Circular-dichroism studies on two murine serum amyloid A proteins

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C.d. studies have shown that mouse SAA₂ (serum amyloid A₂) protein has about one-half of the α -helix content of the SAA₁ (serum amyloid A₁) analogue (15 as against 32%), although secondary-structure prediction analyses based on sequence data do not suggest such a large difference between the forms. The decreased helical content may be a reflection or indication of a stronger propensity to aggregation of the SAA₂ form compared with SAA₁. The main elements of secondary structure in both proteins are β -sheets/turns. Interactions with Ca²⁺ are accompanied by small losses in α -helix content, whereas binding to chondroitin-6-sulphate in the presence of millimolar Ca²⁺ also decreases the amount of secondary structure. However, SAA₂ binding to heparan sulphate increases its β -sheet structure, whereas with SAA₁ secondary structure is not apparently altered by its interaction with heparan sulphate. Computer-generated surface profiles show slight differences in accessibility, hydrophilicity and flexibility between the proteins. Understanding these differences may help to explain why SAA₂ is found in amyloid fibrils whereas SAA₁ is not. In particular, a stronger tendency to aggregation might be the reason why SAA₂ is deposited exclusively in these fibrils.

INTRODUCTION

Murine SAA (serum amyloid A protein) is an acute-phase protein whose serum concentration increases dramatically (500–1000 fold) during the first 24 h of an inflammatory process (McAdam & Sipe, 1976; Sipe *et al.*, 1977; McAdam *et al.*, 1978; Sipe, 1978). SAA is made primarily in the liver (Benson & Kleiner, 1980; Selinger *et al.*, 1980; Szein *et al.*, 1981, 1982; Yatsuta *et al.*, 1982). In the circulation SAA behaves to all intents and purposes as an apoprotein of high-density lipoprotein (HDL) (Benditt & Eriksen, 1977; Benditt, 1979; Skogen *et al.*, 1979). Three murine isoforms of SAA have been identified (Taylor & Rowe, 1984; Ramadori *et al.*, 1985; Lowell *et al.*, 1986a,b; Meek & Benditt, 1986; Stearman *et al.*, 1986; Yamamoto *et al.*, 1987), but only one, circulating SAA₂, is the precursor of the inflammation-associated amyloid protein, the AA peptide (Meek *et al.*, 1986; Shiroo *et al.*, 1987; Tape *et al.*, 1987). The mechanism whereby SAA is converted into AA amyloid fibrils is unknown. The relative sizes of SAA and AA, 12–14 kDa and 8.5 kDa respectively, indicate that proteolytic cleavage of SAA to AA occurs, with AA representing the N-terminal two-thirds of SAA (Levin *et al.*, 1972; Rosenthal *et al.*, 1976; Parmelee *et al.*, 1982). There is, however, growing evidence that intact SAA is a substantial part of AA amyloid deposits, and in some cases intact SAA makes up 100% of the fibril peptide (Ericsson *et al.*, 1987; Westermark & Sletten, 1982). Whether the proteolytic step(s) takes place before or after AA-peptide organization into crossed β -sheeted fibrils (the characteristic organization of all amyloids regardless of the underlying peptide) is therefore not clear.

The tissue deposition of AA amyloid fibrils occurs coincidentally with heparan sulphate proteoglycan (HSPG) deposition (Snow & Kisilevsky, 1985; Snow *et al.*, 1987a), and perhaps chondroitin sulphate (Linker & Carney, 1987). The HSPG is ultrastructurally intimately associated with AA amyloid fibrils (Snow & Kisilevsky, 1987). Since highly sulphated GAGs have been shown to be an integral part of every amyloid deposit (Snow *et al.*, 1987b), it became important to examine the secondary structure of an amyloidogenic peptide, SAA, and what influence GAGs have on this folding. Two closely related SAA isoforms were therefore examined, SAA₁ and SAA₂, the latter the precursor to the amyloid deposits, the former not.

MATERIALS AND METHODS

Generation of circulating SAA

High concentrations of plasma HDL-SAA were induced in 200 Swiss white mice by producing a small sterile dorsal subcutaneous abscess. The latter was initiated by injecting 0.5 ml of 2% (w/v) AgNO₃ solution under the skin of the back (Axelrad *et al.*, 1982). After 24 h, under Metaphane (Pitman-Moore) anaesthesia, the mice were exsanguinated from the retro-orbital sinus with citrated Pasteur pipettes.

