In order to determine the exact glycosylation site, wild-type apoM and N135Q, N148Q mutants were expressed in vitro and the products were digested by PNGase. The absence of the glycosylated band at 26 kDa before and after the PNGase treatment in the N135Q lane strongly supports that N135 in wild-type apoM is glycosylated and that the N135Q mutation results in the loss of the single glycosylation side chain in apoM

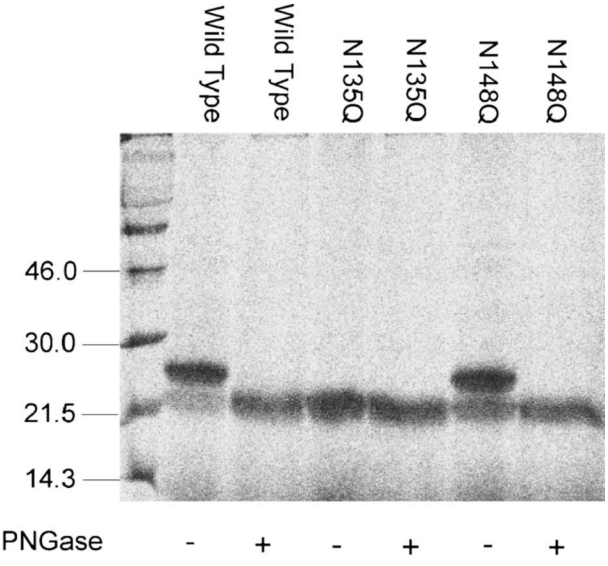


Fig. 2. Glycosylated Asn residue in apoM. To elucidate the exact position of the glycosylation site in apoM, two mutants, N135Q and N148Q, were constructed and expressed. The products were then treated with PNGase overnight. Both treated (PNGase +) and untreated (PNGase −) products were analyzed by 12% reducing SDS–PAGE.

