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Original Articles

Human Serum Amyloid P Component is a Single Uncomplexed Pentamer in Whole Serum

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Abstract

Background: Serum amyloid P component (SAP) is a universal constituent of amyloid deposits and contributes to their pathogenesis. SAP also has important normal functions in the handling of chromatin in vivo and resistance to bacterial infection. The atomic resolution crystal structure of SAP is known, but its physiological oligomeric assembly remains controversial. In the absence of calcium, isolated human SAP forms stable decamers composed of two cyclic disk-like pentamers interacting face to face. However, in the presence of its specific low molecular weight ligands and calcium, SAP forms stable pentamers. In the presence of calcium, but without any ligand, isolated human SAP aggressively autoaggregates and precipitates, imposing severe constraints on methods for molecular mass determination.

Materials and Methods: Gel filtration chromatography and density gradient ultracentrifugation were used to compare SAP with the closely related molecule, C-reactive protein (CRP; which is known to be a single pentamer) and the effect of human serum albumin on SAP autoaggregation was investigated.

Results: In most physiological buffers and with the necessary absence of calcium, SAP, whether isolated or from whole serum samples, eluted from gel filtration columns clearly ahead of CRP. This is consistent with the existence of a monodisperse population of SAP decamers, as previously reported. However, in Tris/phosphate buffer, SAP was pentameric, suggesting that decamerization involved ionic interactions. On density gradients formed in undiluted normal human serum, SAP sedimented as single pentamers not complexed with any macromolecular ligand, regardless of the presence or absence of calcium. The calcium-dependent autoaggregation of isolated SAP was completely inhibited by physiological concentrations of albumin and the SAP remained pentameric.

Conclusions: Human SAP exists within serum as single uncomplexed pentamers in the presence or absence of calcium. This oligomeric assembly, thus, does not require a calcium-dependent small molecule interaction. The usual >2000-fold molar excess of albumin over SAP in plasma is apparently sufficient to keep SAP in its physiological conformation.

Introduction

Serum amyloid P component (SAP) is a normal plasma glycoprotein which, together with the classical acute-phase protein, C-reactive protein (CRP), forms the pentraxin family of calcium-dependent ligand binding plasma proteins (1). These molecules are phylogenetically conserved, indicating that they have important functions (2,3). Human SAP and CRP have 51% strict residue-for-residue sequence homology, rising to 66% when conservative substitutions are taken into account (4,5). Their protomers have a flattened β-jellyroll fold, which they share with a diverse group of other proteins belonging to what we have called the "lectin fold" superfam-

ily (6–10). The pentraxins also share a characteristic oligomeric assembly of protomers that are noncovalently associated in a disk-like configuration with cyclic pentameric symmetry (1,11–13). Isolated human CRP molecules consist of a single such pentamer; whereas, isolated human SAP in solution in calcium-free physiological saline buffers is a stable decamer, composed of two pentamers interacting face to face (14). We and others have assumed that the native state of SAP under physiological conditions in plasma is also decameric. However, we recently reported that, in its calcium-dependent complex with specific low molecular weight ligands, at physiological pH and ionic strength, human SAP becomes pentameric (15). Also, Sørensen et al. (16) reported, on the basis of gel filtration studies, that SAP is pentameric in serum.

Interest in, and the importance of, SAP have increased sharply because of its role in amyloidosis, a disorder of protein folding and aggregation associated with systemic amyloidosis, Alzheimer's disease, and the transmissble spongiform encephalopathies (17). SAP is universally associated with amyloid deposits in vivo. SAP levels correlate with amyloidogenesis in animal models and binding of SAP protects amyloid fibrils against proteolysis in vitro. Finally, mice with targeted deletion of the SAP gene do not develop amyloid normally (18–20). The SAP-amyloid interaction is, thus, a novel and intriguing therapeutic target. Furthermore, SAP is the single plasma protein that shows specific, calcium-dependent, binding to DNA and chromatin in vitro, and it binds to extracellular chromatin and apoptotic bodies in vivo (21–24). SAP is also a lectin that binds to various bacteria (25). We previously predicted that these binding reactions were likely to mediate important functions for SAP. Recently, we found that SAP knockout mice spontaneously developed severe antinuclear autoimmunity and glomerulonephritis, as well as altered innate resistance to bacterial infection (26,27). Thus, in addition to its pathological importance, SAP has very significant physiological functions that have merited its conservation in evolution. The precise organization and structure function relationships of the SAP molecule are, therefore, of both fundamental and practical interest.