Isolation of HDL-SAA

HDL was isolated from the pooled plasma by sequential centrifugation. Briefly, the density of the plasma was adjusted to 1.063 g/l with solid KBr, followed by centrifugation at 10 °C for 16 h at 75000 *g* in a Beckman 50Ti rotor. The top quarter, which contained VLDL and

Abbreviations used: SAA, serum amyloid A protein; AA, amyloid A protein; GAGs, glycosaminoglycans; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; HSPG, heparan sulphate proteoglycan; DMPC, dimyristoyl phosphatidylcholine.

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LDL, was aspirated and discarded. The pooled infranats, adjusted to solvent density 1.21 g/l by addition of KBr, were re-centrifuged for 48 h at 106000 *g* in a 50Ti rotor. The top layers (HDL) were aspirated, pooled and dialysed against 0.15 M-NaCl/0.1% EDTA, pH 6.4, for 24 h at 4 °C, then against 10% formic acid for 24 h.

Gel chromatography of HDL apoproteins

The dialysed HDL preparation was fractionated on a column (2.5 cm × 100 cm) of Sephacryl S-200 previously equilibrated with 10% (v/v) formic acid at a flow rate of 25 ml/h, and 5 ml fractions were collected. Proteins were separated into two peaks. The second major peak, containing mainly protein species of about 12–14 kDa and AA immunoreactivity, was pooled and freeze-dried.

Anion-exchange chromatography

The freeze-dried sample obtained from the gel-filtration step was dissolved in 10 mM-Tris (pH 8.2)/8 M-urea and was applied to a column (1 cm × 30 cm) of DEAE-cellulose (Whatman DE-52) previously equilibrated with the same buffer. After loading, the column was washed further with 80 ml of initial buffer. The column was then eluted with a linear gradient of 150 ml each of 10 mM-Tris (pH 8.2)/8 M-urea and 150 mM-Tris (pH 8.2)/8 M-urea. The flow rate was 12 ml/h and 2.3 ml fractions were collected. At the end of the gradient, the column was further washed with 7.5 ml of 1 M-Tris (pH 8.2)/8 M-urea.

Polyacrylamide-gel electrophoresis

Column fractions were analysed for their protein components on 15% (w/v) polyacrylamide gel in SDS/urea systems as described by Neville (1971).

Determination of absorption coefficient

This involved the determination of protein concentration by the microbiuret method (Bailey, 1967), with bovine serum albumin as a standard. The absorption coefficient was then estimated from measured A_{280} against protein concentration.

GAGs

Chondroitin 6-sulphate, heparin and heparan sulphate were purchased from Sigma (St. Louis, MO, U.S.A.) Sigma, and Miles ((Elkhart, IN, U.S.A.) respectively.

C.d. measurements

These were effected on a Jasco J-500C spectropolarimeter interfaced with a DP500N data processor. The cell was maintained at 25 °C using a Lauda RM6 circulating water bath. Near-u.v. (320–250 nm) scans were performed in a micro cell (with a pathlength of 1 cm) that required only 90 µl of solution. Far-u.v. (250–190 nm) runs were done in either a 0.01 or a 0.05 cm cell. The computer-averaged trace of either four or eight scans was employed in all calculations. Signal due to solvent was subtracted. For experiments involving addition of Ca²⁺ or GAGs, small portions from concentrated solutions were added directly to the amyloid protein in the cell. The instrument was routinely calibrated with D-(+)-10-camphorsulphonic acid at 290 nm, and with pantoyl-lactone at 219 nm, according to procedures

outlined by the manufacturer. The data were normally plotted as mean-residue-weight ellipticity ($[\theta]$; units degrees·cm²·dmol⁻¹) versus wavelength in nm, calculated via the following equation:

$$[\theta]_{\lambda} = \frac{\theta_{\text{obs.}} \times \text{m.r.w.}}{10 \times d \times c} \quad (1)$$

where λ is the wavelength, $\theta_{\text{obs.}}$ is the observed ellipticity in degrees, m.r.w. is the mean residue weight, c is the concentration in g/ml and d is the path length in cm.

Computer-assisted analyses

The secondary structures of the amyloid proteins have been determined under a variety of conditions using the mainframe-driven FORTRAN program CONTIN, developed by Provencher & Glöckner (1981), which analyses c.d. spectra as a sum of data collected from 16 proteins whose structures are known from X-ray crystallography. The input to the program was the mean-residue-weight ellipticities in 1 nm intervals from the minimum value measured to 240 nm. Predictions for the secondary structure of the amyloid proteins, from amino acid sequence data, were based upon the Dufton & Hider (1977) modification of the Chou & Fasman (1974) method. A program to handle these calculations was written by Mr. D. Ross and was run on an IMB PC XT computer.

