

BINDING OF SERUM AMYLOID A TO PERLECAN AND THE EARLY
DETECTION OF AA AMYLOID

by

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ABSTRACT

Previous studies demonstrated a spatial relationship between the basement membrane protein, perlecan (and its glycosaminoglycan heparan sulphate) and amyloid, an extracellular, fibrillar, protein deposit of diverse origin. In the inflammatory-associated experimental amyloid model (amyloid A model), there is co-deposition of these components with the amyloid A fibril protein, and perlecan mRNA levels increase before amyloid A deposits are detected histologically. Heparan sulphate causes serum amyloid A (SAA), the precursor of the amyloid A fibril protein, to have more β -sheet structure, which is the conformation of the protein in the deposit. The potential role of perlecan in amyloid A amyloidogenesis was approached by: monitoring conformation changes in SAA in the presence of various glycosaminoglycans; and measuring the affinity of perlecan for SAA. Tryptophan fluorescence showed an SAA conformation change in the presence of heparan sulphate even when SAA is in a synthetic lipoprotein. Using enzyme linked immunosorbent assay procedures, the SAA-perlecan binding interaction has a K_d of 10^{-5} M or more. Modification of the *in vitro* conditions did not decrease the K_d of the interaction. Binding assays which coupled SAA or perlecan to Sepharose 4B beads also determined a low affinity interaction. The temporal association between perlecan mRNA levels, amyloid enhancing factor activity, and amyloid A deposition is based on Congo red staining of histological sections. Studies with Thioflavine T, a fluorescent dye that undergoes specific excitation and emission wavelength shifts when bound to amyloid, was used to quantify type A amyloid deposition over a four day period, and to show that murine amyloid A deposits are present 12 h after induction with silver nitrate and amyloid enhancing factor. These findings make it difficult to determine which of amyloid deposition or perlecan mRNA levels precedes the other. Furthermore, they suggest that amyloid enhancing factor activity could be the results of early, histologically undetectable amyloid fibrils.

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LIST OF ABBREVIATIONS

AA	amyloid A
A β	Alzheimer's amyloid
AEF	amyloid-enhancing factor
AH	heavy chain amyloid
AL	light chain amyloid
β PP	β -protein precursor
BSA	bovine serum albumin
CcR	cytochrome <i>c</i> reductase
CD	circular dichroism
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate
CR	Congo red
DEAE	diethylaminoethyl
DTT	dithiothreitol
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EDTA	ethylenediaminetetraacetic acid
EHS	Engelbreth-Holm-Swarm
ELISA	enzyme linked immunosorbent assay
FAP	familial amyloid polyneuropathy
FT-IR	Fourier transform infrared
GAG	glycosaminoglycan
HDL	high density lipoprotein
IL	interleukin
LCAT	lecithin cholesterol acyl transferase
Lp	lipoprotein particle
NEM	<i>N</i> -ethyl-maleimide
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PC	phosphatidyl choline
PMSF	phenylmethanesulfonyl fluoride
RT-PCR	reverse transcription-polymerase chain reaction
SAA	serum amyloid A
SAP	serum amyloid P
sulfo-NHS	sulfo- <i>N</i> -hydroxysuccinimide
TBS	Tris-HCl buffered saline
TES	Tris-HCl EDTA saline
TGF- β	transforming growth factor- β
ThT	Thioflavine T
TNF- α	tumour necrosis factor- α
Tris-HCl	2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride
TTBS	Tween-20/Tris-HCl buffered saline

1. INTRODUCTION

1.1 Definition of Amyloid

Amyloid is a term that describes an extracellular, proteinaceous deposit which possesses very specific morphologic, staining, and structural characteristics (1). When stained with traditional dyes, amyloid appears amorphous. However, after being stained with Congo red (CR) and viewed through polarized light, amyloid takes on a green-birefringent appearance, indicating some type of organized, underlying structure (2). Ultrastructurally, amyloid appears as a non-branching group of fibrils, each having a diameter of 7 to 10 nm and having an indefinite length (3). Fourier transform infrared spectroscopy (FT-IR) and X-ray crystallography have determined that amyloid has a protein conformation similar to that of silk protein, which consists of stacks of anti-parallel β -sheets, known as a cross- β pleated sheet (4,5). In addition, biochemical analysis has shown the amyloid fibrils to be insoluble and resistant to proteolysis (6).

1.2 Staining of Amyloid

Histochemical stains have been used to help differentiate amyloid from surrounding tissue, and to provide clues to additional constituents and to how deposition may occur. Comparisons of staining of amyloid with CR with other dyes have shown that viewing CR stained tissue through a polarizing filter is the most specific way of differentiating amyloidotic and non-amyloidotic tissue (7). Determining the binding site on the fibril for the dye may help determine the structure of amyloid. Turnell and Finch (8) have found that with globular insulin, CR binds between anti-parallel β -sheets, lending support to the

proposed cross- β sheet structure of amyloid (8). A possible dye binding sequence was found, but it is not present in all fibril precursor proteins. Therefore, CR binding may be dependent on common tertiary or quaternary structures in amyloid, or on some other common structural element that is part of the amyloid fibril structure. This has led some workers to believe that the dye may bind by intercalation or binding in parallel to the cross- β sheet of the fibril. However, recent work has shown that the dye displays green birefringence with the α -helical and β -sheet forms of poly-*L*-lysine, and no birefringence is seen when CR is bound to poly-*L*-serine, a polypeptide that is all β -sheet. This suggests that CR binding and birefringence do not derive specifically from β -sheet structures, so that the dye cannot be used reliably to probe the molecular structure of amyloid (9).

Another amyloid binding dye is Thioflavine T (ThT), a fluorescent dye that displays a shift in excitation and emission maxima when bound to amyloid (10,11). ThT staining of histological sections causes ThT bound to elastic fibrils and nuclei to fluoresce in addition to ThT bound to amyloid, so that ThT staining was thought to be non-specific for amyloid. The problem relates to the excitation light not being a single wavelength, and the inability of the eye to discriminate a single wavelength of emitted light. When stained sections are differentiated with acetic acid or pre-stained with hematoxylin, elastic fibril and nuclear ThT fluorescence is prevented and the stain is considered specific for amyloid.

Unlike light microscopic techniques, ThT experiments on amyloidotic tissue homogenates with a fluorometer have had more success, since the excitation and

emission light are single wavelengths. The dye has recently been used to quantify the amount of murine apoA-II amyloid fibril in tissue homogenates, and to measure the kinetics of *in vitro* fibrillogenesis of apoA-II and the Alzheimer β -protein. (12,13).

1.3 Amyloidosis

Amyloidosis is associated with various diseases, though in some cases it occurs without any other apparent disease(s). Amyloidosis itself, by interfering with organ function, usually leads to organ failure and death. As is shown in Table 1, a different type of fibril protein is present depending on the disease or the pathological process. In the past, classification of amyloid has divided the disorders according to their clinical manifestations (14), but this has now been superseded by a classification according to amyloid protein type (15).

Table 1. Fibril Proteins

Amyloid Protein	Fibril Protein Precursor
AA	SAA
AL	κ , λ light chains
AH	IgG heavy chains
ATTR	Transthyretin
AApoA1	Apo-AI
AGel	Gelsolin
ACys	Cystatin C
A β	β -protein precursor
A β 2M	β 2-microglobulin
AScr	Scrapie protein
ACal	(Pro)calcitonin
AANF	Atrial natriuretic factor
AIAPP	Islet amyloid polypeptide
AIns	Insulin
AApoAII	ApoA-II

Approximately 20 different amyloidogenic proteins have been described, but there is no obviously similar primary sequence, secondary structure, or function (6). This diversity suggests that amyloidosis is not a single disease, but rather a final common pathway by which these diverse proteins are deposited in a fibrillar manner.

1.4 Possible Mechanisms in the Formation of Amyloid

Since amyloidosis describes a heterogeneous group of diseases, different pathogenetic mechanisms might initiate the formation of amyloid. Several mechanisms have been implicated, including modified precursor protein because of a mutation; increased synthesis of the precursor; or altered proteolytic cleavage of the precursor (6).

1.4.1 Primary Sequence and Mutation of Amyloid Precursor Proteins

Because the amyloid fibril has a predominantly cross- β pleated sheet structure, it was thought that some common sequence motif would explain the tertiary structure of all amyloid fibril proteins. Based on nuclear magnetic resonance spectroscopy (NMR) and FT-IR work, specific sequence motifs have been proposed for some fibril proteins, but no motif has been found that is common to all amyloid fibril proteins (5,16).

Primary sequence is apparently important in transthyretin amyloid, the protein found in familial amyloid polyneuropathy (FAP). The first FAP families studied were shown to have a point mutation in residue 30 (15). Since then, 41 mutations have been found in transthyretin, which are spread over the whole protein. The mutated transthyretin is present from birth, yet amyloid is not seen until 50 to 60 years later. Furthermore,

different kindreds with the same mutant variant do not necessarily get amyloid (17).

Moreover, in senile systemic amyloidosis, the transthyretin precursor is normal. It is apparent from these observations that while a mutation is a permissive for certain types of amyloid, it is insufficient in itself to cause deposition.

1.4.2 Precursor Quantity

Amyloid A (AA) amyloid deposition requires the presence of an abundance of precursor (6). It occurs in individuals with chronic inflammation, where there is an elevated level of the AA precursor, serum amyloid A (SAA). However, SAA levels have been increased experimentally by the injection of the cytokine tumour necrosis factor- α (TNF- α), and no AA amyloid was detected (18). A similar situation occurs in humans with rheumatoid arthritis, where SAA levels are elevated but AA amyloid is associated with only 2-3% of the individuals (19). The reverse case, where amyloid occurs at lower rather than higher level of precursor, occurs in senile systemic amyloidosis. The levels of the transthyretin are lower in older people than in the young, yet it is the former that develop amyloid (6). This suggests that additional factors are required to develop amyloid.

1.4.3 Proteolysis

In several types of amyloidosis, including AA, light chain (AL), and Alzheimer's amyloidoses, the fibril protein is a proteolytic fragment of the precursor protein. In AL amyloid, the deposit contains a fragment of immunoglobulin light chain. When present in the urine the light chains are named Bence Jones proteins (20). It has been shown that

tryptic cleavage of the Bence Jones protein formed amyloid fibrils *in vitro*, which led to the hypothesis that proteolysis of the precursor causes the formation of amyloid.

Proteolysis of the amyloid fibril precursor, however, is not found in every amyloid. In apoA-I, apoA-II, β -2-microglobulin, cystatin-C, insulin, and transthyretin amyloids the precursor and fibril protein are identical in size and sequence (6,15). In duck AA amyloid deposits, only intact SAA is found, and in rheumatoid arthritic patients with AA amyloid, both the cleaved (AA) and uncleaved protein (SAA) have been found (21-24). Thus proteolysis might occur post deposition, as has been demonstrated by immunohistochemistry for murine AA deposits (25). Using antibodies against AA (the amino-terminus of SAA) and the carboxyl-terminus of SAA, intact SAA was found at the outer surface of the deposit, which is presumably where deposition has occurred most recently, while AA is found in the older, central part of the deposit (25).

1.5 AA Amyloid

To study the common properties of amyloid which may explain its formation, an animal model of amyloidosis is required. For the work presented here, the murine AA amyloid model was chosen since it has been extensively characterized. Because AA amyloid occurs in individuals with chronic inflammation, amyloid in mice is induced by the subcutaneous injection of silver nitrate followed by amyloid enhancing factor (AEF). Silver nitrate is used since it is non-immunogenic, so that unrelated effects of the immune system on amyloid are avoided. The main role of silver nitrate is to induce inflammation and activate the acute phase response, which increases the synthesis of SAA several

hundred fold (26). AEF is injected to reduce the lag time required to detect AA deposits, and to ensure that all the animals form amyloid by 36 h. Without it, in CBA/J mice AA amyloid deposition is detected 7 to 10 days after the induction of inflammation (27).

Regardless of the induction scheme used, AA amyloid is first detected by CR staining in the spleen, specifically in the perifollicular areas, where the deposit is closely associated with capillaries and reticuloendothelial cells (1). Deposition is next seen in the liver, and is followed by deposition in the kidney. The reason why this occurs is not known, but it has been speculated that it is partly due to the fenestrated vascular lining in such areas, allowing easy access of SAA to extravascular spaces, where amyloid deposition can occur.