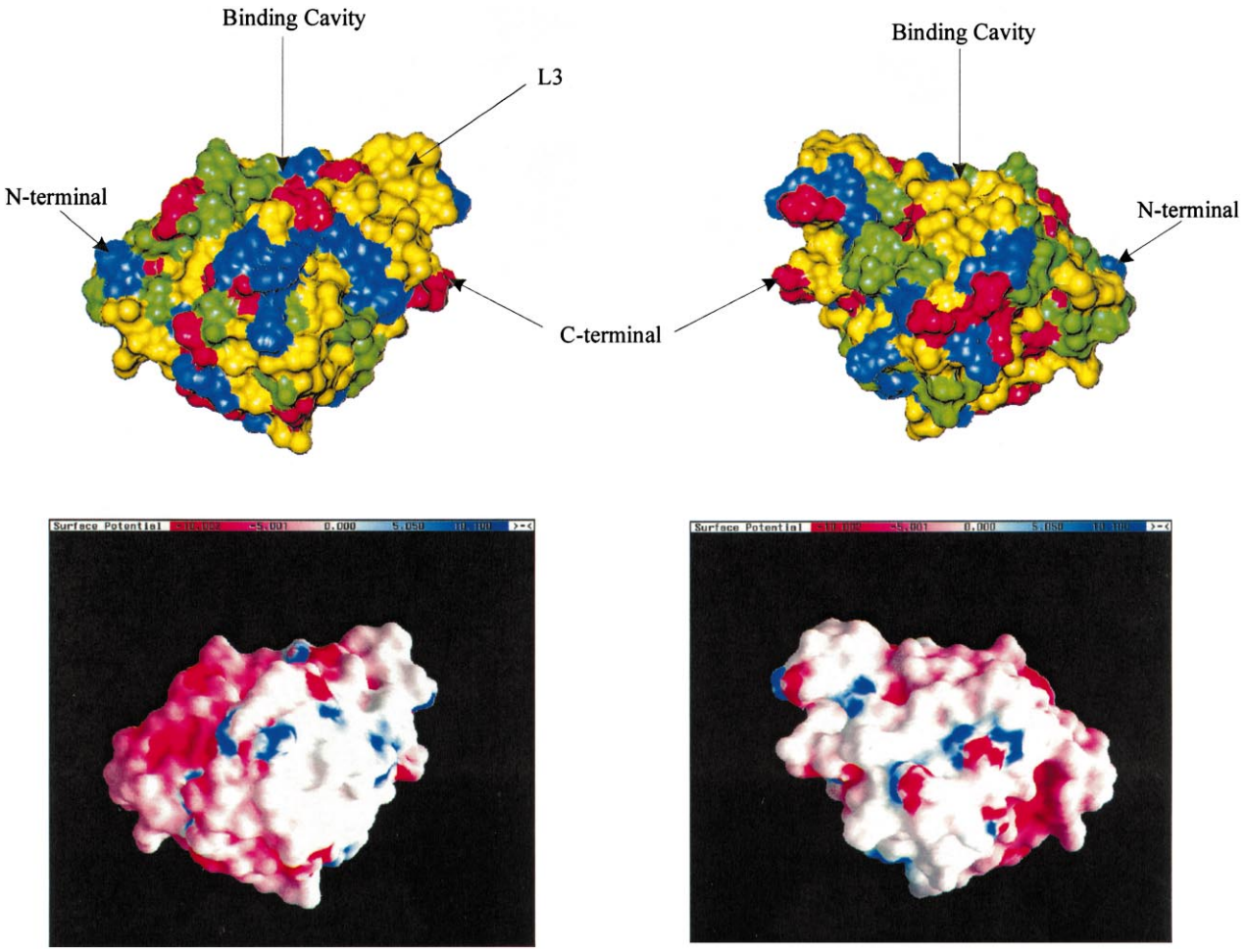


Fig. 3. Outline of the molecular surface of apoM. Top: The solvent accessible surface of apoM is shown with the same orientation (left) as for Fig. 1B and rotated (right) in order to see the other face of the protein. Side chains are colored as follows: blue (positively charged residues), red (negatively charged), green (polar, and backbone atoms) and yellow (hydrophobic and aromatic). An exposed hydrophobic/aromatic patch, involving the residues Pro65, Phe63, Cys95–Cys183, Ile88, Met90, Leu94 and Pro97, at the level of the L3 loop is indicated. Bottom: Surface electrostatic potential of apoM. Negative potentials are in red and positive potentials in blue. Two acidic patches can be seen (see text).

(Fig. 2). The segment including Arg121 of RBP, corresponding to apoM Asn135, is solvent exposed and it was therefore used to build this region of apoM.

3.3. Putative structure–function relationships of apoM

ApoM retains an uncleaved N-terminal signal peptide, which most likely anchors the molecule into single layer lipids on HDL. The major phospholipid in HDL is phosphatidylcholine, which has a positively charged choline group exposed to the solvent [43]. Two electronegative regions are striking in the apoM model and are located around the N-terminus and the opening of the binding pocket (Fig. 3). Interaction between the N-terminal electronegative region of apoM and the phosphatidylcholine of HDL is possible and could be of functional importance. Moreover, strong negative potentials were also found at the opening of the binding pocket and could play a role for interactions with putative ligands or membrane receptors. The opening of the binding pocket is large in the model to accommodate the glycosylated Asn135. The key residues lining the pocket are: Thr113, Leu111, Tyr102, Met119, Leu82, Met73, Thr109, Val70, Asn68, Phe49, Arg143, Tyr147, Leu145, and Met133. In addition, an exposed hydrophobic patch is present at the surface of apoM and could be involved in intermolecular interactions.

4. Conclusion

ApoM is a new apolipoprotein of unknown function for which we provide structural and functional information. Based on threading, sequence searches and homology modeling, we propose that apoM adopts the lipocalin fold. Therefore, the known functions of this family of protein can be investigated for apoM. Three disulfide bridges are suggested to be present in apoM, which would make it a member of the lipocalin subgroup of proteins with three S–S bonds. ApoM has a glycosylated Asn residue at position 135. The amino acids lining the cavity have been listed and are essentially hydrophobic/aromatic. These residues can thus be mutated to probe the putative ligand binding pocket of apoM. Electronegative regions are seen at the N-terminus of apoM and near the entrance of the ligand binding pocket while a hydrophobic patch is seen next to the L3 loop. These regions might be of functional importance and can now be evaluated rationally based on the 3D model structure.

Acknowledgements: This work was supported by grants from the Swedish Medical Council, the Network for Cardiovascular Research funded by the Swedish Foundation for Strategic Research, the Louis Jeantet Foundation of Medicine, research funds from the University Hospital in Malmö, the Fondation pour la Recherche Médicale, and a pre-graduate fellowship from Biomedicinska Forskarskola in Lund funded by the Swedish Foundation for Strategic Research (J.D.). Coordinate file will be available at the following URL: http://www.klkemi.mas.lu.se/dahlback and Links.

