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α_2-μ-Globulin fragment (a2-f) from kidneys of male rats

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Abstract:
The structure of α_2-μ-Globulin fragment (A2-f) is not known.α_2-μ-Globulin fragment (A2-f) is a 15.5 kDa protein that binds equimolar amount of fatty acids in male rat kidneys. The expression of this protein has been shown to change in response to drug-induced and genetic hypertension which suggests that it plays an important role in renal fatty acid metabolism under pathological conditions as well as normal conditions. A2-f has sequence homology with amino acid 28-178 of α_2-μ-globulin (A2U) that is synthesized pre-dominantly in the male rat liver and is present in the urine. It is believed that unusual structural features permit A2-f to be targeted to the proximal tubule cell; to escape lysosomal degradation in liver and to enter the cytosol of proximal tubule cells of the kidneys. Homology modeling has been employed to determine the structural elements of this protein and they have been compared with the published structure of A2U. Results suggest differences between the structure of A2-f and its precursor protein A2U.

Keywords: α_2-μ-Globulin fragment, Kidney fatty acid binding protein, Chemically-induced nephropathy.

Background:
α_2-μ-Globulin fragment (A2-f) is an unusual protein because unlike most other physiologically known proteins, it is synthesized in the male rat liver but enters another organ. Some of the unusual properties of A2-f are: (i) it escapes degradation by proximal tubule lysosomes; (ii) it selectively accumulates in the proximal tubule, where it may represent the most abundant cytosolic protein; and (iii) it crosses a biological membrane to enter the cytosol. The structural features that mediate this unusual behavior have not been identified **[1]**. A2-f is also thought to be a unique kidney fatty acid binding protein for several reasons: (a) it has a similar molecular size as all known FABPs; (b) it is found in the soluble protein fraction; and (c) it is abundant in cells with high fatty acid flux and binds long chain fatty acids *in vitro* **[2]**. The potential role of this protein in the proximal tubule was determined in isolated proximal tubule segments **[1]**. The presence of A2-f in proximal tubule, the site of the highest fatty acid flux, further supported the idea that A2-f is a unique kidney fatty acid binding protein **[2]**.

Complete sequence analysis of A2-f revealed that it is derived by the proteolysis of A2U. Proteolytic removal of amino acids from A2U (18.6 kDa) produces A2-f, which has an apparent Molecular weight of 15.5 kDa **[3, 4]**. The proteolytic conversion is believed to be mediated by lysosomal cysteine proteases. However, the specific enzyme(s) responsible for converting A2U to A2-f have not been identified **[5]**. The amino acid sequence of the signal peptide of A2U has been determined using a combination of nucleic acid and protein sequencing techniques **[6]**. Like most secreted proteins, the majority (13 out of 19) of the amino acid residues in the N-terminus are hydrophobic (or uncharged). Using a combination of protein and nucleic acid sequencing, the amino acid sequence of signal peptide of A2U has been determined **[6]**. Like many secreted proteins, the majority of N-terminus residues (13-19) are hydrophobic or unchanged. There is no clear resemblance between this signal sequence and the one identified for pre-albumin, a protein produced and secreted by the same cells in liver.

A2-f binds long chain fatty acids *in vitro* with a 1:1 stoichiometry and with a high affinity (K_d ranges from 0.1-2 μM) **[2]**. Metabolic data obtained from suspensions of fresh proximal tubule segments showed that A2-f facilitates fatty acid oxidation and promotes ATP production **[1]**. A2-f helps maintain a large, intracellular pool of readily available substrates which allows support of a high rate of fatty acid oxidation **[1]**. In addition to its effect on metabolism, A2-f can also prevent injury caused by free fatty acids by partitioning fatty acids away from membranes and preventing them from disrupting membrane function and causing organelle injury **[7]**. A2-f can also modulate a "second messenger" effect of fatty acids on specific ion channels **[8]**.