1.5.1 AA

In the mouse the AA amyloid fibril protein is mainly the 76 amino acid residue *N*-terminal fragment of SAA. However, fragments larger and smaller than the 76 residue AA have been found in the same deposit. In all cases the *N*-terminal end is the same, indicating that this region may be important for fibrillogenesis. As described below, SAA exists in several isoforms, but as determined by sequence analysis, in mice the AA protein is a fragment only of SAA₂ (28). Differences between these two isoforms are shown in Figure 1.

AA deposition is not the result of more SAA₂ mRNA being produced than the SAA₁ mRNA during amyloidosis, nor is it the result of increased levels of SAA₂ protein in the circulation (28). It has been shown that SAA₂ is selectively removed from the circulation during amyloidosis. Because of this, and because there is no splenic SAA₂ mRNA synthesis, AA was proposed to come from the circulatory pool of SAA (28). To prove the hypothesis, one experiment showed that the injection of human SAA and lipopolysaccharide into mice led to human AA in the amyloid deposits (29). This has been difficult to repeat. A more conclusive experiment showed that when murine ³[H]-SAA was injected into mice, ³[H]-AA was detected in the amyloid deposit, proving that the fibril precursor comes from the circulation (30).

Figure 1. Protein Sequence of SAA₁ and SAA₂

Amino-acid sequence of two isoforms of SAA. Sequences are identical except where noted. The conserved region, calcium binding sequence, and proposed GAG-binding sequence are indicated in the figure.

1.5.2 SAA and High Density Lipoproteins

SAA is associated with the serum high-density lipoprotein (HDL) (31). HDL is a sphere of 7-10 nm in diameter, and has an apparent molecular weight of 200 kDa (32). The particle consists of a core of esterified cholesterol and triglycerides surrounded by apolipoproteins, unesterified cholesterol, and phospholipids (33). HDL also contains the enzymes lecithin cholesterol acyl transferase (LCAT), which esterifies cholesterol, and cholesterol ester transfer protein, which transfers cholesterol esters between lipoproteins (34,35). This latter protein is not found in mice (36). The apolipoproteins give the particle stability, and direct the metabolism of cholesterol and triglycerides. They include apoA-I, which is both the most abundant protein on HDL, and also a co-factor for LCAT. This protein is believed to direct the uptake of HDL by cells through an apoA-I receptor. ApoE is believed to direct HDL to the liver for cholesterol excretion, or to other cells requiring cholesterol, where cell surface apoE receptor-mediated endocytosis occurs.

One major role proposed for HDL is reverse cholesterol transport, which is removing cholesterol from peripheral tissues to the liver for catabolism and secretion. This is vital, since the liver is the major site of cholesterol degradation and removal. HDL's role in reverse cholesterol transport is supported indirectly by the observation that HDL concentrations are negatively associated with the risk of coronary heart disease from atherosclerosis (37).

Under non-inflammatory conditions, HDL contains very low levels of SAA (33). Once inflammation occurs, SAA levels increase dramatically. SAA synthesized in the liver

associates predominantly with HDL (85-90% of total SAA), and has been shown to displace apoA-I from the HDL particle *in vitro* (38). Presumably a similar mechanism occurs *in vivo*. Most of the SAA is present in the denser HDL₃ subclass, and accounts for 20% to 50% of HDL's total apolipoprotein (32); only 5-10% of total plasma SAA is associated with the other lipoproteins. The remainder is free or associated with albumin (39).

1.5.3 SAA Isoforms and Expression

In the mouse, SAA is a 12 kDa protein of 104 amino acid residues in length, and has three main isoforms: SAA₁, SAA₂, and SAA₃ (39). Comparisons of these isoforms with SAA from other species shows a highly conserved region from residues 38 to 58.

In situ mRNA detection has shown that the main site of synthesis of SAA₁ and SAA₂ is in the liver (40-43), where during inflammation it can account for 5% of total hepatic protein synthesis (32). The release of interleukins such as interleukin-1 (IL-1), IL-6, and TNF- α during inflammation increase the synthesis of SAA₁ and SAA₂ to levels as high as 1000 times above normal (26,44). This has been shown both by induction of SAA synthesis in hepatocyte cultures (45), and also *in vivo* (18,44). The increase in SAA levels and the time these levels remain elevated depend on the inflammatory stimulus, with systemic bacterial infections being the most effective.

1.5.4 Possible Functions of SAA

Many diverse functions have been proposed for SAA. These include inhibition of LCAT activity (46), suppression of the lymphocyte reaction to antigens (47), action as a chemoattractant for monocytes and polymorphonuclear cells (48), mediation of a decrease of fever during inflammation (49), inhibition of oxidative burst by binding to neutrophils (50), and protection of LDL from oxidation (51). It is thought that the latter three functions are related in that they may limit the extent of damage during an inflammatory response. Finally, since considerable amounts of SAA are bound to HDL, which itself is proposed to be involved in reverse cholesterol transport, SAA might play a fundamental role in cholesterol metabolism during inflammation (42).

1.6 Common Properties of Amyloid

Although the various amyloids differ in their pathogenetic origin (mutation, serum levels, or cleavage of the precursor), all amyloids studied thus far have in addition to their common structural appearance several common elements, which may help explain how amyloid is formed. They include amyloid enhancing factor (AEF), serum amyloid P (SAP), and glycosaminoglycans (GAGs).

1.6.1 Amyloid Enhancing Factor

First discovered in 1966 by Werdelin and Ranlov, AEF is defined as a substance that reduces the lag time required to induce experimental deposition of AA amyloid. AEF itself does not cause AA amyloid deposition (27), an inflammatory stimulus is also necessary. AEF is obtained from the supernatant of amyloidotic tissue homogenates, and

has been found in several types of amyloid, indicating that it is not a protein specific to a particular amyloid type (27,52-54). Time course analysis of AEF supernatants has shown that the activity is present in AA amyloidotic tissue 24 h before the deposit is detected by CR staining, and that the effect of AEF can persist for several days after injection (27).

Characterization of AEF over the last 20 years has resulted in contradictory conclusions. Spleen cells from mice induced to form AA amyloid were shown to have AEF activity, as do peritoneal resident macrophages (55). AEF activity has also been found in normal mouse spleen cell suspensions. The number of normal cells required to exert AEF activity was however many orders of magnitude larger than for cells from an amyloidotic animal (56). This was not verified by another group (57). Lipases and nucleases failed to destroy AEF activity, while proteolysis or denaturing conditions (urea or guanidine-HCl) did, indicating that AEF is a protein or a group of proteins which perhaps requires a particular conformation (27).

Attempts at characterizing the protein(s) involved in AEF have not been conclusive. Recently, ubiquitin, an intracellular protein involved in marking proteins for proteolytic degradation, has been detected in murine AA amyloid and in Alzheimer amyloid deposits (21,58,59). Ubiquitin extracted from both of these tissues has been shown to have AEF activity in mice, while normal ubiquitin did not (21,59). It is not precisely known why this difference exists, nor has this work been repeated.

Another possibility is that AEF may be amyloid fibrils in the AEF extract. It has been shown that AEF contains green birefringent material after staining with CR, and that mice injected with islet amyloid polypeptide or Alzheimer's amyloid fibrils formed *in vitro*, together with an inflammatory stimulus, form AA amyloid rapidly (54). In this case, the injected fibril may be acting as a seed for the formation of the new fibril. The experiment is far from conclusive because of recent data obtained with the amyloid resistant CE/J mouse strain. In these mice, a chronic inflammatory stimulus with or without AEF does not result in AA amyloid deposition. The supernatants of splenic homogenates from CE/J mice receiving repetitive inflammatory stimuli, did however demonstrate AEF activity when they were injected into an amyloid susceptible strain along with an inflammatory stimulus (60). This suggests that the CE/J strain is able to form a fibril-free AEF, but there is no evidence as to what that may be. Thus, AEF describes a group of poorly defined tissue extracts, which accelerate the formation of amyloid.

1.6.3 Serum Amyloid P

SAP is a glycoprotein synthesized by the liver (61). In humans it has been found associated with the glomerular and vascular basement membrane, but its function is unknown (62). SAP has been found in all amyloid deposits that have been examined, and co-deposits temporally and spatially with amyloid (61,63). Because of these observations, it was thought that SAP might be one of the proteins responsible for AEF activity. This was not substantiated. SAP has since been shown to bind to many types of non-amyloid fibrillar proteins (27,64). The binding of SAP to amyloid occurs in a calcium dependent

manner, and the protein has been found to bind to a specific carbohydrate linkage which is found in GAGs (65).

1.6.4 Glycosaminoglycans

Carbohydrates were found in amyloid over 125 years ago by Virchow after staining amyloidotic tissue with iodine (6). Because iodine in the presence of sulphuric acid stains starch or cellulose dark blue, Virchow described the stained area as being 'amyloid' or 'starch-like'. This term, however, is incorrect, insofar as the carbohydrate component has since been shown to be GAGs, linear polysaccharide chains built of multiple disaccharide units of a uronate and a modified hexosamine. Using two cationic dyes specific for GAGs, Cuproinic Blue and Ruthenium Red, it was shown that there is a close structural association of these polysaccharides with the AA amyloid fibrils at the electron microscopic level (66).

To determine the type of GAG present, several experiments have been performed. At the histochemical level, 0.7 M MgCl₂ was added to the Cuproinic Blue stain which makes it specific for highly sulphated GAGs, and the staining did not disappear (66). Treatment of amyloidotic tissue with chondroitinase ABC and AC, enzymes which specifically digest chondroitin sulphate, did not cause a change in the staining pattern, indicating that the GAG was not chondroitin sulphate. Treatment with nitrous acid, which digests heparin and heparan sulphate, destroyed Ruthenium Red and Cuproinic Blue staining. To conclusively determine the specific type of GAG present, the GAGs from AA amyloid were extracted and identified by electrophoresis (67). The results demonstrated that it is

the heparan sulphate GAG that is present in all types of amyloid examined (For a review see (6)).

1.6.5 Perlecan

Heparan sulphate is usually attached to a protein core *in vivo*, forming a proteoglycan. To see whether the protein was the basement membrane form of heparan sulphate proteoglycan, immunohistochemistry of amyloidotic tissue was performed (67, 68).

Tissues examined after AA amyloid induction demonstrated that the protein was perlecan, and that deposition of the AA fibril and this proteoglycan were coincidental or within a few hours of each other. The study found that along with perlecan, the basement membrane proteins laminin, type IV collagen, and fibronectin were also present in the deposit, and that these components were deposited at approximately the same time as AA (68). These data suggest that amyloid may be associated with a general disturbance in basement membrane protein metabolism (6).

1.7 The Basement Membrane and its Proteins

Normal basement membranes are thin layers (20-250 nm wide) of extracellular matrix material composed of proteins such as laminin, entactin, type IV collagen, fibronectin and perlecan (69). Located adjacent to many cell types, they are involved in cell support and stability, selective filtration, compartmentalization of organs, and cellular regulation of migration, growth and differentiation (70,71). The proteins are often produced and deposited by the cells that subsequently attach themselves to the basement membrane. The interaction is mediated by specific cell surface receptors such as integrins (72).

Laminin, type IV collagen, and perlecan have been shown to self-polymerize, and the entire basement membrane is believed to self-assemble through high affinity, non-covalent interactions and subsequent covalent binding, a complicated process due to the large size of the proteins involved (130 - 850 kDa) (70). The sequence and structure of these proteins have been determined from the mouse Engelbreth-Holm-Swarm (EHS) solid tumour (69,73).

1.7.1 The Basement Membrane Proteoglycan Perlecan

Mouse perlecan from the EHS tumour has a protein core that is 400 kDa in weight, with three heparan sulphate chains of 65 kDa each attached near the *N*-terminal end (74,75). Attachment of heparan sulphate is mainly by *O*-glycosylation, though *N*-glycosylation has not been ruled out. There is no post translational modification before incorporation of perlecan into the basement membrane, other than the addition of heparan sulphate (76).

Using cell culture systems and cDNA cloning, the expression of mouse perlecan has been examined. Chromosomal *in situ* hybridization and somatic cell hybridization have shown that there is only one mouse and human perlecan gene (77,78), and that perlecan mRNA is expressed in connective tissue and endothelial cells, with the former being the main site of expression (79). Transforming growth-factor- β (TGF- β) is believed to be an important up-regulator of perlecan expression. When added to cultured colon carcinoma cells, there

was an upregulation of core protein expression which was attributed to mRNA stabilization (80).

Sequencing of perlecan cDNA revealed that the protein has a secretion leader sequence followed by five domains (75). Domain I contains the putative heparan sulphate attachment sequence SGD, and is the only region where there is no sequence homology with other basement membrane proteins. Domain II contains the sequence DGSDE, which has a high sequence homology to the LDL-receptor, suggesting a region that may be involved in apolipoprotein binding. The remaining domains have no known function, but have motifs similar to other proteins: domain III is similar to the globular-rod domain of the laminin short arms, domain IV has N-CAM like repeats, and domain V repeats are similar to the globular regions in laminin.