Composite surface profiles were drawn showing the regions of sequence most likely to lie on the surfaces of these proteins. These computer-generated profiles are composites of three approaches which include h.p.l.c. hydrophilicity parameters (Parker *et al.*, 1986), accessibility (Janin, 1979) and flexibility (Karplus & Schulz, 1985). The program used to generate these data was obtained from the Alberta Peptide Institute (Department of Biochemistry, University of Alberta).

Absorption spectra and protein-concentration determination

Absorption spectra for amyloid proteins were measured on a Perkin-Elmer Lambda 5 recording spectrophotometer in a solvent system of 100 mM-NaCl/25 mM-Tris/HCl, pH 8.0. The spectra were characterized by maxima near 278 nm; shoulders near 290 nm confirmed the presence of tryptophan, whereas fine structure in the region 270–250 nm arose from phenylalanine residues (results not shown). Specific absorption coefficients ($A_{278}^{0.1\%}$) of SAA isomers were 2.2 for SAA₁ and 2.5 for SAA₂.

RESULTS

Protein purification

Ion-exchange chromatography of Swiss white mouse SAA in a Tris concentration gradient resulted in the identification of three SAA isomers (Fig. 1).

Peak I, which was eluted at 45 mM-Tris, was a major isomer and migrated slightly faster on SDS/urea gel than did Peak II and Peak III (Fig. 2).

All three isomers appeared to be homogeneous and possessed AA immunoreactivity. It should be pointed out that the unbound and 1 M-Tris-wash fraction (Fig. 1) did not contain protein bands of 12–14 kDa, nor did they possess AA immunoreactivity.

From migration patterns on SDS/urea gels, Peak I

Fig. 1. DEAE-cellulose chromatography of SAA-rich fractions, pooled from a Sephacryl-S200 column

The column was elated with a linear Tris gradient in 8 M-urea, pH 8.2, at a flow rate of 12 ml/h. Fractions of volume 2.3 ml were collected. Double-ended arrows indicate the pooled fractions of each analogue.

Fig. 2. SDS/urea/polyacrylamide-gel electrophoresis of SAA analogues

Lane 1, 6 μ g of peak III from the DEAE-cellulose column; lane 2, 6 μ g of protein from peak II of the DEAE-cellulose column; lane 3, 6 μ g of protein from peak I of the DEAE-cellulose column (Fig. 1); lane 4, standard proteins: phosphorylase *b* (M_r 92500); bovine serum albumin (M_r 66200); ovalbumin (M_r 45000); carbonic anhydrase (M_r 31000); soybean trypsin inhibitor (M_r 21500); and lysozyme (M_r 14400).

was identified as SAA₂, Peak II as SAA₁ and Peak III was probably SAA₃. The yields from 200 mice were 15.7 mg for SAA₁, 25.3 mg for SAA₂ and 4.2 mg for SAA₃.

C.d. spectra

The initial far-u.v. c.d. spectra generated for SAA₂ were obtained on solutions of low protein concentration obtained by direct dissolution of freeze-dried protein in

Fig. 3. Far-u.v. c.d. spectra for mouse SAA₂ in 100 mM-NaCl/25 mM-Tris/HCl, pH 8.0

A protein concentration of 0.088 mg/ml and a 0.05 cm-path-length cell were used. The temperature was 25 °C. —, Native protein; ---, plus 2 mM-Ca²⁺; ···, plus 2 mM-Ca²⁺ plus chondroitin 6-sulphate (0.38 mg/ml); — · —, spectrum corrected for the optical activity of chondroitin 6-sulphate.

100 mM-NaCl/25 mM-Tris/HCl, pH 8.0. The spectrum is characterized by a single minimum centred near 204 nm with $[\theta]_{204} = -11500^\circ$. A broad shoulder beginning around 224 nm is also evident. In the presence of 2 mM-Ca²⁺, a diminution is noted in the strength of the negative signal, $[\theta]_{204} = -10700^\circ$, and the shoulder near 224 nm is less well defined. Addition of chondroitin 6-sulphate, to a final concentration of 0.38 mg/ml, to the SAA₂-Ca²⁺ complex produces a considerable reduction in signal, even when correction is made for the ellipticity of the GAG alone at the same concentration as was used in the protein sample. The single minimum is blue-shifted 3 nm with $[\theta]_{201} = -9060^\circ$. A new shoulder around 222 nm is now clearly defined ($[\theta]_{222} = -3416^\circ$). These spectra are shown in Fig. 3.