A study has shown that direct lysosomal uptake of A2-f contributes to chemically-induced nephropathy **[9]**. The increment in lysosomal levels of A2-f cannot be explained by a decrease in its proteolytic susceptibility. In rats exposed to TMP, the specific lysosomal transport of A2-f increases, as well as the ability of lysosomes to directly transport other substrates for this pathway. This result suggests that the chemically induced accumulation of cytosolic A2-f in lysosomes is mediated by an increased rate of direct uptake into lysosomes. Since nothing is known about the structure of A2-f, homology modeling has been employed in the present study to determine its structural elements and they have been compared with the published structure of A2U.

Methodology:

The sequence of A2U was acquired from the SWISS-PROT data base using the accession code P02761. The published amino acid sequence of A2-f was used as the target protein **[10].** Secondary and tertiary structures of A2-f were determined by the use of 3D-JIGSAW program **[11, 12, & 13].** This program is modular in design with each module centered on a particular algorithm required in the modeling process. It is based on rigid-body assembly and can either be run in fully automatic mode via a web server or the program modules may be executed separately and the intermediate files saved. The homology modeling comprised three steps: (i) template selection, (ii) target-template alignment and (iii) model building. Essentially, this is an iterative procedure until a satisfactory model is obtained. 3D-JIGSAW predicts protein structure on the basis of each amino acid in the protein sequence based on the probability that it will form an α-helix or a β-strand, etc. In the predicted secondary structure, each amino acid is given a score between 0-9, where 0 means that system cannot predict it and the amino acid residue can form any structural element whereas a score of 9 means it has a very high probability of forming a particular structural element.

Discussion:

In the present study, the secondary and tertiary structures of A2-f were determined by the use of the program, 3D-JIGSAW. This program has been designed to work on targets found to have at least one parent structure with no less than 40% sequence identity between target and parent(s). 3D-JIGSAW superimposes proteins to create a multiple sequence alignment. It uses rigid-body fragments to construct the complete backbone and uses fragments that vary in size between those of loop replacements to multiple secondary structure motifs when more than one template is used **[14, 15]**. Side-chains are then added using knowledge-based information and refined by a mean field approach in which the effects of solvation are considered.