Perlecan has been located in skin, breast, placenta, kidney glomeruli and tubules, liver and splenic perisinusoidal space, and blood vessels with the aid of monoclonal antibodies (79). Because perlecan is found in many different organs, it likely has multiple functions. Since the heparan sulphate chains bind to both laminin and entactin, perlecan may play a central role in basement membrane assembly (81). It also binds to cells, probably through its RGD sequences, to integrin cell receptors (82). Perlecan may also have a role in skeletal muscle structure, as has been shown by its primitive ancestor in the nematode. A null mutation of the protein resulted in a severe disruption of the skeletal musculature (83). Because heparan sulphate has a highly negative charge, perlecan is involved in charge dependent selective filtration in glomerular basement membranes (84).

1.8 Potential Role of Heparan Sulphate in AA Amyloid

Since a spatial relationship exists between perlecan and amyloid, the question was asked whether perlecan has a fundamental role in the formation of amyloid. Several pieces of evidence support a causative role for heparan sulphate in amyloidogenesis. It was shown that the appearance of heparan sulphate in the tissue occurred at the same time as the fibril was first detected by immunohistochemistry (67). To examine what happens to GAGs before AA amyloid is deposited, the rate of GAG synthesis and GAG levels in the serum were monitored after AEF and/or an inflammatory stimulus was injected into mice (85). The inflammatory stimulus alone increased synthesis of all of the GAGs, but AEF caused an increase in the synthesis of heparan sulphate before amyloid deposition was detected by CR staining.

Another experiment showed a temporal relationship between perlecan mRNA synthesis and AA amyloid deposition. Murine perlecan mRNA levels were measured by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) after induction of AA amyloid (86). By amplifying total splenic mRNA with primers internal to perlecan mRNA, the mRNA levels were shown to be above those of the controls 24 h after induction, 12 h before AA amyloid was detected by CR staining. If an inflammatory stimulus or AEF was given separately, no significant increase in perlecan mRNA was seen. These results suggested that increased perlecan synthesis may be occurring before AA amyloid deposition. In so doing the pool of nascent perlecan may be involved in changing the secondary structure of SAA₂ (see below).

A physical interaction between an amyloid precursor and perlecan was shown by Narindrasorasak *et al.* (87). Using an enzyme linked immunosorbent assay (ELISA), it was shown that perlecan binds to the β -protein precursor (β PP) in a saturable, specific manner with high affinity (10^{-8} M range). The interaction could be inhibited by heparin, but not by chondroitin sulphate or dermatan sulphate, demonstrating that binding probably occurred between β PP and the heparan sulphate chain (87).

McCubbin *et al.* examined whether heparan sulphate may cause a fundamental change of the amyloid precursor SAA, which would facilitate the formation of amyloid (88). In the mouse, there are two main isoforms of SAA in the circulation, SAA₁ and SAA₂, but only SAA₂ forms AA fibrils. In the experiment, SAA conformation was determined by circular dichroism (CD) measurements before and after the addition of various GAGs. It was shown that the addition of heparan sulphate, but not heparin nor chondroitin sulphate, caused SAA₂ to have more β -sheet structure. The addition of heparan sulphate to SAA₁, which is not found in the AA fibril, did not cause an increase in the percentage of β -sheet in the protein, showing that the change is specific to the fibril precursor.

Additional work has focused on the SAA from the amyloid resistant CE/J strain of mouse. In this strain there is only one SAA, which is a hybrid of SAA₁ and SAA₂, and has a pI of 6.15 (89). When the CD experiments described above were repeated, it was shown that the SAA_{pI=6.15} did not acquire more β -sheet structure after the addition of heparan sulphate (89), supporting the hypothesis that the conformational change to more β -sheet in SAA₂ is a requirement for AA amyloid deposition.

2. RESEARCH OBJECTIVES

The evidence presented suggests that perlecan may have a fundamental role in amyloidogenesis. The purpose of the work was three-fold, firstly, further characterization of the conformational change in SAA by heparan sulphate, secondly, characterization of the binding of perlecan to SAA, and thirdly, the early detection of AA amyloid with more sensitive techniques. The later could have implications on the interpretations of the role of perlecan in amyloidogenesis, and secondly, on the nature of AEF. These objectives were approached in the following ways:

- 1) To determine whether the conformational change of SAA by heparan sulphate occurs while SAA is within a lipid environment, the protein was incorporated into a synthetic lipoprotein particle, and conformational changes were monitored by tryptophan fluorescence after the addition of various GAGs.
- 2) To determine the affinity of the interaction between perlecan and SAA, three different solid phase assays were used: a) an ELISA; b) a method similar to immunoprecipitation; and c) competition of HDL-SAA in the high affinity basement protein interaction between perlecan and fibronectin.
- 3) To detect AA amyloid as early as possible, ThT fluorescence, a more sensitive amyloid detection technique, was used to quantify the amount of AA amyloid in murine spleen homogenates at various times after amyloid induction.

3. MATERIALS AND METHODS

3.1 Animals

Swiss white CD₁, 6-8 week old female mice from Charles River were used for all amyloid experiments. C57BL mice from Charles River were used for EHS tumour production.

3.2 Materials

Chemicals were purchased from BDH (Poole, UK), Fisher Scientific (Ottawa, ON), or Sigma Chemicals (St. Louis, MO). All chemicals were of analytical grade or better. [³⁵S]-sulphate, [¹²⁵I]-chloride, and Universol were purchased from ICN (Costa Mesa, CA). All column materials were from Pharmacia (Uppsala, Sweden). The glycosaminoglycans, lipopolysaccharide, cytochrome *c* reductase (CcR), and phosphatidylcholine (PC) were from Sigma Chemicals. Aquacide was obtained from Calbiochem (San Diego, CA). The BioRad DC Protein assay kit, a modification of the Lowry assay, was from BioRad (Hercules, CA).

ELISA plates were obtained from Dynatech (Chantilly, VA), ICN/Flow (Costa Mesa, CA), and Nunc (Roskilde, Denmark). Sulfo-*N*-hydroxysuccinimide (sulfo-NHS) was purchased from Pierce (Rockford, IL). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was purchased from Sigma Chemicals. Fibronectin was obtained from Gibco-BRL (Gaithersburg, MD). Sheep anti-rabbit IgG alkaline phosphatase was obtained from Boehringer Mannheim (Mannheim, Germany). Anti-fibronectin and all of the bovine serum albumin powders were purchased from Sigma Chemicals. ThT was obtained from Aldrich (Milwaukee, WI).

3.3 HDL and HDL-SAA Purification

HDL and HDL-SAA were purified according to the method of McCubbin *et al.* (88). Inflammation was induced by subcutaneous injection of 0.5 mL 2% (w/v) silver nitrate. After 18 h, the mice were killed by placing them in a carbon dioxide atmosphere and bled by cardiac puncture. Plasma was separated from the blood by centrifugation at 10 000 *g* for 30 min. KBr was added to the plasma so that the final density of the solution was 1.063 g/L. The sample was centrifuged at 75 000 *g* for 16 h at 4°C in a Beckman 50Ti rotor. The VLDL and LDL in the top fourth of the tube were aspirated and discarded. The infranatants were pooled and the density was adjusted to 1.21 g/L with KBr, and the sample was re-centrifuged at 106 000 *g* for 48 h at 4°C in a Beckman 50Ti rotor. The top yellow layer containing HDL or HDL-SAA was aspirated, pooled, and dialyzed against 0.15 *M* NaCl /0.1% EDTA, pH 6.4 for 24 h at 4°C, and then against 10% (v/v) formic acid for 24 h.

3.4 SAA Purification

SAA was purified according to the method of McCubbin *et al.* (88). The HDL-SAA preparation was separated on a Sephacryl S-200 column in 10% (v/v) formic acid at a flow rate of 25 mL/h and 5 mL fractions were collected. The second major peak, containing 12-14 kDa protein, was pooled and lyophilized. Purity of the SAA was determined by SDS-PAGE on a 15% acrylamide gel.

3.5 Synthetic Lipoprotein Preparation

Synthetic lipoprotein particles (Lp) were prepared according to the method of Jonas (90). Briefly, SAA was dissolved in 0.15 *M* NaCl/5 *mM* EDTA/10 *mM* Tris-HCl, pH 8.2 (TES)/4 *M* urea, while CcR was dissolved in TES. The SAA solution was dialyzed

against TES. The protein solution was adjusted to 2.0 mg/mL by concentration against aquacide. Lps were formed by first shell drying 2.7 mg egg phosphatidylcholine per mg protein in a 15 mL Corex tube under a stream of nitrogen gas. For each mg protein, 1.5 mg sodium cholate was added. The tube was repeatedly vortexed and cooled on ice for 30 min, and left on ice until the dispersion became clear. The SAA or CcR protein solution was dripped into the dispersion and stirred at 4°C for 18-20 h. Sodium cholate was removed by dialyzing the protein-lipid dispersion against TES overnight. To minimize lipid oxidation the dialysis buffer was pre-equilibrated with nitrogen gas. Synthetic Lps were sized on a Sepharose CL-4B column with TES, at a flow rate of 0.22 mL/min and 1.4 mL fractions were collected. The second large peak was pooled and concentrated against aquacide to a volume of approximately 1 mL. To determine the phosphatidylcholine to protein ratio (PC:protein), an inorganic phosphate assay (91) and a DC protein assay was performed. For the phosphate assay, acid washed test tubes with 0.1 mL sample and 0.4 mL oxidizing acid (7% (v/v) perchloric acid/0.9 M H₂SO₄) were heated to 250°C for 1 h. After cooling to room temperature, 4 mL H₂O and 2 mL 2.5% (w/v) sodium molybdate/2.5 M H₂SO₄ were added. Then 0.5 mL 1% (w/v) *p*-methylamino phenolsulphate/3% (w/v) sodium metabisulphite was added to the tube and the colour was allowed to develop for 30 min. The A₇₀₀ from the samples and the standard curve were used to determine the concentration of inorganic phosphate.

3.6 Tryptophan Fluorescence

The GAGs heparin, heparan sulphate, and chondroitin sulphate were dissolved in TES at an initial concentration of 10 mg/mL. Various concentrations of GAGs were added to a stirring solution containing Lp-SAA or Lp-CcR at a concentration of 1 or 100 µg/mL. Tryptophan fluorescence was measured by exciting at 280 nm and reading the relative

intensity at 350 nm on a Perkin-Elmer LS50B. The excitation slit width was 5 nm; the emission slit width was 10 nm.

3.7 Perlecan Purification

Perlecan was purified according to the method of Ledbetter *et al.* with the addition of a low salt extraction step to remove serum proteins (92). EHS tumour was prepared by serial passage in C57BL mice according to the method of Orkin (73). The GAGs were labelled by injecting approximately 1 mCi [³⁵S]-sulphate in 1/10 of the mice 24 h before harvest. The tumour was removed and flash frozen in liquid nitrogen. Unlabelled, frozen tumour was thawed in 5 volumes (w/v) of 0.15 M NaCl/0.1 M 6-aminohexanoic acid/8 mM *N*-ethyl-maleimide (NEM)/ 2 mM phenylmethanesulfonyl fluoride (PMSF)/50 mM Tris-HCl, pH 7.4. The solution was stirred vigorously for 15 min. The tumour was homogenized with a polytron for 1 min and then centrifuged at 8000 g for 20 min. The supernatant was discarded while the pellet was re-homogenized in 3 volumes (w/v) of the 0.15 M NaCl solution and re-centrifuged at 8000 g. The pellet was then homogenized in the 0.15 M NaCl solution in the presence of 10 mM EDTA.

Both labelled and unlabelled tumours were added to 6 volumes (w/v) of 3.4 M NaCl/ 0.1 M 6-aminohexanoic acid/4 mM EDTA/8 mM NEM/2 mM PMSF/50 mM Tris-HCl, pH 6.8. The mixture was homogenized for 1 min, and then stirred for 1 h at 4°C.

Centrifugation at 12 000 g for 15 min separated the insoluble tissue from the supernatant. The supernatant was discarded and the pellet was re-extracted with 2 volumes (w/v) of the 3.4 M NaCl solution and centrifuged at 12 000 g for 10 min.