The effects of addition of Ca²⁺ and heparan sulphate (final concn. 0.38 mg/ml) to a solution of SAA₂ are similar. For the native protein $[\theta]_{203} = -11800^\circ$ and $[\theta]_{222} = -5290^\circ$, whereas in the protein spectrum corrected for the ellipticity of heparan sulphate, $[\theta]_{203} = -10650^\circ$ and $[\theta]_{222} = -4440^\circ$. In other words a significant decrease in ellipticity has occurred upon interaction of SAA₂ with the GAG, heparan sulphate in the presence of millimolar Ca²⁺. Equivalent data have been generated for the effects produced by the third GAG studied, namely heparin. A similar decrease in ellipticity has been noted. Since the changes produced by

Fig. 4. Far-u.v. c.d. spectra for mouse SAA₁ in 100 mM-NaCl/25 mM-Tris/HCl, pH 8.0

A protein concentration of 0.337 mg/ml and a 0.0103 cm-path-length cell were used. The temperature was 25°C. —, Native protein; ---, + 5 mM-Ca²⁺ + chondroitin 6-sulphate (0.38 mg/ml); - · - ·, + 5 mM-Ca²⁺ + heparin (0.38 mg/ml); · · · ·, + 5 mM-Ca²⁺ + heparan sulphate (0.38 mg/ml). In the interests of clarity the spectrum for the effect of Ca²⁺ alone has been omitted and the spectra presented have been corrected for the optical activity of the specific GAG used. The protein was initially dissolved in a solution containing 4 M-urea, which was subsequently removed by dialysis.

these latter two GAGs were quite similar to those induced by chondroitin sulphate, in the interests of brevity, these data are not shown.

As mentioned above, later studies employed SAA₂ samples that had been dissolved initially in urea solutions. Although the general shape of the spectrum obtained was very similar, the minimum was now located near 206 nm, with $[\theta]_{206} = -8600^\circ$. The response to the presence of Ca²⁺ and Ca²⁺/GAGs was, however, similar to that of the non-urea-treated sample (results not shown).

Analogous spectra have been produced for the mouse SAA₁ protein (initially dissolved in 4 M-urea solution) in 100 mM-NaCl/25 mM-Tris/HCl, pH 8.0, and in the presence of millimolar Ca²⁺ and the three GAG samples used in Fig. 3. These data are shown in Fig. 4. Firstly it should be noted that, although the spectrum of native SAA₁ is similar in overall shape to that for the SAA₂ isoform, the absolute ellipticity values are considerably higher {e.g. $[\theta]_{204} = -16900^\circ$ for SAA₁, compared with values of $\approx -11500^\circ$ for SAA₂ and -8600° ($[\theta]_{206}$) for urea-treated SAA₂}. Heparin and heparan sulphate pro-

duce quite similar decreases in ellipticity with basically no shift in the wavelength of the single minimum near 204 nm, $[\theta]_{204} = -14600^\circ$ and -15480° respectively. Chondroitin 6-sulphate produces a 4 nm blue shift of the minimum, but with little change in the absolute value at the new minimum, $[\theta]_{200} = -17000^\circ$. A considerable decrease in ellipticity is noted at longer wavelengths 205–230 nm.

It has been reported that a 45-residue variant of AA₂ will form stable complexes with phospholipid (Segrest *et al.*, 1976), and it was later shown that human SAA₄ underwent a considerable increase in α -helix when incubated with DMPC (Bausserman *et al.*, 1983). On the basis of this analogy, it was decided to examine the far-u.v. c.d. of SAA₁ and SAA₂ in solutions of the detergent SDS both at pH 8 and at pH 2. The spectrum for SAA₂ in the presence of 1.1% (w/w) SDS in 100 mM-NaCl/25 mM-Tris/HCl, pH 8.0, shows the bi-modal pattern typical for a protein with a moderate amount of α -helix. The minima are noted near 207 nm and 220 nm, with ellipticity values of -18700° and -14600° respectively. In 2.5% (w/w) SDS at pH 2 the ellipticity values at the corresponding wavelengths are slightly increased to -19600° and -17600° respectively. The spectrum of SAA₂ has also been measured in 0.075 M-H₃PO₄ at pH 2.0. This low pH has been reported to produce a stable monomeric form of human SAA₁ protein (Bausserman *et al.*, 1983). The spectrum is characterized by a single minimum centred near 199 nm with $[\theta]_{199} = -15600^\circ$. These data are presented in Fig. 5(a).

Similar data have been generated for SAA₁. These are shown in Fig. 5(b). Again the spectra are typical of an α -helix-containing protein. In 1.25% (w/w) SDS at pH 8.0, $[\theta]_{207} = -26200^\circ$ and $[\theta]_{221} = -22400^\circ$. In 2.5% SDS at pH 2, $[\theta]_{207} = -27550^\circ$ and $[\theta]_{219} = -25440^\circ$.