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Analysis of its oligomeric assembly and binding reactions is greatly complicated by the fact that, uniquely among pentraxins, isolated human SAP undergoes rapid autoaggegration when exposed to calcium (13,28). Furthermore, once it is aggregated, human SAP acquires avid and specific binding to plasma fibronectin and C4-binding protein, interactions not shown at all by nonaggregated SAP (29). These phenomena often have been unappreciated, leading to conflicting reports in the literature (30–33). The core of the problem is that measurement of the size or mass of SAP by any technique involving fractionation of serum must necessarily remove the SAP from its normal physiological milieu, in which it coexists at a concentration of around 30 mg/l, with a more than 2000-fold molar excess of serum albumin at 40 g/l, and a further 30 g/l of other serum proteins. Physiological albumin concentrations inhibit the calcium-dependent autoaggregation of isolated SAP (28). However, at lower albumin concentrations, SAP begins to aggregate, acquiring a higher molecular weight and the capacity to bind the other proteins not recognized by nonaggregated SAP.

Based on these considerations, we concluded, in a recent critical review of these issues and the relevant published literature in the field, that the size and molecular organization of SAP within the physiological milieu of whole serum was "unknown and probably unknowable" (18). However we report here that density gradient ultracentrifugation of whole serum, in gradients established in whole undiluted normal serum, demonstrates that native SAP in this matrix exists as single pentamers rather than decamers and that its oligomerization state is not calcium-dependent. We also reconfirm (28,29) that the SAP is not complexed with any macromolecular ligand.

Materials and Methods

Proteins and Other Reagents

Human SAP and CRP, as well as mouse C3 were isolated at >99% purity as previously described (34–36). SAP was oxidatively iodinated using *N*-bromosuccinimide (Sigma, Poole, Dorset, U.K.) (37) and functional integrity of the labeled protein was demonstrated by full retention of its capacity for calcium-dependent binding to phosphoethanolamine covalently immobilized on Sepharose® (Pharmacia Biotech, St. Albans, Herts., U.K.) (34,38). Methyl 4,6–*O*-(1-carboxyethylidene)-β-D-galactopyranoside, here-

after referred to as MOβDG, was synthesized as previously described (39). The mononucleotide dAMP was from Sigma.

Gel Filtration Chromatography

Fast protein liquid chromatography (FPLC; Pharmacia, St. Albans, Herts., U.K.) was run at 0.2 ml/min on a 22 ml Superose 12HR® 10/30 column (Pharmacia Biotech, St. Albans, Berks., U.K.), previously equilibrated with at least 4 column volumes of buffer, and loaded with 200 μl samples of either isolated proteins at 1–2 mg/ml in the running buffer or whole undiluted normal human serum spiked with ^{125}iodine-SAP at 2 μg/ml and CRP at 400 μg/ml final concentrations. After spiking, serum was incubated for 2 hr before analysis. Serum samples were applied at 0.05 ml/min. The buffers used in different runs were as follows (all chemicals were analytical grade from Sigma, Poole, Dorset, U.K.): TE buffer, comprised of 10 mM Tris, 140 mM NaCl, 10 mM EDTA, 0.1% weight-per-volume (w/v) NaN_3, at pH 8.0; Tris/borate, comprised of 90 mM Tris, 80 mM sodium tetraborate, 2 mM EDTA, at pH 8.4; Tris/carbonate, that is 90 mM Tris, 80 mM Na_2CO_3, 2 mM EDTA, at pH 8.4; phosphate buffered (10 mM) physiological saline, at pH 7.4; Tris/phosphate, comprised of 90 mM Tris, 80 mM NaH_2PO_4/Na_2HPO_4, 2 mM EDTA, at pH 6.4, 7.4 and 8.4 in different experiments; triethanolamine, comprised of 170 mM triethanolamine, 2 mM EDTA, at pH 8.4. For experiments with SAP ligand combinations, the column eluant was TN buffer, comprised of 10 mM Tris, 140 mM NaCl, 0.1% w/v NaN_3, at pH 8.0, containing the ligand and $CaCl_2$ concentrations shown in the "Results" section. Protein elution was monitored by absorption at 280 nm, specific protein assays, and counting radio-activity. The SAP profiles from counting and immunoassay were identical in all experiments. Normal human serum samples (3–12 ml) containing SAP at 38 μg/ml, and spiked with a trace of ^{125}iodine-SAP and purified CRP to 400 μg/ml final concentration, were also fractionated at 12 ml/hr on conventional columns of Sephacryl S-300HR® (493 ml) and Sephacryl S-300 (460 ml) (Pharmacia) equilibrated and eluted with TE buffer, both at pH 8.0 and pH 7.4.