The pellet was suspended in 6 volumes (w/v) of 6 M urea/0.1 M 6-aminohexanoic acid/4 mM EDTA/2 mM PMSF/50 mM Tris-HCl, pH 6.8 and homogenized for 30 s. The

mixture was stirred for 2 h at 4°C, and homogenized for 1 min before centrifuging at 12 000 *g* for 10 min. Both the pellet and the supernatant were saved. The pellet was re-extracted with 6 volumes (w/v) of the 6 *M* urea solution and centrifuged at 12 000 *g* for 10 min. The supernatant was combined with that from the previous centrifugation.

The supernatant was dialyzed against 5 volumes (w/v) of 6 *M* urea/0.15 *M* NaCl/50 mM Tris-HCl, pH 6.8 at 4°C. Triton X-100 to a final concentration of 0.5% (v/v) was added to the extract after dialysis.

The proteoglycan was purified by first mixing the urea extract with DEAE-Sephacel equilibrated in 6 *M* urea/0.15 *M* NaCl/0.5% (v/v) Triton X-100/50 mM Tris-HCl, pH 6.8 at 4°C for 1 h. The DEAE-Sephacel-protein complex was washed three times with 2 volumes of the 6 *M* urea/0.5% (v/v) Triton X-100 solution and poured into a column. A salt gradient from 0.15 *M* to 1.0 *M* NaCl in 6 *M* urea/50 *M* Tris-HCl, pH 6.8 was used to separate the proteoglycan. From each fraction 50 µL of solution was added to 4 mL Universol scintillation fluid and the radioactivity was measured on a Beckman LS 1701 scintillation counter. Fractions containing [³⁵S]-sulphate were pooled and precipitated in 4 volumes (v/v) of methanol at 4°C overnight.

The precipitate was centrifuged at 12 000 *g* for 15 min, and dissolved in 1 volume of the 6 *M* urea buffer. The solution was chromatographed on the DEAE-Sephacel column with a salt gradient from 0.15 *M* to 0.7 *M* NaCl in 6 *M* urea/50 mM Tris-HCl, pH 6.8. From each fraction 50 µL of solution was sampled for radioactivity. Fractions containing [³⁵S]-sulphate were pooled and precipitated in 4 volumes (v/v) of methanol at 4°C overnight. The precipitate was centrifuged at 12 000 *g* for 15 min and dissolved in a solution of 4 *M* guanidine-HCl containing 0.5% (v/v) Triton X-100, 20 mM Tris-HCl, pH 7.0.

Solid CsCl was added so that the final concentration of the solution was 50% (w/w). The sample was centrifuged at 33 000 rpm in a Beckman 50.2Ti rotor for 68 h at 25°C. The solution in the tubes was divided into 10 parts, and each part sampled for [³⁵S]-sulphate radioactivity. Samples with counts four times that of background or more were pooled and dialyzed against 0.2 M NaCl and precipitated in 4 volumes methanol at 4°C overnight.

The pellet was resuspended in a minimal volume of 4 M guanidine-HCl/1.0% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS)/20 mM Tris-HCl, pH 7.0 and chromatographed on a Sepharose CL-4B column. Samples were eluted with 4 M guanidine-HCl/0.1% (w/v) CHAPS/20 mM Tris-HCl, pH 7.0. Fractions containing [³⁵S]-sulphate were pooled and dialyzed against distilled water at 4°C, and then lyophilized.

3.8 Equilibrium ELISA Binding Assay

The basic ELISA protocol used is that of Narindrasorasak *et al.* (87). Changes to the protocol are listed in Table 2.

The wells of Immulon 4 plates were loaded with 0.1 mL of 1 µg/mL murine SAA in 20 mM NaHCO₃, pH 9.6 and incubated overnight at 4°C. After washing the wells three times with 0.15 M NaCl/20 mM Tris-HCl, pH 7.4 (TBS), 0.15 mL of 1% (w/v) bovine serum albumin (BSA)/TBS, pH 7.4 was incubated in the plate for 2 h at 37°C. The plates were washed three times with TBS/0.05% (v/v) Tween-20, pH 7.4 (TTBS) between each solution added. Starting at a maximum concentration of 10 µg/mL, serial dilutions of the murine perlecan ligand in TTBS were added to the wells. The plates were stored overnight at 4°C. Next, 0.1 mL of the rabbit anti-murine perlecan antibody diluted in

0.1% (w/v) BSA/TTBS, pH 7.4 was incubated in the plate for 2 h at 37°C. The sheep anti-rabbit IgG conjugated with alkaline phosphatase was diluted in 0.1% (w/v) BSA/TTBS, pH 7.4 and 0.1 mL of the solution was incubated in the plate for 2 h at 37°C. For colour development, 0.1 mL of 20 mg/mL *p*-nitrophenol phosphate/20 mM glycine, pH 10.0 was added to each well. The reaction was allowed to develop until a maximum absorbance at 405 nm of 2.0-2.2 was reached, at which point 50 µL of 2 M NaOH was added to stop the reaction. The ELISA plate reader is capable of reading a maximum absorbance of 2.5.

When the pH of the coating buffer was changed, the SAA coating efficiency was tested by ELISA using anti-AA antibodies.

Table 2. ELISA Conditions**Coating Conditions**

Type of plate	Immulon 2, Immulon 4, Linbro, Covalink
Coating pH	2.9, 7.0, 7.5, 8.0, 9.6
BSA	1%, 3% (w/v)
PC	0x, 1x, 10x, 100x molar excess over SAA
PC and sodium cholate	PC with 1% (w/v) sodium cholate
Urea	4 M

Ligand Buffer

CD buffer	10 mM Ca ²⁺ /0.1 M NaCl/25 mM Tris-HCl, pH 8.0
Glycerol	10% (v/v)
BSA	0.1%
DTT	5 mM
NaCl	0, 50, 100 mM
pH	7.0, 7.5, 8.0, 8.5

Table 2 (continued)

Limiting Reagents

Coated protein concentration	1 $\mu\text{g/mL}$ - 1 mg/mL
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BSA	RIA, Fraction V, fatty acid free, heat denatured, base denatured
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Polyamino-acid	poly-leucine, poly-phenylalanine
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Primary Antibody Concentration	1/400 to 1/1 dilution
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Secondary Antibody Concentration	1/5000 to 1/500 dilution
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3.9 Coupling of SAA to Covalink Plates

Coupling of SAA to Nunc Covalink plates was performed according to the manufacturer's specifications. Briefly, 0.1 mL of 1 µg/mL SAA in 85 mM sulfo-NHS was added to the Covalink plate. The coupling reaction was started with 50 µL of 65 mM EDC and allowed to continue for 2 h at room temperature. The reaction was stopped by washing with 0.15 M NaCl/20 mM phosphate/0.05% (v/v) Tween-20/1% MgSO₄. The blocking procedure and onwards followed the basic ELISA protocol.

3.10 Competition by HDL or HDL-SAA of Fibronectin-Perlecan Binding

Using the ELISA procedure described above, fibronectin was coated to Immulon 4 plates. The ligand was perlecan, to which approximately 10 µg/mL of HDL or HDL-SAA was added. The ligand buffer had 8 mM KBr because the HDL and HDL-SAA was not further purified. The KBr did not significantly alter the ionic strength of the buffer since there was already 0.15 M NaCl in the binding buffer.

3.11 Coupling of SAA or Perlecan to CNBr-activated Sepharose 4B Beads

Perlecan or SAA was coupled to CNBr-activated Sepharose 4B beads according to the manufacturer's specifications. Briefly, 4 mg of perlecan or 10 mg of SAA were covalently linked through primary amino groups with 2 mL CNBr-activated Sepharose 4B gel in a solution of 0.5 M NaCl/0.1 M NaHCO₃, pH 8.3. Unreacted groups were blocked by incubating the beads in 0.2 M glycine, pH 8.0 overnight at 4°C. Uncoupled protein was washed away with 0.1 M sodium acetate/0.5 M NaCl, pH 4.0.

3.12 HDL-SAA Iodination

HDL-SAA was iodinated according to the method of McFarlane (93). The specific activity of the sample was not determined. The [¹²⁵I]- HDL-SAA was diluted serially two fold into 0.15 M NaCl/0.05% (v/v) Tween-20/20 mM phosphate, pH 7.4.

3.13 Sepharose 4B Solid Phase Binding Assay

SAA-Sepharose 4B (50 µL) was incubated with 0.1 mL of perlecan in TTBS at 37°C for 2 h. The sample was centrifuged at 10 000 g for 15 min. The supernatant was aspirated and coated onto a plate to quantify free perlecan by ELISA.

A two fold serial dilution of the iodinated HDL-SAA was added to 10 µL of the perlecan-Sepharose 4B beads or uncoupled beads. The mixture was incubated at 37°C for 2 h on a rotating platform. The entire mixture was then layered on top of phthalate oil and centrifuged at 10 000 g for 15 min. The bottom of the tube containing the pellet of beads was cut off and the radioactivity of the pellet was measured.

3.14 AEF Preparation

AEF was induced in the mice according to the method of Axelrad *et al.* (27). Briefly, AEF was extracted from amyloidotic tissue by homogenization of amyloidotic spleens in 0.15 M NaCl at 4°C using a polytron. The mixture was centrifuged at 10 000 g for 30 min. The pellet was repeatedly washed, re-homogenized and re-centrifuged with 0.15 M NaCl until the supernatant $A_{280} < 0.1$. The pellet was then homogenized in distilled water at 4°C and centrifuged again at 10 000 g. The supernatant was discarded. This step was repeated with the pellet and the second supernatant is defined as AEF by its biological activity.

3.15 Amyloid Induction

Amyloid was induced according to the method of Axelrad *et al.* (27). Briefly, 0.1 mg AEF (by protein concentration) was administered by intravenous injection using the dorsal tail vein of the mouse. Inflammation was induced by subcutaneous injection of 0.5 mL of 2% (w/v) silver nitrate. After the specified period of time, the mice were killed by cervical dislocation. The spleens were removed and frozen at -20°C until use.

3.16 AA Amyloid Fibril Preparation

AA amyloid fibrils were prepared according to the method of Pras *et al.* (94). AA amyloid was induced according to the above method, but instead of silver nitrate, 0.2 mL of 0.25 mg/mL lipopolysaccharide was injected intraperitoneally every other day. After 21 days, the spleens were removed and frozen at -20°C until use. The purification procedure was identical to that of AEF preparation, except that on top of the pellet in the second water wash there were AA-fibrils, which were removed by aspiration. The AA-fibrils were flash frozen in liquid nitrogen and lyophilized. For storage the AA-fibrils were kept at -20°C in the presence of a desiccant.

3.17 ThT Fluorescence of Spleen Homogenates from AA Amyloid Induced Mice

Murine spleens were hand homogenized with a smooth tipped pestle in 10 mL of 0.15 M NaCl per spleen and centrifuged at 10 000 *g* for 30 min. Splenic capsule (which remained on the pestle) and supernatant were discarded. This step was repeated. The pellet was sonicated for 15 seconds in 1 mL of distilled water. The pellet solution was frozen at -20°C. Samples were dissolved in 0.25 M NaOH prior to their use. The sample (20 µL) was added to the fluorescence buffer, which consisted of 10 mM ThT/50 mM glycine, pH 9.0. ThT fluorescence was measured within one hour on a Perkin Elmer LS-50 fluorometer. The excitation wavelength was 450 nm with a slit width of 5 nm. Emission wavelength

scans from 470 to 490 nm were made with a slit width of 10 nm. The protein concentration of the sample was determined by the DC Protein Assay according to the manufacturer's specifications. The amount of amyloid in the samples was expressed in terms of (I_{482} /protein concentration) x splenic weight. A standard curve derived from the addition of known amounts of purified AA-fibrils to untreated spleen homogenates was used to determine the concentration of the AA-fibril protein in the spleen homogenates.

4. RESULTS

4.1 SAA Purification

SAA was purified according to the method of McCubbin *et al.* (88). Animals were bled 18 h after silver nitrate injection, at which point plasma SAA levels had increased 300 fold (39). Sequential density flotation first removed LDL and VLDL, following which the density was adjusted to 1.21 g/mL by the addition of solid KBr, which then allowed HDL-SAA to be removed from the top of the density gradient. To remove the lipid bound to SAA, the HDL-SAA was dialyzed against 10% (v/v) formic acid followed by chromatography on a Sephacryl-200 column to purify the SAA (Figure 2). The second peak, which contained both SAA₁ and SAA₂, was pooled and lyophilized. Purity of the SAA was determined by a 15% SDS-PAGE acrylamide gel. ApoC was usually a minor contaminant.