Near-u.v. c.d. spectra

The c.d. spectrum of murine SAA₁ (initially dissolved in 4 M-urea solution) in 100 mM-NaCl/25 mM-Tris/HCl, pH 8.0, in the wavelength region 310–250 nm is dominated by a large negative trough with its minimum centred near 280 nm, $[\theta]_{280} = -58.9^\circ$. Superimposed on this trough are weak shoulders near 291 and 296 nm, a pronounced shoulder at 268 nm and two well-defined maxima near 263 and 256 nm. The large minimum probably arises from tyrosine residues in asymmetric environments, whereas the weak shoulders on the long-wavelength side of the trough may reflect minor contributions from tryptophan residues. The strong wavy profile seen from 250 to 275 nm is due to phenylalanine chromophores.

When Ca²⁺ is added to the protein solution to a final concentration of 10 mM, slight perturbations are induced in all regions of the spectrum. The minimum noted initially near 280 nm undergoes a 2 nm blue shift to 278 nm, along with a slight increase in intensity, $[\theta]_{278} = -60.5^\circ$. The peaks at 256 and 263 nm are significantly diminished, and there is greater definition to the shoulder/trough noted near 268 nm.

The perturbations produced on the spectrum of SAA₁ by addition of chondroitin sulphate, heparin and heparan sulphate in the presence of millimolar Ca²⁺ are particularly dramatic in the wavelength region 270–290 nm, with considerable decreases in ellipticity being noted. This is particularly well emphasized in the case of

Fig. 5. Far-u.v. c.d. spectra for mouse SAA₂ (a) and SAA₁ (b)

(a) A protein concentration of 0.393 mg/ml and a 0.0103 cm-path-length cell were used. —, Native SAA₂ in 100 mM-NaCl/25 mM-Tris/HCl, pH 8.0; ---, +1.11% (w/w) SDS at pH 8.0; - · - ·, in the presence of 2.5% (w/w) SDS in 0.1 M-H₃PO₄, pH 2.0; · · · ·, SAA₁ in 0.075 M-H₃PO₄, pH 2.0. The temperature was 25 °C. (b) A protein concentration of 0.337 mg/ml and a 0.0103 cm-path-length cell were used. The temperature was 25 °C. —, Native SAA₁ on 100 mM-NaCl/25 mM-Tris/HCl, pH 8.0; ---, in 1.25% (w/w) SDS at pH 8.0; - · - ·, in the presence of 2.5% (w/w) SDS in 0.075 M-H₃PO₄, pH 2.0. Protein was initially dissolved in 4 M-urea solution as described in the legend to Fig. 4.

Table 1. Far-u.v. c.d. data for murine SAA₁ and SAA₂

Protein	Solvent conditions	Proportion of structure as:			
		α -Helix	β -Sheet	β -Turn	Remainder
SAA ₁	100 mM-NaCl/25 mM-Tris/HCl, pH 8*	0.32	0.27	0.12	0.30
	pH 8 + 10 mM-Ca ²⁺ †	0.29	0.28	0.13	0.31
	pH 8	0.35	0.33	—	0.32
	pH 8 + Ca ²⁺ + chondroitin sulphate	0.16	0.26	0.16	0.43
	pH 8 + Ca ²⁺ + heparin	0.26	0.21	0.16	0.37
	pH 8 + Ca ²⁺ + heparan sulphate	0.30	0.28	0.11	0.30
	1.25% SDS, pH 8.0	0.62	0.38	—	—
	1.25% SDS, pH 2.0	0.67	0.21	—	0.11
	100 mM-NaCl/25 mM-Tris/HCl, pH 8.0†	0.23	0.41	0.09	0.27
	100 mM-NaCl/25 mM-Tris/HCl, pH 8.0	0.15	0.47	0.15	0.22
SAA ₂	pH 8.0 + Ca ²⁺ + chondroitin sulphate	0.08	0.43	0.20	0.28
	pH 8.0 + Ca ²⁺ + heparin	0.10	0.42	0.21	0.27
	pH 8.0 + Ca ²⁺ + heparan sulphate	0.20	0.58	0.19	0.03
	1.1% SDS, pH 8.0	0.40	0.50	0.10	—
	2.5% SDS, pH 2.0	0.42	0.54	0.04	—
	0.075 M-H ₃ PO ₄ , pH 2	0.09	0.41	0.20	0.31

* These measurements were made over the range 195–240 nm in a 0.05 cm-path-length cell; all other measurements were extended to 190 nm by use of a 0.01 cm-path-length cell.

† This was a non-urea-treated sample; all other SAA₁ and SAA₂ samples were initially dissolved in urea solutions.