Density Gradient Ultracentrifugation

Linear 5–10% w/v Ficoll 400 (Pharmacia) gradients were formed in either whole undiluted normal human serum or human serum albumin (Sigma) solution at 40 mg/ml in phosphate-buffered saline, pH 7.2 (PBS). Volumes of 0.7 ml of 10%, 9%, 8%, 7%, 6%, and 5% Ficoll solutions were sequentially layered into 5.5 ml polycarbonate ultracentrifuge tubes (Beckman Instruments Inc., High Wycombe, Bucks., U.K.) and allowed to diffuse for 5 hr at 4°C. Samples of 200 μl of whole undiluted normal human serum were spiked with ^{125}iodine-SAP and with CRP, to final concentrations of 2 μg/ml and 70 μg/ml respectively, and were then loaded on top of the gradients and centrifuged at 40,000 rpm for 18 hr at 4°C in a type 4l Ti swing-out rotor in a Beckman L8-70 ultracentrifuge (Beckman, High Wycombe, Bucks., U.K.). In some experiments, the sample and the whole gradient contained EDTA at a final concentration of 10 mM, and in others, mouse C3 was incorporated in the sample at 40 μg/ml as a marker of known M_r ~ 180,000. After centrifugation, 100-μl fractions were collected by upward displacement with 50% w/v sucrose introduced from the pierced bottom of the tube. Fractions were assayed for SAP by radioactive counting, and for CRP and mouse C3 by specific immunoassays.

Calcium Dependent Autoaggregation of SAP

Volumes of 2 μl concentrated $CaCl_2$ solution were added to duplicate 100-μl volumes of whole normal human serum containing 30 mg/l of SAP and in a parallel experiment, to duplicate 100-μl volumes of purified SAP at 30 mg/l in TN buffer containing 40 g/l of human serum albumin, in order to make the final calcium concentration to 2, 5, 10 and 20 mM. Control tubes received water, instead of $CaCl_2$. After mixing and incubation at 37°C for 1 hr, all tubes were centrifuged at 10,000 g for 10 min and the supernatants assayed for SAP. In another experiment, performed in duplicate throughout, purified SAP was added (to a final concentration of 31.3 mg/l) to solutions in TN buffer of human serum albumin containing concentrations of 0, 1, 5, 10, 20 and 40 g/l; and $CaCl_2$ was then added (2 μl per 100 μl to provide a final concentration of 2 mM). Control tubes received water instead. After mixing and incubation at 37°C for 1 hr, all tubes were centrifuged at 10,000 g for 10 min and the supernatants assayed for SAP. In a third study, performed in duplicate throughout, purified SAP in volumes of up to 10 μl was added to 100 μl volumes of whole normal human serum

to increase the final SAP concentration by 100, 200, 300, 400, 500, and 1000 mg/l. In a strictly parallel experiment, the SAP was added to human serum albumin at 40 g/l in TN buffer and 2 μl volumes of 100 mM $CaCl_2$ solution were then added to each tube to bring the final calcium concentration to 2 mM. Controls for both these series consisted of the same mixtures in which 200 mM EDTA, pH 7.2, was added to make a final EDTA concentration of 10 mM. After mixing and incubation at 37°C for 1 hr, all tubes were centrifuged at 10,000 g for 10 min and the supernatants assayed for SAP. Finally, mixtures of equal volumes of whole serum or TN buffer, purified SAP in TN buffer, and either $CaCl_2$ or EDTA, were made so that the final concentration of SAP was 500 mg/l, in either 2 mM calcium or 10 mM EDTA. After mixing and incubation at 37°C for 1 hr, all tubes were centrifuged at 10,000 g for 10 min and the supernatants assayed for SAP.

Specific Protein Assays

SAP, CRP and mouse C3 were quantified by electroimmunoassay using monospecific sheep antisera and serum standards previously calibrated against the isolated pure proteins (36, 40,41).