4.2 Synthetic Lipoprotein Particle Preparation

To construct HDL-SAA-like particles without other lipoproteins, Lps were prepared using PC with SAA or CcR protein. To ensure that particles of a particular PC:protein ratio and size range were used, the Lps were chromatographed on a Sepharose CL-4B column. With Lps-SAA (Figure 3a), most of the protein was incorporated into particles with a PC:protein ratio of 1.9 (Table 3) and an approximate molecular weight of 400 kDa (William Bagshaw, personal communication). In the case of the Lps-CcR, the pooled fractions had a PC:protein ratio of 1.3, but most of the protein was found to have eluted in the void volume (Figure 3b).

Figure 2. Representative Elution Profile of Delipidated HDL-SAA on a Sephacryl S-200 Column.

After the lipoprotein particle was delipidated by dialysis against 10% (v/v) formic acid, the protein sample was applied to a Sephacryl S-200 column (2.5 x 90 cm). The column was eluted with 10% (v/v) formic acid at a flow rate of 25 mL/h with 5 mL fractions being collected. Protein content was measured by absorbance at 280 nm. The peak fractions labelled SAA were pooled.

Figure 3. Elution Profile of Lp-SAA and Lp-CcR on a Sepharose CL-4B column.

The Lps were sized by chromatography on a Sepharose CL-4B column (1 x 80 cm). The column was eluted with TES at a flow rate of 0.22 mL/min with 1.4 mL fractions being collected. Fractions were monitored at A_{280} for protein content. The peak fractions labelled were pooled. *Top*, Lp-SAA elution profile. *Bottom*, Lp-CcR elution profile.

Table 3. PC:Protein Ratio

Lp	PC:Protein (mass:mass)
Lp-SAA	1.9
Lp-CcR	1.3

4.3 Tryptophan Fluorescence of Lp-SAA and Lp-CcR

Tryptophan fluorescence is a technique that can be used to indicate changes in a protein's conformation. By providing excitation light at 280 nm and measuring the emitted light at 350 nm, the effect of changes in the microenvironment around a protein's tryptophan(s), such as changes in exposure to the solvent, can be measured.

Previous CD work had added chondroitin sulphate, heparan sulphate, or heparin to SAA₁ or SAA₂ to measure changes in the amount of α -helix, β -sheet, and random coil in the protein (88). In experiments reported here, Lps were used because they more closely mimic the native HDL-SAA particles than SAA alone, and they allow the SAA to be in an unaggregated state at physiological pH and salt concentration. The heparan sulphate was the same GAG as that found on the perlecan protein, while chondroitin sulphate and heparin were used as representative control GAGs. Lps stability was shown by the steady tryptophan fluorescence baseline in Figure 4a (Lp-SAA) and 4c (Lp-CcR) after its addition to TES.

The addition of Lp-SAA or Lp-CcR to the TES solution caused a large increase in tryptophan fluorescence (Figure 4a,c), while the addition of 500 μ g heparan sulphate, chondroitin sulphate, or heparin to TES alone did not cause any change, showing that the fluorescence changes were not caused by protein contamination or scattering from the GAG solutions (Figure 4b). The addition of 10, 100, or 500 μ g heparan sulphate to 1 μ g/mL Lp-SAA, and the addition of 10 or 100 μ g heparan sulphate to 100 μ g/mL Lp-SAA

Figure 4. Changes in Tryptophan Fluorescence of Lp-SAA and Lp-CcR in the Presence of Various GAGs.

Lps were added to TES followed by various concentrations of heparan sulphate, chondroitin sulphate, and heparin. The excitation wavelength was 280 nm; emitted light was measured at 350 nm. *A, top line*, 25 s after the addition of 100 µg/mL Lp-SAA, increasing amounts of heparan sulphate were added at successive 30 s intervals. *A, bottom line*, same as *top line* except 1 µg/mL Lp-SAA was added. *B, top line*, 25 s after the addition of 100 µg/mL Lp-SAA, chondroitin sulphate and heparin were added at successive 30 s periods. *B, bottom line*, chondroitin sulphate, heparin, and heparan sulphate were added at successive 30 s intervals to TES. *C*, 25 s after the addition of 100 µg/mL Lp-CcR, heparan sulphate was added.

did not cause any detectable change, demonstrating that the increase in volume did not cause the change in fluorescence, and that the effect was titratable (Figure 4a). The addition of 500 µg heparan sulphate caused a decrease in tryptophan fluorescence. The addition of 500 µg chondroitin sulphate to 100 µg/mL Lp-SAA did not cause a change, while 500 µg heparin decreased the tryptophan fluorescence (Figure 4b). Heparin added to Lp-SAA in the absence of chondroitin sulphate caused a similar shift. To show indirectly that the changes are specific to SAA and not to any other protein incorporated into an Lp, 500 µg heparan sulphate was added to the outer mitochondrial membrane protein CcR, which did not show any detectable change in fluorescence (Figure 4c).

4.4 Equilibrium ELISA Binding Assay

ELISA is traditionally used to quantify the amount of antibody, but by adding a step where a solution containing a ligand is loaded onto the coated wells, it can be used to measure the equilibrium dissociation constant (K_d) and the maximal binding constant (b_{max}) of a binding interaction between a coated protein and a soluble ligand protein. It is also an efficient assay, since nanogram quantities of protein can be coated onto a well, and microgram quantities of ligand can be detected (87,95).

To fit the ELISA data to a single class of binding site formulation, the non-linear regression equation used was:

$$A_{405} = a + bx + [cx/(d + x)] \quad (87).$$

Calculations were made by Sigmaplot 5.0 Software (Jandel Scientific). The fitted value 'a' represented the theoretical zero, that is, the signal obtained in the absence of ligand,

'b' represented the linear component of the ligand binding to the coated protein, 'c' was the

Figure 5. Representative ELISA Binding Curves.

Binding curves of fibronectin to perlecan (*open circles*), SAA to perlecan (*open squares*), and BSA to perlecan (*open triangles*). In all cases perlecan was the soluble ligand and fibronectin, SAA, or BSA was the coated protein. The ELISA assay was performed as described, and the curve was calculated using a non-linear curve fit program. *Symbols*, experimental data; *lines*, curve-fit generated from experimental data.

theoretical b_{\max} , while 'd' was the theoretical mass dissociation constant, which can be converted to the molar dissociation constant K_d by dividing it by the molecular weight of the ligand. The variable 'x' was the ligand concentration added. To be able to detect a binding interaction that is of high affinity and specificity, the K_d must be 10^{-6} M or lower, since interactions of lower affinity are washed away in the assay (96).

Along with the experiment of SAA and perlecan binding, the binding of fibronectin to perlecan was used as a positive control, while the binding of BSA to perlecan was used as a negative control for the detection portion of the assay, and as an empirical estimate of the non-specific binding of perlecan to SAA. Since SAA aggregates and becomes insoluble at physiological pH and salt concentration, it was coated on the wells, and perlecan was added as the ligand.

The fibronectin-perlecan binding assay (Figure 5) determined a K_d identical to the accepted value (2×10^{-9} M as measured by ELISA and by binding in the soluble phase) (97). Under the same ELISA conditions, the SAA-perlecan and the BSA-perlecan binding assays appeared similar, suggesting that the binding is of low affinity (Figure 5). To approximate what may occur *in vivo*, various conditions in the SAA-perlecan binding assay were changed as listed in Table 2. These were grouped into conditions that affect the coated protein (Table 4), ligand binding (Table 5), or those that ensured that the reagents in the assay were not limiting (Table 6).

Changes in coating efficiency when coating buffers of different pH values were used were checked by ELISA. No quantitative difference was seen.

Under all conditions tested, no reproducible, high affinity, saturable binding with low non-specific binding between SAA and perlecan was detected.

Table 4. Coating Conditions

Condition	Parameter	High Affinity Binding
ELISA Plate	Immulon 2,4; Linbro; Covalink	–
Coating pH	2.9, 7.0, 7.5, 8.0, 9.6	–
BSA	1%, 3% (w/v)	–
PC	0x, 1x, 10x, 100x molar excess over SAA	–
PC and sodium cholate	PC with 1% (w/v) sodium cholate*	–
Urea	4 M	–

*Plates were washed either with TTBS as outlined above or TBS was substituted.

Table 5. Ligand Buffer

Condition	Parameter	High Affinity Binding
CD buffer	10 mM Ca ²⁺ /0.1 M NaCl/ 25 mM Tris-HCl, pH 8.0	–
Glycerol	10% (v/v)	–
BSA	0.1%	–
DTT	5 mM	–
NaCl	0, 50, 100 mM	–
pH	7.0, 7.5, 8.0, 8.5	–

Table 6. Limiting Reagents

Condition	Parameter	High Affinity Binding
Protein concentration	1 µg/mL - 1 mg/mL	–
BSA	RIA, fraction V, fatty acid free, heat-denatured, base-denatured	–
Polyamino-acid	poly-leucine, poly-phenylalanine	–
Primary Antibody Concentration	1/400 to 1/1 dilution	–
Secondary Antibody Concentration	1/5000 to 1/500 dilution	–

4.5 **Competition by HDL or HDL-SAA of Fibronectin-Perlecan Binding**

The effects of HDL and HDL-SAA on fibronectin-perlecan binding using the ELISA were examined, rather than measuring the binding affinity of SAA to perlecan directly (Figure 6). With the addition of approximately 1 mg/mL HDL or HDL-SAA to the ligand binding step, the fibronectin-perlecan binding did not saturate.

4.6 **Sepharose 4B Solid Phase Binding Assay**

Another solid phase support binding assay that was used is similar to immunoprecipitation, where one of the proteins was coupled to Sepharose 4B beads. The change in the amount of ligand bound was quantified to determine the binding affinity. Both SAA and perlecan were coupled through their primary amino groups to CNBr activated Sepharose 4B beads, except that in the case of the perlecan-Sepharose 4B beads [¹²⁵I]-HDL-SAA was used instead of SAA. This allowed the assay to be performed at a physiological *pH* of 7.4.

As can be seen in Figure 7, there was no significant difference between the amount of [¹²⁵I]-HDL-SAA bound to the perlecan-coupled and the uncoupled beads. The SAA-coupled bead assay (Figure 8) was similar to the ELISA except that SAA was bound to Sepharose 4B beads instead of being coated onto wells, and the concentration of perlecan bound was determined by subtracting the A₄₀₅ of free perlecan from total perlecan added. Problems with this approach are discussed later. The curve fit determined a K_d of approximately $2 \times 10^{-9} M$, but a straight line with $r = 0.937$ can also be fitted to the data indicating a large amount of non-saturable binding.

4.7 ThT Fluorescence of Spleen Homogenates from AA Amyloid Induced Mice

The histological sensitivity of CR staining of AA amyloidotic spleens allows the detection of anatomic amyloid deposition as early as 36 h after induction with silver nitrate and

Figure 6. Binding of Fibronectin to Perlecan in the Presence of HDL or HDL-SAA.

Binding curve of fibronectin to perlecan in the presence of HDL (*open circles*) or HDL-SAA (*open squares*). Fibronectin was the coated protein while perlecan was the soluble ligand protein. The remainder of the procedure was the same as the ELISA.

Figure 7. Perlecan-Sepharose 4B Solid Phase Binding Assay.

Binding curve of [125 I]-HDL-SAA to perlecan-Sepharose 4B beads (*open circles*), and uncoupled Sepharose 4B beads (*open squares*) using a modification of the immunoprecipitation method. * Rejected outlier.

Figure 8. SAA-Sepharose 4B Solid Phase Binding Assay.

Binding curve of perlecan to SAA-Sepharose 4B beads. To determine the amount of bound perlecan, the A_{405} of the free perlecan was subtracted from the A_{405} of the perlecan added. Values are means of $n=4 \pm$ S.E.M. *Solid line*, non-linear regression fit for binding interaction. *Dashed line*, linear fit for binding interaction.

AEF. However, increased basement membrane protein mRNA levels are detected using RT-PCR as early as 12-24 h after induction (86,98). A less commonly used amyloid stain is ThT, which has been used to detect apoA-II-amyloid in murine liver homogenates (11). ThT could be used to detect AA amyloid in spleen homogenates earlier than has been previously possible. Figure 9, an AA-fibril protein standard curve, showed that the signal from ThT fluorescence allowed as little as 50 $\mu\text{g/mL}$ AA-fibril protein to be detected. The ThT fluorescence buffer contained normal spleen homogenate to show that the signal was detectable above splenic cell and protein background ThT fluorescence, and that the signal increased linearly with increasing amounts of AA-fibril protein. Fluorescence was at background when ThT was added to SAA or HDL-SAA, demonstrating that the dye was specific for the fibril form. Furthermore, the addition of an equal volume of 4 M guanidine-HCl destroyed the fluorescence signal at 482 nm.