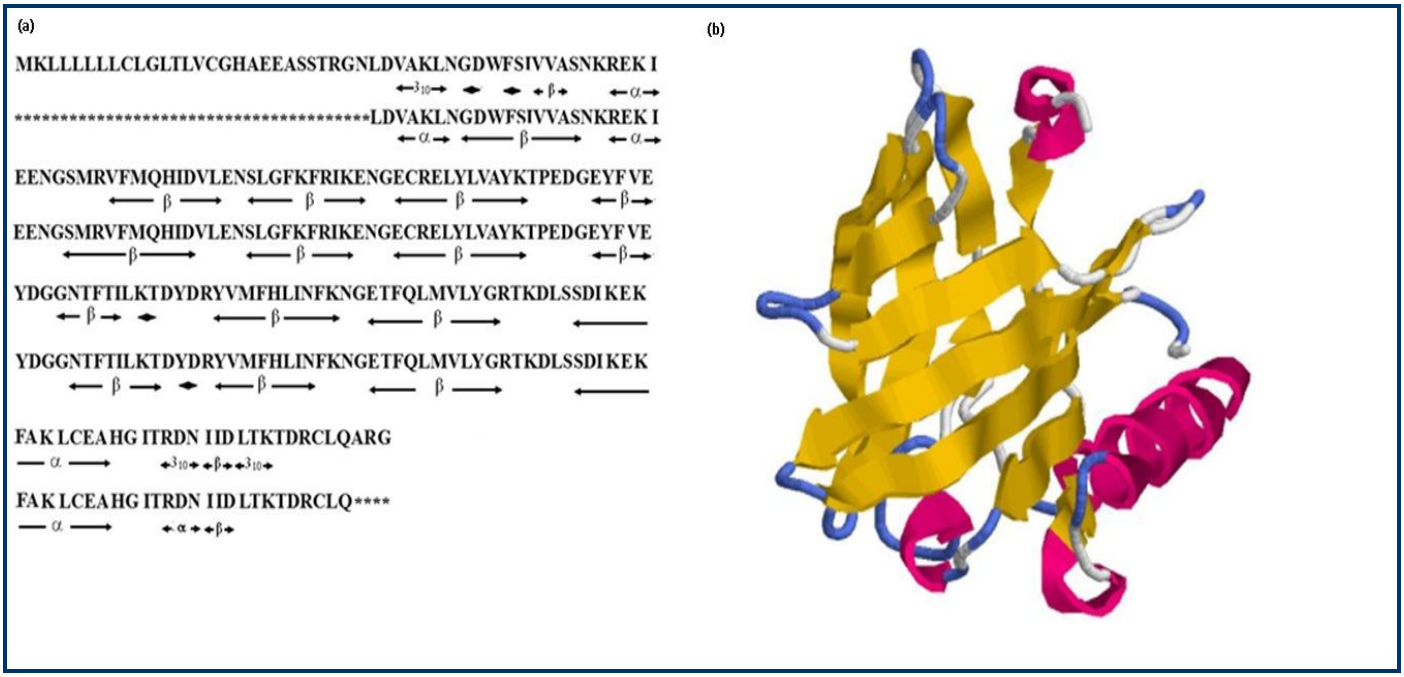

Figure 1: (a) Comparison of secondary structure elements of A2-f with A2U. The secondary structure elements of A2U are based on the crystal structure of A2U; **(b)** Tertiary structure of A2-f obtained by homology modeling.

A2-f is a member of a family of fatty acid binding proteins called lipocalins. These proteins are very diverse at the sequence level yet have highly conserved tertiary structures. Twelve structures of members of the lipocalin family have been

solved experimentally **[16]**. Knowledge about structures has revolutionized the understanding of properties of lipocalins. The structures show that most lipocalins share three conserved sequence motifs (kernel lipocalins) while others share only one or two sequence motifs (outlier lipocalins). The lipocalins are part of a larger protein superfamily, the calycins, which includes the fatty acid binding proteins, avidins, a group of metalloproteinase inhibitors, and triabin. The superfamily is characterized by a similar structure (a repeated +1 topology β-barrel) and by the conservation of a structural signature **[16]**. Analysis of known crystallographic structures of lipocalin family reveal that there is overall structural similarity. The structure of individual proteins is specifically adapted for binding ligands by forming a binding site in the internal cavity (inside the β-barrel) and/or an external loop scaffold. The structure of the lipocalin fold reveals that both the ends and sides of this barrel are different. These differences are also shown by analyses of structural and sequence variation within the family. These differences can be explained by a possible difference in functional role between the two ends of the lipocalin fold. The structurally conserved end of the molecule may be involved binding to cell surface receptors, while the structurally variable end may be involved in binding of small ligands and forming macromolecular complexes **[16]**.

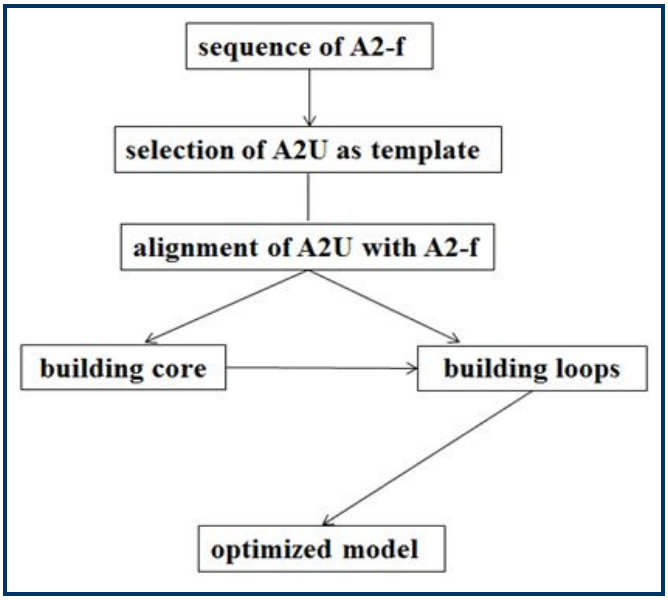

Figure 2: Schematic of the methodology for homology modeling of A2-f.