Fig. 6. Chou-Fasman (1974) prediction of α -helix, β -sheet and β -turn content from sequence data

The cut-off point for conformer formation is indicated by the line joining 1 on the y-axes. —, mouse SAA₂; ---, mouse SAA₁. (a) Prediction of α -helix; (b) prediction of β -turn content (same symbols as for a); (c) prediction of β -sheet content (same symbols as for a).

heparan sulphate. If these data are plotted in the form of difference spectra (data not shown), the maxima in amplitudes are located near 274 and 282 nm. This implies that the GAG binding affects the environment of certain tyrosine and/or tryptophan residues, but has little effect on phenylalanine moieties (since little signal perturbation is noted in the wavelength region 270–250 nm).

The near-u.v. c.d. spectrum of SAA₂ (initially dissolved in 4 M-urea solution) in 100 mM-NaCl/25 mM-Tris/HCl, pH 8.0, is somewhat less complex than that of the SAA₁ analogue. Basically, the spectrum also consists of

a broad minimum centred near 280 nm, but with only minor fine-structure contributions noted on either limb. The spectrum is very responsive to the presence of 2 mM-Ca²⁺, the $[\theta]_{280}$ decreasing from an initial value of -80° to -63° . Addition of extra Ca²⁺ to SAA₂ resulted in immediate precipitation of material, unlike the situation with SAA₁, where Ca²⁺ was added to 10 mM with no apparent adverse effects. Some increase in fine structure accompanied the addition of Ca²⁺, particularly on the low-wavelength side of the spectrum. The initial minimum noted at 280 nm is some 20° more intense than was found for SAA₁. Only a slight decrease in ellipticity is noted when chondroitin 6-sulphate or heparin is added in excess to a solution of SAA₂ in the presence of 2 mM-Ca²⁺. However heparan sulphate produced a significant decrease in the value of the ellipticity noted near 280 nm. This latter effect is similar to the situation found with SAA₁ protein, i.e., heparan sulphate produces the greatest effect. These data are not shown.

Computer analysis of far-u.v. c.d. data

The far-u.v. c.d. data obtained for SAA₁ and SAA₂ was analysed by the computer program CONTIN of Provencher & Glöckner (1981) to estimate the amount of α -helix, β -sheet, β -turns and aperiodic structure present in these proteins under a variety of different conditions. A subroutine built into this program takes into consideration errors in estimation of protein concentration of $\pm 3\%$. Within these limits, the error in estimating the secondary-structure content is on the order of $\pm 0.5\%$. The results are presented in Table 1.

SAA₂ has a low amount of α -helix, some 15% (23% for the non-urea-treated material), and consists mainly of β -sheets and turns. SAA₁, on the other hand, has significant helical content (32–35%) and consequently less β -structure. The effect of Ca²⁺ addition to millimolar levels is to decrease the helical content of both proteins by 3%. In the presence of the three GAGs employed in the present study, the α -helical content of SAA₁ is decreased; chondroitin 6-sulphate seems to be most potent in this respect. In the case of SAA₂, chondroitin 6-sulphate had an effect similar to that seen with SAA₁. Regarding β -sheet/turn structure, there is an apparent difference in the behaviour of SAA₁ and SAA₂ in the presence of heparin and heparan sulphate. Relatively few differences were induced in SAA₁ by heparin or heparan sulphate; however, with SAA₂, heparan sulphate led to a large increase in β -structure, with a concomitant decrease in its aperiodic component. The secondary structures of both SAA₁ and SAA₂ are responsive to the presence of the lipid analogue, the detergent SDS, both at pH 8 and at pH 2, undergoing considerable increase in α -helical content under these conditions.

The α -helical content of SAA₂ in 0.075 M-H₃PO₄ at pH 2, some 9%, is decreased from the 'native' value of 15%. It had already been reported that human SAA₁ was monomeric at pH 2.9 and contained essentially no helical structure (Bausserman *et al.*, 1983), and was somewhat unstable in alkaline solution owing to its considerable propensity to aggregation.

Computer-predicted secondary structure

The secondary-structure predictions of the spectroscopic analysis were compared with those obtained by using the Chou-Fasman (1974) empirical analysis from sequence data for murine SAA₁ and SAA₂ (Yamamoto

Fig. 7. Composite plot of hydrophilicity, surface accessibility and polypeptide flexibility for mouse SAA₂ (—) and SAA₁ (---). The algorithm described by Parker *et al.* (1986) was used.

& Migita, 1985). The amounts of secondary structure derived from the data in Figs. 6(a), 6(b) and 6(c) are as follows: 43% and 47% α -helix for SAA₁ and SAA₂, 10% β -sheet for both analogues and 25% and 20% β -turns for SAA₁ and SAA₂ respectively. The experimentally observed helical content is much less than these predicted values.