In the time course (Figure 10), the results are shown from experiments with spleen homogenates from mice injected with silver nitrate and AEF, silver nitrate only, or AEF only. The fluorescence signal from silver nitrate and AEF treated mice steadily increased until 48h, at which point it leveled off. When the $I_{482}/[\text{protein}]$ measurement is multiplied by the splenic mass, the rate of amyloid formed over the early time period was more rapid than at later times (Figure 11).

Mice receiving either silver nitrate, or AEF, had no significant increase in splenic ThT fluorescence except at 12 h after injection. Treatment with 2 M guanidine-HCl (final concentration) destroyed the fluorescence of AEF treated mice, but not mice receiving silver nitrate, showing that the signal there was due to increased background and not AA amyloid (Figure 10, *inset A*). When AEF was injected 24 h prior to the inflammatory stimulus, a time sufficient to allow for the AEF ThT "signal" to disappear, spleen homogenates showed ThT fluorescence slightly above background 12 h after the silver

nitrate injection, which indicated AA amyloid deposition occurred 12 h after completion of the induction protocol. Treatment of this sample with 2 M guanidine-HCl decreased the fluorescence to background levels, demonstrating that the signal was due to amyloid fibrils. Examination of the pooled salt washes from the silver nitrate and AEF group showed no significant ThT fluorescence signal above background, which demonstrated that no detectable amount of AA-fibril was lost during the fibril isolation procedure (Figure 10, *inset B*).

Figure 9. AA-Fibril Protein Standard Curve.

Purified AA-fibril was added to ThT fluorescence buffer containing control tissue homogenate with (*closed circles*) and without (*open and closed triangles*) 2 M guanidine-HCl (final concentration). The excitation wavelength was 450 nm, emitted light was measured at 482 nm. The signal was normalized by dividing I_{482} by the relative protein concentration of the sample, then subtracting the control tissue I_{482} /relative protein concentration. Values are $n=1$ (*closed circles*), and $n=2$ (*open and closed triangles*). *Dashed lines*, data from each trial. *Solid lines*, mean of both trials.

Figure 10. A Time Course of ThT Fluorescence of Spleen Homogenates from Mice Induced to form AA Amyloid.

Spleen homogenates from animals induced to form AA amyloid (silver nitrate and AEF, *open circles*), or controls (silver nitrate, *open triangles*; AEF, *open squares*) were added to ThT fluorescence buffer. The excitation wavelength was 450 nm; emitted light was measured at 482 nm. The signal measured was normalized by dividing I_{482} by the relative protein concentration of the sample, then subtracting control tissue I_{482} /relative protein concentration. Values are means of $n=4 \pm$ S.E.M. (silver nitrate and AEF), and means of $n=3 \pm$ S.E.M. (silver nitrate or AEF). *Inset A*, ThT fluorescence of spleen homogenates in the presence of 2 M guanidine-HCl (final concentration). Values are means of $n=3 \pm$ S.E.M. *Inset B*, ThT fluorescence of salt washes from spleen homogenates of silver nitrate and AEF injected mice.

Figure 11. ThT Fluorescence of Spleen Homogenates from Amyloid Induced Mice and the Increase in Spleen Mass.

Combining the increase in spleen weight after silver nitrate and AEF injection with ThT fluorescence provided a measure of the amount of amyloid expressed as (μg AA-fibril (dry weight) \times mg spleen)/spleen. The fluorescence signal after treatment with 2 *M* guanidine-HCl (final concentration) was subtracted from these values. To convert I_{482} /relative protein concentration to (μg AA-fibril \times mg spleen) per spleen, the standard curve (Figure 9) was used. The 12 h time point of mice receiving AEF and silver nitrate was from mice where the two injections were separated by 24 h. Silver nitrate and AEF, *open circles*; silver nitrate, *open triangles*; AEF, *open squares*. *Inset*, splenic weight during amyloid induction protocol. Values are means of $n=4 \pm$ S.E.M. (silver nitrate and AEF), and means of $n=3 \pm$ S.E.M. (silver nitrate, or AEF).

5. DISCUSSION

5.1 Overview

Amyloid refers to proteinaceous tissue deposits of different origins, which have a common appearance at the light and electron microscopic level, and a cross- β sheet structure at the X-ray diffraction level. Twenty different precursor proteins have been described that have no similar primary sequence or secondary structure that could account for the common organization of amyloid. Therefore additional component(s) may be involved in amyloid deposition, of which one possibility is the proteoglycan perlecan. Perlecan is a basement membrane protein that has been found in all types of amyloid deposits examined thus far. The perlecan GAG, heparan sulphate, has been shown to be intimately associated with the deposit. In addition to this spatial relationship, experiments in a mouse model have shown that the synthesis of heparan sulphate and perlecan mRNA precede AA amyloid deposition *in vivo* as detected by CR staining. *In vitro* studies have shown that the addition of heparan sulphate to a solution of SAA₂, the only isoform found in mouse AA amyloid, imparts to this SAA a greater proportion of β -sheet. It has been proposed that perlecan, possibly through increased synthesis, may facilitate or even induce amyloid deposition.

To study further the role of perlecan in AA amyloid, several approaches were used to examine the interaction of heparan sulphate or perlecan with SAA. 1) The influence of heparan sulphate on SAA tryptophan fluorescence was studied while the protein was incorporated into a synthetic lipoprotein particle. The Lp was used for two reasons, a) SAA does not normally exist at a significant concentration free in plasma, and thus a Lp particle is similar to its physiologic environment; b) since native HDL-SAA contains many different apolipoproteins, correlating a shift in tryptophan fluorescence to a specific

apolipoprotein in its native particle would be impossible. 2) The binding between perlecan and SAA was analyzed by ELISA. Perlecan was used as a ligand instead of heparan sulphate since heparan sulphate is normally part of a proteoglycan, and perlecan has been found in the amyloid deposit. The ELISA technique was chosen because it is a simple assay and requires only nanogram quantities of protein. Additionally, hydrophobic SAA is immobilized on a solid surface rather than being free in solution. 3) Rather than measuring the direct binding of SAA to perlecan, HDL-SAA was used to examine its effect on the normal interaction between fibronectin and perlecan. If SAA does interact with perlecan, it might inhibit the binding between these two basement proteins.

Lastly, both AEF activity and increased levels of perlecan mRNA seem to precede AA amyloid deposition, but this observation could be due to a difference in sensitivity of the techniques used to detect perlecan mRNA (RT-PCR) and tissue amyloid (CR staining). A more sensitive way of detecting amyloid would allow a more precise temporal relationship to be established between these events. The presence of AA amyloid is commonly determined by CR staining, therefore a potentially more sensitive method of amyloid tissue detection using ThT was investigated. The amount of AA amyloid in murine spleen was determined in a four day time course. The spleen was used since it has been shown that AA deposits are detected there first (99).

5.2 Tryptophan Fluorescence of Lp-SAA after the Addition of GAGs

Past work with CD experiments showed that the addition of heparan sulphate caused an increased proportion of β -sheet in SAA₂, while SAA₁ acquired slightly more α -helix structure. This experiment had been performed with free SAA. The work was repeated with Lp-SAA to see if similar changes occur when the SAA is in a lipid environment, the

environment in which it is normally found in plasma. Tryptophan fluorescence was used as an indicator of changes in SAA conformation.

The results showed that heparan sulphate caused a decrease in tryptophan fluorescence only at the higher concentration of GAG and of Lp-SAA (Figure 4a). No change was seen at the lower Lp-SAA concentration. If chondroitin sulphate was substituted for heparan sulphate, no such decrease was seen. The conformation change in SAA after the addition of heparan sulphate can therefore be considered specific to that GAG, since the lack of change after the addition of chondroitin sulphate shows that it was not simply due to dilution of Lp-SAA, or because of a change in ionic strength in the Lp-SAA buffer.

Heparin, a GAG that is very similar in structure to heparan sulphate, caused a decrease in fluorescence similar to that of heparan sulphate. These results were consistent with the CD experiments, since tryptophan fluorescence cannot show the type of conformational change that occurred. The CD experiment showed that heparin caused SAA₁ and SAA₂ to have more α -helix structure. The difference in effect is probably due to the subtle differences in structure between heparin and heparan sulphate, but this distinction is not very precise (100). Heparin is accepted to be more uniformly sulphated than heparan sulphate, and contains more iduronate than glucuronate (100). These differences can lead to different heparin and heparan sulphate structures, so that when interacting with proteins unique conformational changes are effected.

As a final control for conformational changes in lipid-associated proteins, heparan sulphate was added to Lp-CcR, another membrane protein, instead of Lp-SAA. CcR is a protein that is associated with the outer mitochondrial membrane. No changes in

tryptophan fluorescence were seen with Lp-CcR, suggesting that the change in Lp-SAA was specific to SAA and heparan sulphate.

The results from these tryptophan fluorescence experiments were consistent with the CD data, in that heparan sulphate changed the conformation of SAA. The results showed that a change occurs even when SAA is in a lipid environment. If this interaction were of low affinity (reversible), the fluorescence shift should have been unstable, implying that there may be a tight binding between heparan sulphate and SAA. Because heparan sulphate is usually attached to perlecan *in vivo*, and because perlecan has been found in many types of amyloid deposits, the binding of perlecan to SAA was investigated by ELISA to determine the affinity between these proteins.

5.3 Affinity of SAA-Perlecan Binding by ELISA

ELISAs have been used in several other experiments to model and determine the affinity of the binding between various basement membrane proteins such as perlecan and fibronectin (81,101). The fibronectin-perlecan binding was repeated in the present work and had an affinity of $2 \times 10^{-9} M$ (Figure 5), which is in agreement with the published value as determined by ELISA and other techniques (97). This showed that ELISA could be used to study protein-protein binding. Furthermore, using the ELISA procedure the K_d of the β PP-perlecan interaction and SAA-laminin interaction have been shown to be of high affinity (in the $10^{-9} M$ range). These experiments demonstrated that in such an assay both proteins (SAA and perlecan) exhibit properties as a ligand to a coated protein. This suggested that an ELISA could be constructed to determine the K_d between perlecan and SAA.

The results of a typical SAA-perlecan binding curve showed no high affinity binding interaction (Figure 5). Most of the results had only a slightly higher b_{\max} than that of BSA-perlecan binding, suggesting that the binding was of low affinity. Because the ELISA technique is capable of accurately measuring molar affinities of 10^{-6} M or less (96), the SAA-perlecan affinity in the assay conditions used was probably of 10^{-5} M or higher. Therefore modifications of the conditions were studied.

5.3.1 Changes in ELISA Conditions

The lack of binding may have been caused by the *in vitro* conditions in the assay being different from those *in vivo*. Thus, a variety of changes were made with respect to the coating conditions, ligand buffer, blocking agent, and antibody concentration. The rationale for these changes and their effect will be discussed stepwise in detail.

5.3.1.1 Plates

Immulon 2 and 4, Linbro plates, and Covalink plates were used to see if different brands of plates would make a difference in the binding. In ELISAs, the coating of the protein to the polystyrene wells is believed to occur through hydrophobic binding (Van der Waals interactions) of the protein to the plate surface (102,103). It was thought that subtle differences in the manufacturing process may lead to different efficiencies in binding among the different brands. Desorption of the coated protein during subsequent buffer changes was not a problem (104), but coating may have led to steric blocking of the binding site or denaturation of the protein. Theoretically, the Covalink plate does not rely on hydrophobic binding of SAA, but links the protein to a primary amino group (attached to an eleven carbon spacer arm), which keeps SAA away from the surface. The linkage to SAA was through primary amino-groups, which ensured that the potential GAG binding sequence was not blocked (105). However, the potentially fibrillogenic *N*-

terminal region is sterically blocked (39). It is not known whether this had an effect on the binding interaction.

No difference in SAA-perlecan binding was seen when the different polystyrene plates were used. The Covalink plates, in addition, had a high background, suggesting that the blocking of the plate was not as efficient as with the standard plates.

5.3.1.2 **pH**

Even with the Covalink plate, a hydrophobic protein such as SAA may still have been able to bend the spacer arm and bind to the plate surface. Several other changes were therefore made to the coating conditions attempting to keep SAA unaggregated. The standard coating buffer was at pH 9.6, since the coating of antibodies is most efficient at this pH. A small protein such as SAA may be more susceptible to denaturation than an immunoglobulin, so a variety of pHs were used to coat SAA onto the plate (Table 4). At pH 2.9, SAA is in an unaggregated state (106), so that monomers rather than aggregates may be coated. This could allow more SAA to be available for perlecan binding.