The tertiary structure of the precursor protein, A2U in complex with a hyaline droplet inducer, was deduced earlier by X-ray crystallography **[17]**. The A2U monomer has a folding topology of an eight-stranded β-barrel made up of two antiparallel orthogonal β-sheets, with both N- and C-terminal extensions (supplementary material). The larger extension at the C-terminus of the barrel consists of an α-helix, which packs onto one face of the barrel, followed by a loop which brings the C-terminal part of the protein back to the other face of the barrel. In both A2U and mouse urinary protein (MUP), the N-terminal extension forms a short β-strand not present in Retinol Binding Protein **[18]**. The crystal structure identified Tyr-124 as involved in hydrogen bonding with the carboxyl group of the ligand fatty acid **[19]**. According to the investigators, the hydroxyl group of Try-124 interacts with the ionized form of the carbonyl group of the fatty acid. Comparison of the cavities in A2U and in the corresponding MUP reveal that the former is tailor-made for small oval hydrophobic ligands such as *d*-limonene 1,2-epoxide. The cavity in MUP is more shallow and elongated and cannot easily accommodate such ligands.

Comparison of the amino acid sequence of A2U and A2-f revealed that the difference in the number of amino acids between the two proteins is thirty one. A2U has 181 amino acids while A2-f has 150 amino acids. In A2-f, twenty eight amino acids have been omitted at the N-terminus and three amino acids at the C-terminus. The additional amino acids in A2U at N-terminus and C-terminus are present either as random coil or as a bend. The homology modeling determined tertiary structure of A2-f is typical for lipocalins **(Figure 1a).** It is an eight-stranded, antiparallel, symmetrical β-barrel fold, which is actually a β-sheet which has been rolled into a cylindrical shape. Inside this is a ligand binding site, which plays an important role in the transport properties of A2-f. The secondary structure **(Figure 1b)** shows that A2-f posseses five α-helices and nine β-strands. There are also 3 single amino acid β-turns, 1 two amino acids β-turn, 1 three amino acids β-turn, 5 four amino acids β-turns, 1 five amino acids β-turn and a single six amino acids β-turn. There are three Glycine residues present in the secondary structure elements. Gly-8 is present at the beginning of β_A; Gly-44 is present in the middle of β_C and Gly-112 is at the end of β_H. The rest of the Glycine residues and all of the Proline residues are present in the β-turns. **Table 1 (see supplementary material)** summarizes the ten observed structural differences between A2-f and A2U. It shows that there are differences between A2-f and A2U in both the position and length of α_I, α_{III}, α_V, β_A, β_B, β_C, β_F and β_G. There is also an additional β-strand and 3_{10}-helix present in A2U.

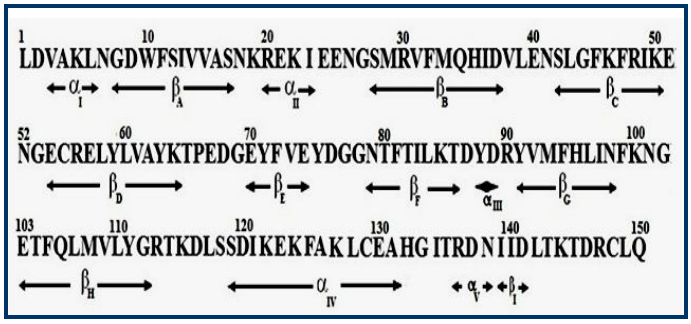

Figure 3: Secondary structure elements of A2-f obtained by homology modeling.