By utilizing a composite algorithm for hydrophilicity, accessibility and flexibility (Parker *et al.*, 1986), surface profiles were drawn showing the regions of sequence most likely to lie on the surfaces of these two proteins. These are shown in Fig. 7. The residues which actually comprise the putative surface sites are listed in Table 2. An important point that comes out of these data is that, in both proteins, the first 20 or so residues from the N-terminus are buried, whereas the regions near the C-terminus are exposed. In addition it should be noted that regions which show a strong propensity for β -turn formation by Chou-Fasman (1974) analysis are all located on surface sites.

Table 2. Computer-predicted surface regions for mouse SAA₁ and SAA₂

Surface site	Surface regions (residue nos.)	
	SAA ₁	SAA ₂
1	21–24	21–24
2	27–33	26–33
3	35–35	35–35
4	39–49	39–49
5	56–63	56–62
6	71–95	71–73
7		75–95
8		97–100

DISCUSSION

Since there is a great deal of interest in the mechanism whereby a soluble SAA or AA protein is laid down as an insoluble amyloid fibril, and since this process is probably controlled by the nature of the secondary and tertiary folding of the SAA or AA protein, any information that pertains to their folding would be valuable. It was felt worthwhile to measure the complete c.d. spectra of the murine SAA₁ and SAA₂ proteins, to establish the secondary structures of these materials, and also to monitor any conformational changes that might accompany their interaction with Ca²⁺ and GAGs, two components known to be present at sites of amyloid deposition.

Secondary-structure predictions on human SAA₁ by the procedure of Chou & Fasman (1974) indicated that this protein contained 40% α -helix and 45% total β -structure (Turnell *et al.*, 1986a,b). It had already been shown experimentally that an analogous human protein SAA₄ had some 32% α -helix in the native state at pH 7.4. Interestingly, when this protein was incubated with the phospholipid DMPC at a molar ratio of 1:100, the α -helix content of the complex was increased to 40%. Insolubility and aggregation problems precluded equivalent measurements being performed on human SAA₂ protein, but c.d. studies at pH 2.9, where the protein was reported to be stable and to exist in the monomeric state, indicated that the protein contained hardly any α -helical structure (Bausserman *et al.*, 1983). The rationale for looking at the structures of these proteins in the presence of a high concentration of Ca²⁺ (namely 2–5 mM) stems from two comments made in a paper by Turnell *et al.*, (1986a). The first concerned the fact that amyloid deposits are rich in calcium (Kulla *et al.*, 1977) and the second that the sequence of SAA₁ showed that a region, Asp⁴³–Ala⁵⁵, highly conserved in all reported SAA and AA sequences (e.g., Yamamoto & Migita, 1985) contained

the completely conserved tetrapeptide Gly⁴⁸-Pro⁴⁹-Gly⁵⁰-Gly⁵¹, which is homologous with Gly³⁰-Xaa³¹-Gly³²-Gly³³ found in all phospholipase A₂ sequences (Dennis, 1983) and which forms the main-chain Ca²⁺-binding site in the crystal structure of the bovine enzyme. Thus the protein may exhibit low-affinity Ca²⁺ binding at this site and may also show conformational sensitivity as a result of binding this cation. In addition, the sites of amyloid deposition are extracellular, where the concentration of Ca²⁺ is of the order of 2.5 mM.

The c.d. results obtained in the present study showed that considerable differences in secondary structure are obtained for murine SAA₁ and SAA₂. The helical content of SAA₁, namely 32–35%, is comparable with that already noted for human SAA₄ (Bausserman *et al.*, 1983). SAA₂, on the other hand, has an α -helix content of ~15%. Chou-Fasman (1974) analysis of the sequences of both these proteins indicates an α -helical content of ~43% and 47% for SAA₁ and SAA₂ respectively. The decreased helical content found experimentally for SAA₁ may be a reflection of instability/aggregation/incomplete refolding after urea dissolution at the pH value of 7.5 used for the c.d. measurements of human SAA₁ (Bausserman *et al.*, 1983). Unfortunately lack of material precluded sedimentation-equilibrium studies to monitor the molecular mass of SAA₁ in solution at pH 7.5. In addition, it is possible that the conformational parameters derived from globular proteins may not be applicable to proteins that are highly aggregated.

The c.d. studies with SDS were intended to mimic the earlier observations on the increase in helix produced in SAA₄ protein upon interaction with phospholipid (Bausserman *et al.*, 1983). Both SAA₁ and SAA₂ analogues undergo a considerable increase in α -helix, particularly at pH 2.0. It has been found that acidic solutions of SDS can be much more potent than neutral pH ones in ordering structure in certain polypeptides and proteins (Wu *et al.*, 1981). Of note is the result obtained for SAA₂ in solution at pH 2, where the helical content was decreased to 9%.