Under all pHs tested, the efficiency of coating was not affected, and no high affinity binding was detected.

5.3.1.3 **Protein Stability - Addition of Phosphatidyl Choline or BSA**

Since SAA exists in the circulation attached to HDL, PC was added in an attempt to mimic the lipid environment of the particle. A molar equivalent, ten-fold molar equivalent, and hundred-fold molar equivalent of PC were added to ensure that a sufficient quantity was present. All concentrations of PC were above the critical micelle concentration, so that sodium cholate was added to disrupt large, multilamellar micelles and allow small micelles to bind SAA. During the remaining steps of the procedure, TBS

was substituted for TTBS so that Tween-20 would not disrupt any lipid-protein interactions. Another way that the SAA can be stabilized while being coated is to add BSA to the coating buffer. It has been shown that fibronectin-cell binding is considerably enhanced when BSA is present (107), suggesting that BSA could limit denaturation due to protein coating. Despite the changes, the binding affinity was the same in the presence and absence of PC or BSA.

5.3.1.4 Urea

Urea used during the coating of SAA, to keep the protein in a less aggregated and more uniform state, has been shown to enhance the SAA-laminin binding interaction (John B. Ancsin, personal communication). This denaturant, which would normally inhibit protein-protein binding, was however present only in the coating step since it was washed away in the subsequent rinsing steps. When used to study SAA-perlecan binding, no enhancement was seen in terms of a high affinity interaction.

5.3.1.5 Ligand Buffer

If the amyloid microenvironment influences SAA-perlecan binding, changes in the ligand buffer should have the greatest effect on the interaction (Table 5). The standard ELISA ligand buffer was 0.15 M NaCl, 0.05% (v/v) Tween-20, 20 mM Tris-HCl, pH 7.4. Changes were therefore made to the salt concentration, pH, and detergent in the buffer.

5.3.1.6 Sodium Chloride and pH

The NaCl concentration was varied from 0 to 0.15 M, and the pH was varied from 7.0 to 8.5. The reduced salt concentration elevated both the signal detected in the SAA-perlecan binding and in the BSA-perlecan binding. Altering the pH caused no significant change in binding. The results suggested that if the amyloid microenvironment has a different ionic strength or pH, it does not influence an SAA-perlecan interaction.

5.3.1.7 CD Buffer

One ligand buffer which combined changes in *pH* and NaCl concentration was the buffer from the CD experiments, which had shown that heparan sulphate changed the conformation of SAA₂ (88). The buffer had 0.1 *M* NaCl, less than that in the ELISA buffer, and a slightly elevated *pH* of 8.0, both of which ensured that the free SAA remained soluble for the CD measurements. Calcium (10 *mM*) was added at four times physiological levels, because amyloid deposits have been shown to be rich in calcium, and because of a putative calcium binding domain in SAA (88). In the ELISA, the CD buffer resulted in an elevated background, probably due to the lack of the detergent Tween-20, and the lower salt concentration.

The low salt concentration may have caused aggregation of proteins, by not masking charges on the surface of the protein. The presence of Tween-20 in the standard ligand buffer may be considered non-physiological, but in ELISA it disrupts non-specific hydrophobic binding between the proteins (108), by binding to hydrophobic regions on proteins and preventing aggregation with other proteins. Detergents may also help by acting as an additional blocking agent, filling gaps left by BSA.

5.3.1.8 HDL-SAA or SAA as a Ligand

In all previous attempts at establishing an ELISA to study SAA-perlecan binding, SAA was the immobilized protein. HDL-SAA or SAA could however be used as the free ligand and perlecan as the coated protein. With this arrangement, the large perlecan protein would be immobilized on the well's surface, and HDL-SAA or SAA would be free to bind to it, possibly mimicking the situation *in vivo*. However, high backgrounds were observed in wells coated with BSA alone - a required control (R. Kisilevsky,

personal communication). Presumably, the SAA is in an aggregated state, and because of its hydrophobic nature may have bound to BSA.

5.3.1.9 Limiting Reagents in the ELISA

Previous studies have shown that SAA binds to BSA coated to the microtitre wells (R. Kisilevsky, personal communication). The specificity of the interaction was not determined. Since this interaction may have obscured any SAA-perlecan binding, where SAA was the immobilized protein, the blocking agent (BSA) was denatured, or BSA was substituted with ovalbumin or poly-leucine and poly-phenylalanine. The poly-amino acids were used as a non-albumin protein blocking agent, but their use was limited since they were not soluble in any of the buffers, and required an organic solvent that dissolved the polystyrene plate.

No noticeable effect on either the SAA-perlecan or SAA-BSA interaction was seen when using denatured BSA or ovalbumin. Attempts at dissolving poly-amino acids in physiological buffers resulted in a very high background.

Blotto (5% skim milk powder in TBS), normally an excellent blocking agent, was not used because it is poorly characterized and contains materials that may interfere with the assay (109). Gelatin (mainly type I collagen) has been shown to effectively inhibit the fibronectin-perlecan binding assay (87), and may similarly interfere in a SAA-perlecan interaction.

Several reagents were tested in excess to ensure that sufficient quantities of coated protein and antibodies were present in the assay (Table 6). Previous studies have shown that the

sensitivity of the ELISA can rise exponentially after increasing the amount of protein coated (110), SAA was therefore added in 1000x excess over the standard procedure. Similarly, primary and secondary antibodies were added in excess over recommended working concentrations to maximize the signal of the colorimetric assay. However, it is doubtful that loss of bound antibody was a problem, since antibody binding is practically irreversible in the ELISA procedure (111). None of these changes resulted in the detection of a high affinity interaction.

5.3.3 Competition by HDL or HDL-SAA of Fibronectin-Perlecan Binding

Despite the many changes made to the ELISA protocol, the techniques showed that the affinity of SAA for perlecan was not significantly greater than that of BSA for perlecan. An attempt was made to observe an effect of SAA indirectly through the competition of HDL-SAA in the fibronectin-perlecan interaction where fibronectin was the coated protein. To control for the other apolipoproteins, HDL was also used as a competitor. The results indicated that HDL and HDL-SAA were equally effective, and that in the presence of the lipoproteins the binding of perlecan to fibronectin became linear and did not saturate. Other studies have shown that apoA-I and apoE, which are on HDL, and in the case of apoA-I on HDL-SAA, bind to heparan sulphate (112). Thus, apoA-I and apoE may be the proteins interfering with perlecan binding to fibronectin. This type of protein interaction may suggest a possible reason for amyloid formation, which is discussed below.

5.3.4 Coupling of SAA or Perlecan to Sepharose 4B Beads

It was still possible that SAA was denatured in binding to the polystyrene plates despite changes in the coating conditions. Another solid phase medium, Sepharose 4B, was therefore used to try to determine the affinity between SAA and perlecan. In a technique

similar to immunoprecipitation, SAA was coupled to the beads through their amino-groups, which ensured that some of the protein was attached through the *N*-terminal end, keeping the potential GAG binding sequence available for perlecan binding.

The amount of bound perlecan was calculated by measuring the changes in the amount of free protein by ELISA. However, it can be argued that the assay was not sensitive enough to detect a small amount of perlecan removed from the ligand buffer. Assuming a 1:1 binding stoichiometry of SAA to perlecan and an affinity of 10^{-9} M, the amount of perlecan remaining free should be measurably less than the amount added. If the stoichiometry is much higher than 1:1, this indirect method of measurement may not be sensitive enough to quantify the amount of bound perlecan.

The curve fit from the perlecan to SAA-bead binding gave an affinity in the 10^{-9} M range (Figure 8), but because of the large error in the amount of perlecan measured, interpretation of the results is difficult. The error cannot be from the trapping of perlecan during centrifugation, since the amount of perlecan bound to uncoupled beads has been subtracted from these values. The error comes from the difficulty in reproducibly aliquoting equal amounts of SAA-beads, due to the difficulty in creating an even suspension of the beads. This approach may however be worth exploring further.

The reverse situation, where perlecan was bound to the beads and SAA was the free ligand, was also investigated. Perlecan was coupled through its amino-groups to Sepharose 4B. Coupling through carboxyl-groups would be the preferred orientation, since the heparan sulphate chains are located in the *N*-terminal region. Nevertheless, the perlecan-beads may be the most accurate *in vitro* mimic of the situation *in vivo*, because perlecan is normally associated with a solid phase matrix, namely as part of the basement membrane. SAA in these studies was in the native HDL-SAA particle. The results show

that the amount of HDL-SAA bound to perlecan-beads was not significantly greater than that bound to uncoupled beads, indicating that there was no high affinity binding between HDL-SAA and perlecan.

After this exhaustive approach further work was concentrated on the early detection of AA amyloid.

5.4 ThT in the Early Detection of AA Amyloid

The hypothesis that perlecan may be involved in amyloidogenesis comes from the observations that heparan sulphate is deposited concomitantly with amyloid, that it is part of the AA fibril as assessed by electron microscope techniques, and that perlecan mRNA levels, as assessed by RT-PCR, are increased before AA amyloid is detected in murine spleens. This temporal relationship was deduced using CR to detect amyloid. Although highly specific, CR may not be sensitive enough to detect small, early amyloid deposits, and thus it would appear as if the increase in perlecan mRNA levels preceded actual amyloid deposition.

An alternative method of amyloid detection is its interaction with ThT, a fluorescing dye that undergoes a specific excitation and emission wavelength shift when bound to amyloid (11,13,113). The fluorescence intensity of a representative spleen sample were measured, and when evaluated against a standard curve, can be used to quantify the amount of amyloid present. With this more sensitive technique, AA amyloid was detected earlier than was possible with CR.

5.4.1 AA Amyloid Fibril Standard Curve

Before the early detection of AA amyloid was examined, the specificity and sensitivity of ThT fluorescence was determined with a standard curve of increasing amounts of extracted AA fibrils (Figure 9). The graph shows that over the range examined the amount of AA fibril protein detected was linear, and the sensitivity of the assay was at least 50 $\mu\text{g}/\text{mL}$. Normal spleen homogenates were added to the buffer to show that the signal due to fibrils was detectable above background ThT binding to spleen protein. The addition of 2 *M* guanidine-HCl (final concentration) reduced the ThT fluorescence to that of background, demonstrating that the fluorescence was due to some denaturable component, and was not just an increase in light scatter as more fibril protein was added.

5.4.2 Optimization of ThT Signal

Initial work with ThT on untreated spleen homogenates showed decreased fluorescence intensity with increased amyloid induction time, implying that there was less amyloid than in spleens from uninduced mice. Studies with CR have shown that this is not true, and that AA amyloid deposition does occur during the time period examined. In the ThT experiments, the fluorescence intensity was standardized against protein concentration. During amyloid induction, spleen cells proliferate 72 h later so that the protein concentration of the homogenate increases faster than the rate of AA amyloid formation. Therefore several enrichment steps were performed to correct the problem. The splenic capsule was removed, since the homogenized fragments caused light scatter that would interfere with the signal due to amyloid. The tissue was washed twice with large volumes of 0.15 *M* NaCl to remove salt soluble spleen proteins, but not to remove amyloid which is insoluble at this salt concentration. This increased the sensitivity of the assay. To ensure that fibrils were not lost in the salt washes, the ThT fluorescence of the salt washes was examined (Figure 10, inset *b*). No signal was detected, indicating that if any fibril was lost, the amount was less than the assay could detect. Finally, the homogenates were

dissolved in 0.25 M NaOH before the samples were read, which resulted in more reproducible measurements. The treatment reduced the turbidity of the samples by solubilizing the amyloid without destroying the fibrils (114).

5.4.3 ThT Fluorescence During AA Amyloid Induction

Spleen homogenates from all mice showed a ThT fluorescence intensity above background at 12 h, while at 18 h the intensity returned to zero for silver nitrate or AEF treated animals. The earliest that AA amyloid fibrils could be detected with ThT was at 18 h, which is 18 h before AA amyloid can be detected by CR (36 h), and is the earliest that AA amyloid has ever been detected in mice.

Further investigation and modifications were made to see whether AA amyloid could be detected at 12 h post-induction, and whether the signal seen in mice receiving either AEF or silver nitrate alone was an artifact. To see whether the increased fluorescence at this time point was due to AA fibrils, guanidine-HCl (2 M final concentration) was added to the samples. In the homogenates from mice injected with silver nitrate, the intensity did not decrease significantly, demonstrating that the increase in fluorescence was due to some component other than amyloid fibrils. In mice receiving AEF, or silver nitrate and AEF, the fluorescence at 12 h was reduced to background by treatment with guanidine-HCl, showing both contained AA fibrils. AEF is known to contain fibrils, suggesting that injected amyloid fibrils in the AEF are being cleared by the spleen, which is the major circulatory filtering organ. At 24 h the fluorescence from the homogenates of AEF treated mice returned to control levels, indicating that the fibrils have probably been degraded and/or secreted. Thus, using the AEF accelerated amyloid induction protocol, AA amyloid cannot be detected any earlier than 18 h, since the technique cannot differentiate between injected and new fibrils.