Kimura *et al*. **[20]** earlier showed that A2-f possesses a disulfide bond between Cys-55 and Cys-148. A free Cys-129 was also found to be present by reaction with iodoacetamide under denaturing and non-reducing conditions. From structural studies on many lipocalins it is likely that the disulfide bond may be important to maintain the 3-D structural integrity of the protein for binding of hydrophobic ligands **[21]**. The role played by the disulfide bond has also been examined in rat cutaneous fatty acid-binding protein by chemical and mass spectrometric analyses. These analyses revealed two disulfide bonds between Cys-67 and Cys-87, and between Cys-120 and Cys-127. Cys-43 had a free sulfhydryl group. A homology modeling study of the

protein also provided evidence for the presence of the disulfide bonds. These disulfide bonds apparently are not directly involved in fatty acid-binding activity, because a recombinant rat protein expressed in *E. coli* in which all five cysteine residues were fully reduced still showed fatty acid-binding activity. The fact that the evolutionarily distant shark liver fatty acid-binding protein also has a disulfide bond corresponding to the one between Cys-120 and Cys-127, suggests that some functions of cutaneous fatty acid-binding protein might be regulated by the cellular redox state through formation and reduction of disulfide bonds **[22]**.

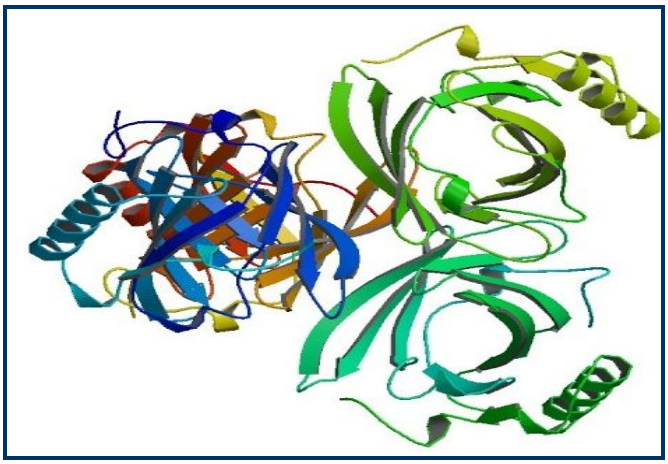

Figure 4: Ribbon diagram of A2U based on the crystal structure solved at 2.5 Å resolutions **[18].**

Conclusion:

The structure of A2-f predicted by homology modeling contains a β-barrel formed by two sets of four anti-parallel β-strands and an α-helix consisting of 12 residues near the C-terminus. An internal cavity and an external loop scaffold is present within the β-barrel which gives rise to different binding modes for ligands of different size and chemical structure. The determined structural elements of A2-f do not readily explain its properties such as escape from degradation by proximal tubule lysosomes, the crossing of a biological membrane to re-enter the cytosol and selective accumulation in the proximal tubule cells. However, the structural elements do reveal differences between A2-f and its precursor protein, A2U.

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Supplementry material:

Table 1: Total Heterotrophic and hydrocarbon degrading bacterial count

Secondary Structure Element	*Differences between A2-f and A2U*
α_I	In A2-f, an α-helix is present. In A2U, a 3_{10} helix is present at the very same position.
β_A	In A2-f a longer and uninterrupted single β-strand is present but in A2U, in this position, 3 short β-strands are present.
β_B	Length of β_B is *longer* in A2-f. It also starts earlier and ends earlier.
β_C	Length of β_C is *shorter* in A2-f. It ends earlier at Lys-50.
β_F	Length of β_F is *longer* in A2-f. β_F starts later and ends later at Thr-86 in A2-f.
Additional β-strand	A2U has an extra β-structure between Lys-113 and Thr-114. This structure is missing in A2-f.
α_{III}	A short α-helix is present in A2-f between Tyr-88 and Asp-89. This element is missing in A2U.
β_G	Length of β_G is *shorter* in A2-f. It starts later at Tyr-91 and ends earlier at Asn-98.
α_V	In A2-f, α_V is present between Arg-136 and Asn-138. In A2U, a 3_{10} helix is present at this position.
Additional 3_{10}-helix	An *additional* 3_{10}-helical element is present in A2U from Leu-170 to Lys-172.