Changes in the c.d. spectrum produced by Ca²⁺ were only noted when high concentrations (2–10 mM) were employed. This is in agreement with the earlier suggestion that these proteins would bind Ca²⁺ in a manner similar to the phospholipase A₂ system, i.e., it would be a low-affinity-type binding (Dennis, 1983). Again, it is possible that the decrease in ellipticity noted for both SAA₁ and SAA₂ is part due to changes in aggregation accompanying the interaction with the cation.

Rather dramatic changes in ellipticity were noted for both SAA analogues when they were allowed to interact with several representative GAGs in the presence of millimolar Ca²⁺. These were looked at since GAGs are always found in the amyloid deposits as an integral complex (Snow & Kisilevsky, 1985; Linker & Carney, 1987; Snow & Kisilevsky, 1987; Snow *et al.*, 1987a,b). These changes always showed a decrease in negative ellipticity, equated with a loss in secondary structure. This effect was most prominent with addition of chondroitin sulphate, and implies a somewhat different mode of binding with the GAG. Alternatively, it might indicate an altered degree of aggregation in the presence of chondroitin sulphate.

The SAA₁ and SAA₂ proteins show additional structural dissimilarities as reflected in differences in the c.d. signal across the wavelength region 310–250 nm. Al-

though the spectra of both forms are dominated by a large minimum near 280 nm, subtle alterations are noted on the limbs of this minimum, implying altered contributions from tryptophan and phenylalanine residues. The SAA₁ form has more of these contributions. In addition, altered responses are noted to the presence of Ca²⁺ and Ca²⁺/GAGs.

Turnell *et al.*, 1986a) produced a secondary structure predicted on sequence data for human SAA₁. This included the identification of the *N*-terminal 11 residues as a buried trans-membrane region, possibly composed of a weak amphipathic α -helix and which is functionally important as the lipid-binding site of SAA. A coil-helix transformation of the *N*-terminal sequence upon lipid binding would increase the predicted helical content by 11%. This agrees quite closely with the experimentally observed increase of 14% in α -helix content measured by c.d. when SAA₄ is incubated with DMPC (Bausserman *et al.*, 1983). A point of interest is that a functionally important Ca²⁺-binding loop is predicted in the region of residues 47–55.

Can the computer-generated surface profiles drawn for SAA₁ and SAA₂ yield any additional information on structural differences likely to have a bearing on the question of selective deposition or retention of SAA₂? The predicted amino acid sequences of mouse SAA₁ and SAA₂ (Yamamoto & Migita, 1985) differ at nine positions; most of these substitutions are relatively conservative. However, whereas mouse SAA₁ has glycine at position 60, the SAA₂ analogue contains alanine at that position. At position 7, mouse SAA₁ contains histidine, whereas in mouse SAA₂ this residue is glycine. Another substitution is located at a likely proteolytic cleavage site (residues 75–76). It has been suggested that human SAA is cleaved at the Ser-Leu bond giving rise to the 76-residue AA protein. From the size of mouse AA protein (8.6 kDa) (Hoffman *et al.*, 1984), it is also likely that the Trp⁷⁵-Met⁷⁶ bond in mouse SAA₂ is cleaved during amyloidosis; mouse SAA₁ has threonine and isoleucine in these positions and is also potentially susceptible to proteolytic cleavage. It should be noted, though, that this latter protein does not deposit in tissues as amyloid A protein (Hoffman *et al.*, 1984). Thus mouse SAA₁ may be cleaved, and the amyloid A protein formed may not deposit or may not be retained in tissues. From the increased amount of β -sheet/turn for SAA₂ after its interaction with Ca²⁺ and heparan sulphate, compared with SAA₁, it is tempting to suggest that the SAA₂ isoform may be more resistant to degradation than is SAA₁. SAA₂ may therefore be more available for fibril formation. Alternatively, interactions with other GAGs may, by decreasing the quantity of α -helix in both proteins, make them more readily degradable at additional points and thus not available for fibril formation. Interestingly, the Chou-Fasman (1974) analysis gives little indication of probability for α -helix formation in either analogue beyond residue 80. Both proteins show a strong tendency to form β -turns near the *C*-terminus, with the maximum probability occurring around residue 89.

The sequence substitutions mentioned above do result in minor differences in the computer-generated plots of hydrophilicity, accessibility and flexibility and consequently also in the composite surface plots for murine SAA₁ and SAA₂. The composite plots show quite clearly the buried nature of the *N*-terminal region and the

exposed region near the C-terminus, residues 70–100 or thereabouts. It has been suggested that one possible role for this region is involvement in the rapid clearance from plasma of native apo-SAA (Benditt *et al.*, 1982).

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