However, with the following modification, AA amyloid was detected at 12 h. Several laboratories have shown that AEF activity persists for several days to two weeks after injection. By injecting AEF and waiting 24 h before administering silver nitrate, the ThT fluorescence signal due to injected fibrils disappears, and any remaining signal would be due to the newly synthesized fibrils. Treatment of this spleen homogenate with 2 M guanidine-HCl reduced the fluorescence signal to background, demonstrating that the signal was due to amyloid fibrils. Under these conditions it was possible to detect AA amyloid 12 h post-induction.

5.5 Implications of ThT Fluorescence for AA Amyloidogenesis

5.5.1 Significance of Early Detection of AA Amyloid

The data have shown that AA amyloid can be detected at 12 h after induction. This is at least 24 h earlier than has been possible with CR and histological techniques (99). The perlecan mRNA levels have been measured only as early as 24 h after induction. At present the detection of AA amyloid precedes the detection of perlecan mRNA by 12 h. It is therefore possible that AA amyloid deposition is occurring before increased levels of perlecan mRNA are detected, suggesting that the upregulation of perlecan and other basement membrane proteins is a secondary phenomenon, and that perlecan does not facilitate amyloid deposition. However, work with the α_1 -chain mRNA of type IV collagen has shown that it is increased as early as 18 h (98), and it thus may be that perlecan mRNA levels may be detected earlier than 24 h. Regardless of which event precedes the other, it is clear that they are very closely related temporally, but the issue of which occurs first remains unresolved.

Additional techniques are required to answer the question of whether perlecan (and other basement membrane proteins) cause(s) AA amyloid *in vivo*, rather than simply comparing

the timing of these events. The most definitive experiment would be to inhibit basement membrane protein synthesis specifically during amyloid induction, to see whether amyloid deposition can still occur. Ongoing research is attempting to answer this question.

5.5.2 Quantification of AA Amyloid

Quantification of AA amyloid in Figure 10 showed that deposition seemed to plateau at 48 h, suggesting either that there was no further *de novo* deposition of amyloid; that deposition and degradation had reached equilibrium; or that deposition still occurred but was surpassed by increases in spleen weight. The latter appeared to be the case. When the amount of fibril present was multiplied by the weight of the spleen (Figure 11, *inset*), total AA deposition was still increasing to at least 96 h. This was consistent with histological measurements of splenic AA amyloid, which showed in this model continued to increase for a period of at least two weeks (99). Deposition also seemed to occur at different rates over the time course, with the most rapid amount of deposition occurring between 12 h and 24 h. This may be a result of the levels of SAA being maximal over this time, since studies have shown that SAA protein levels peak at 18 h and begin to decrease slowly thereafter (115).

5.5.3 Appearance of AEF in Relation to AA Amyloid

When using standard AA amyloid induction protocols, such as the daily subcutaneous injection of azocasein in CBA/J mice, AEF can be shown to appear 24 - 48 h before histologically detectable amyloid (99). This early work suggested that AEF and amyloid were separate entities. However, it has been shown that amyloid fibrils, whether obtained from tissue or made *in vitro*, have AEF activity. The present work with ThT shows that histologically undetectable amyloid fibrils are actually present at least 24 h earlier than

previously suspected, and thus AEF may be composed of small quantities of amyloid or amyloid protofibrils.

It may however be premature to reach this conclusion. Work with the CE/J strain of mouse, a mouse incapable of forming AA amyloid, still possesses splenic AEF activity after repetitive inflammatory stimuli (60). This suggests either that there may be histologically undetectable amyloid deposits or protofibrils in CE/J mice, or that AEF is a separate entity.

5.5.4 Summary of ThT Experiments

The ThT experiments have shown that AA amyloid deposition can be detected at 12 h post-induction, at which time CR is not sufficiently sensitive to detect these deposits. The studies have also shown that many of the accepted temporal relationships such as that between AEF and AA amyloid, and perlecan and AA amyloid, should be re-examined in view of this new information. However, the utility of temporal approaches to address the question of which comes first appears to be at their limit.

5.6 Implications for the Role of Heparan Sulphate in AA Amyloidogenesis

5.6.1 Conformational Changes in SAA

With respect to the possible causes of amyloid, the Lp-SAA results with heparan sulphate support the hypothesis that perlecan is involved in AA deposition by inducing a conformational change in SAA. However, the tryptophan fluorescence decrease has not yet been shown to be associated with increased β -sheet structure. If this is confirmed, the results will have also shown that these changes can occur while SAA is within the Lp, and can therefore theoretically occur within the HDL-SAA particle. This is important, since it is unclear whether a possible initial step in amyloidogenesis, i.e. the

conformational change of the precursor, happens while the protein is attached to HDL-SAA or later. It is possible that the conformational change itself may actually lead to SAA being removed from HDL-SAA, or only after it is removed from its plasma carrier. One way of testing this possibility would be to monitor shifts in the tryptophan fluorescence excitation maximum of Lp-SAA in the presence of heparan sulphate; if the SAA were to be removed, a blue-shift would show that the tryptophans had moved into a more polar environment, i.e. into the solution, while a red shift would indicate the opposite. Another option is to separate free SAA from Lp-SAA in the presence or absence of the various GAGs, and examine whether heparan sulphate caused more SAA to be removed from the lipoprotein particle than other GAGs.

5.6.2 Low Affinity Interaction between SAA and Perlecan

The inability to demonstrate a high affinity interaction between SAA and perlecan suggests either that this interaction does not occur, is of low affinity, or requires (an) as yet unidentified co-factor(s). The tryptophan fluorescence studies showed that an interaction between the GAG heparan sulphate and SAA does occur, but the interaction may be similar to an enzymatic reaction. The initial binding of SAA to perlecan would be of high affinity, but the so-called product, cross- β sheet SAA, would have a low affinity for perlecan. Such an interaction could not be measured by the ELISA technique, since it examines binding at equilibrium conditions. The tryptophan fluorescence result, however, showed that this was unlikely. The studies with Lp-SAA had shown that 0.1 mg heparan sulphate did not cause a conformation change in SAA, while 0.5 mg did, suggesting that perlecan is not acting as a catalyst.

Alternatively, the SAA may cause amyloid deposition through its interference of basement membrane protein interactions. Such an effect has been recently shown with β PP, the Alzheimer amyloid protein precursor (116). The addition of HDL-SAA or HDL to

perlecan-fibronectin binding did not result in competitive inhibition, but did interrupt the high affinity binding between the two components. The binding curve had a large linear component that did not occur in the absence of lipoprotein, suggesting that HDL and HDL-SAA somehow disturbed the binding interaction. The *in vivo* interaction between basement membrane proteins are much more complex than the perlecan-fibronectin binding created *in vitro*. During inflammation the basement membrane may be disturbed in such a way that these proteins are exposed, so that the presence of lipoproteins may disrupt any repair or re-assembly of the basement membrane. Under most acute inflammatory conditions, such disturbances by HDL or HDL-SAA may be controlled or reversed. However, during chronic inflammation, repair may become difficult or impossible, and exposed heparan sulphate may cause SAA to have more β -sheet structure. β -sheet proteins are in general harder to cleave proteolytically and in amyloid they may form a proteolytically resistant fibril, suggesting that AA amyloid accumulation may be due to an inability to digest fibrils. With this model, no equilibrium high affinity binding is required between SAA and perlecan.

5.6.3 Perlecan from EHS Tumour

Another explanation as to why no high affinity binding was detected between SAA and perlecan may come from the type of GAG and proteoglycan used in the present experiments. The heparan sulphate used in the tryptophan and CD experiments came from normal porcine intestinal mucosa, while the perlecan came from EHS tumours. In tumours, the growth may be so rapid that normal post-translational modifications of newly synthesized proteins do not occur properly. In the case of perlecan this may affect heparan sulphate synthesis. An *in vivo* effect with decreased heparan sulphate sulphation has been shown in mouse mammary epithelial tumour and is associated with poor basement membrane formation (117), demonstrating that proper sulphation is required for

correct assembly of the basement membrane proteins. A similar process may occur in the EHS tumours.

Perlecan can also be obtained from non-tumour tissue, such as Reichert's membrane, an extra-embryonic basement membrane in pregnant rodents (118). Comparisons between perlecan from EHS tumour and from Reichert's membrane have shown that EHS-perlecan has less 3-*O*-sulphated glucosamine, and that it bound with lower affinity to antithrombin III than the Reichert's membrane form (118). Similarly, the tumour form of syndecan-1, a cell membrane form of heparan sulphate proteoglycan, has less 2-*O*-sulphated glucosamine, and bound with 20-fold lower affinity to type I collagen (119).

It is apparent from these results that the lack of sulphation at specific O-groups in heparan sulphate proteoglycans could have resulted in a loss of high affinity binding to their ligands. An alteration of the specific sulphation pattern in EHS perlecan may explain why the ELISA did not detect any high affinity binding while the tryptophan fluorescence experiments showed an interaction between heparan sulphate and Lp-SAA.

In addition, these observations suggest that a specific carbohydrate sulphation sequence in heparan sulphate may be required to induce the SAA conformation change associated with amyloid. It is thus possible that in amyloidosis it is perlecan that is mishandled and not the precursor protein. Support for this idea would come from characterization of the heparan sulphate sequence in amyloid compared to that of the normal GAG.

5.6.4 Summary of Experiments

The purpose of the present work was to study changes in SAA conformation in a lipid environment after the addition of heparan sulphate, the affinity of perlecan for SAA using several approaches, and the possible detection of AA amyloid in tissue before it can be

detected by a histological technique. The following are the results and conclusions of these investigations:

1) SAA undergoes a conformational change after the addition of heparan sulphate even when in a lipid environment. This suggests that the conformational change induced by heparan sulphate can occur while SAA is in the native HDL-SAA particle.

2) The interaction between SAA and perlecan as measured by several techniques is apparently of low affinity ($K_d < 10^{-6} M$). The lack of high affinity binding may come from the use of EHS perlecan, suggesting that the tumour is not able to properly synthesize heparan sulphate.

3) AA amyloid is detected by ThT fluorescence in the AEF accelerated amyloid mouse model 12 h after amyloid induction, which is 24 h earlier than has been possible with histological techniques. This suggests that amyloid deposition precedes detection of perlecan mRNA levels, but the temporal issue remains unresolved, because the question now is whether the ThT technique is more sensitive than RT-PCR.

5.6.5 Other Future Experiments

In the tryptophan experiments, CcR was used as a control for changes in protein conformation after the addition of heparan sulphate. The protein was used since it was readily available, and could be incorporated into the Lp since it is an outer mitochondrial membrane protein. A more appropriate control, however, would be one that is more similar to SAA yet does not form AA deposits. Such is the case for the SAA from the AA amyloid resistant CE/J mouse. CD work has shown that after the addition of heparan sulphate to this isoform there was little change in conformation (89). If a Lp-SAA(CE/J) were made, no change should be seen in tryptophan fluorescence after the addition of heparan sulphate.

In addition, in the present work Lp-SAA contained both SAA₁ and SAA₂ isoforms, making it difficult to tell which isoform contributed to the signal. Recombinant isoforms are available. The experiment could be repeated with Lp-SAA₁ and Lp-SAA₂ to see if there is a difference in their fluorescence intensity when the various GAGs are added.

One proposed future experiment would be to combine the two aspects of the conformational change in SAA after the addition of heparan sulphate and the binding of ThT to amyloid fibrils, to determine whether the change in conformation of Lp-SAA resembles that of a fibril. A simple criterion would be whether ThT fluorescence undergoes the excitation and emission shift characteristic of an amyloid when added to an Lp-SAA/heparan sulphate mixture.

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10 20 30
SAA1 GFFSFVHEAF-QGAGDMWRAY-TDMKEANWKN-
SAA2 IG G D

40 50 60
SAA1 SDKYFHARGN-YDAAQRGPGG-VWAAEKISDG-
SAA2 G * A

70 80 90
SAA1 REAFQEFFGR -GHEDTIADQE-ANRHGRSGKD-
SAA2 s M #

100
SAA1 PNYYRPPGLP-DKY
SAA2 A

* Conserved region
Ca²⁺ binding sequence 48-51
GAG-binding sequence



