References

[1] Patsch, W. and Gotto, A.M.J. (1996) Methods Enzymol. 263, 3–32.
[2] Alaupovic, P. (1996) Methods Enzymol. 263, 32–60.
[3] Francone, O.L., Gurakar, A. and Fielding, C. (1989) J. Biol. Chem. 264, 7066–7072.
[4] Xu, N. and Dahlback, B. (1999) J. Biol. Chem. 274, 31286–31290.
[5] Koehl, P. and Levitt, M. (1999) Nature Struct. Biol. 6, 108–111.
[6] Sali, A. (1998) Nature Struct. Biol. 5, 1029–1032.
[7] Böcskei, Z., Gromm, C.R., Flower, D.R., Wright, C.E., Philips, S.E.V., Cavaggioni, A., Findlay, J.B.C. and North, A.C.T. (1992) Nature 360, 186–188.
[8] Cowan, S.W., Newcomer, M.E. and Jones, T.A. (1990) Proteins 8, 44–61.
[9] Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Nucleic Acids Res. 25, 3389–3402.
[10] Pearson, W.R. and Lipman, D.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2444–2448.
[11] Pearson, W.R. (1990) Methods Enzymol. 183, 63–98.
[12] Henikoff, S. and Henikoff, J.G. (1994) Genomics 19, 97–107.
[13] Scordis, P., Flower, D.R. and Attwood, T.K. (1999) Bioinformatics 15, 799–806.
[14] Nevill-Manning, C.G., Wu, T.D. and Brutlag, D.L. (1998) Proc. Natl. Acad. Sci. USA 95, 5865–5871.
[15] Fischer, D. and Eisenberg, D. (1996) Protein Sci. 5, 947–955.
[16] Bernstein, F.C. et al. (1977) J. Mol. Biol. 112, 535–542.
[17] Sali, A. and Blundell, T. (1993) J. Mol. Biol. 234, 779–815.
[18] Brook, B., Bruccoleri, R., Olafson, B., States, D., Swaminathan, S. and Karplus, M. (1983) J. Comp. Chem. 4, 187–217.
[19] MacKerell, A.D. et al. (1998) J. Phys. Chem. B 102, 3586–3616.
[20] Nakamura, H. (1996) Q. Rev. Biophys. 29, 1–90.
[21] Sheinerman, F.B., Norel, R. and Honig, B. (2000) Curr. Opin. Struct. Biol. 10, 153–159.
[22] Nicholls, A., Sharp, K.A. and Honig, B. (1991) Proteins 11, 281–296.
[23] Gilson, M.K. and Honig, B.H. (1987) Nature 330, 84–86.
[24] Sharp, K.A. and Honig, B. (1990) Annu. Rev. Biophys. Biophys. Chem. 19, 301–332.
[25] Laemmli, U.K. (1970) Nature 227, 680–685.
[26] Moult, J. and Melamud, E. (2000) Curr. Opin. Struct. Biol. 10, 384–389.
[27] Flower, D.R., North, A.C. and Sansom, C.E. (2000) Biochim. Biophys. Acta 1482, 9–24.
[28] Akerstrom, B., Flower, D.R. and Salier, J. (2000) Biochim. Biophys. Acta 1482, 1–8.
[29] Murzin, A.G., Brenner, S.E., Hubbard, T. and Chothia, C. (1995) J. Mol. Biol. 247, 536–540.
[30] Orengo, C.A., Michie, A.D., Jones, S., Jones, D.T., Swindells, M.B. and Thornton, J.M. (1997) Structure 5, 1093–1108.
[31] Flower, D.R. (1996) Biochem. J. 318, 1–14.
[32] Flower, D.R. (1994) FEBS Lett. 354, 7–11.
[33] Coles, M., Diercks, T., Muehlenweg, B., Bartsch, S., Zölzer, V., Tscheche, H. and Kessler, H. (1999) J. Mol. Biol. 289, 139–157.
[34] Logdberg, L. and Wester, L. (2000) Biochim. Biophys. Acta 1482, 284–297.
[35] Xu, S. and Venge, P. (2000) Biochim. Biophys. Acta 1482, 298–307.
[36] Mantyjarvi, R., Rautiainen, J. and Virtanen, T. (2000) Biochim. Biophys. Acta 1482, 308–317.
[37] Bratt, T. (2000) Biochim. Biophys. Acta 1482, 318–326.
[38] Skerra, A. (2000) Biochim. Biophys. Acta 1482, 337–350.
[39] Bishop, R.E. (2000) Biochim. Biophys. Acta 1482, 73–83.
[40] Peitsch, M.C. and Boguski, M.S. (1990) New Biol. 2, 197–206.
[41] Kabsch, W. and Sander, C. (1983) Biopolymers 22, 2577–2637.
[42] Laskowski, R.A., MacArthur, M.W., Moss, D.S. and Thorton, J.M. (1993) J. Appl. Crystallogr. 26, 283–299.
[43] Deguchi, H., Fernandez, J.A., Hackeng, T.M., Banka, C.L. and Griffin, J.H. (2000) Proc. Natl. Acad. Sci. USA 97, 1743–1748.