Results

FPLC Gel Filtration

Isolated pure SAP consistently eluted before isolated CRP when run on a Superose 12HR column in TE buffer, at pH 8.0 (Fig. 1); in PBS, pH 7.2; in Tris/borate/EDTA, pH 8.4; in Tris/carbonate/EDTA, pH 8.4; or triethanolamine/EDTA, pH 8.4 (Table 1). The same result was obtained when the sample loaded was whole human serum containing SAP and CRP (Table 1), although the elution of both proteins was retarded, compared with loading pure proteins in buffer, presumably because of the much greater viscosity of the sample. However, in Tris/phosphate/EDTA at pH 6.4, 7.4, or 8.4, SAP was markedly retarded and eluted just marginally ahead of CRP.

Conventional Gel Filtration

Fractionation of whole serum on conventional Sephacryl® S-300 and S-300HR columns (Table 2, Fig. 2) showed the same clear separation of pentraxins seen in FPLC on Superose 12HR, with SAP eluting well ahead of CRP.

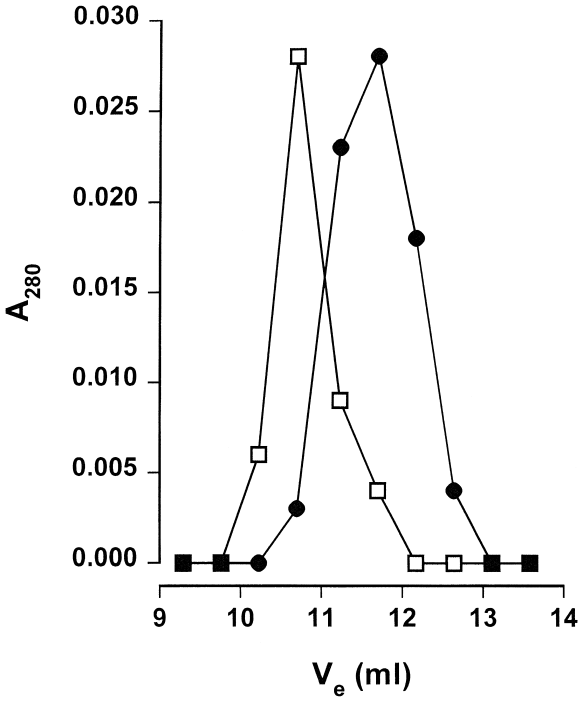

Fig. 1. Fast [protein] liquid chromatography (FPLC) gel filtration on Superose 12HR of isolated SAP (□) and CRP (●) in TE buffer. SAP, serum amyloid P component; CRP, C-reactive protein; Ve, elution volume; A_{280}, absorbance at 280 nm.

Effect of Ligand Binding on SAP Assembly

FPLC gel filtration of SAP in the presence of 2 mM calcium and 10 mM M*Oβ*DG, the best-characterized, calcium-dependent carbohydrate ligand of SAP (39), gave an elution volume (V_e) value very close to that of CRP. SAP is known from solution scattering studies to be pentameric under these conditions (15). In contrast, in the presence of 20 mM calcium and 10 mM dAMP, SAP eluted at its typical V_e well ahead of CRP. The calcium dependent complex of SAP with dAMP is known to be decameric (42).

Density Gradient Ultracentrifugation in Whole Serum or in Human Serum Albumin Solution

When whole human serum was subjected to ultracentrifugation in density gradients composed of Ficoll dissolved in whole human serum, SAP and CRP sedimented to almost the same position, but with SAP always marginally ahead of CRP (Fig. 3A). This sedimentation pattern and the profiles of both proteins were unaffected by the presence of excess EDTA in the sample and throughout the gradient (Fig. 3B), indicating that they were unaffected by

Table 1. FPLC gel filtration of human SAP and CRP on Superose 12HR

Sample	Eluant	V_e (ml)	Oligomerization State
Isolated SAP	TE buffer pH 8.0	10.7	decamer
Isolated CRP	TE buffer pH 8.0	11.7	pentamer
Isolated SAP	Tris/borate pH 8.4	10.1	decamer
Isolated CRP	Tris/borate pH 8.4	12.6	pentamer
Isolated SAP	Tris/carbonate pH 8.4	10.4	decamer
Isolated CRP	Tris/carbonate pH 8.4	11.7	pentamer
Isolated SAP	Triethanolamine pH 8.4	11.2	decamer
Isolated CRP	Triethanolamine pH 8.4	12.9	pentamer
Isolated SAP	PBS	10.8	decamer
Isolated CRP	PBS	11.7	pentamer
Isolated SAP	Tris/phosphate pH 6.4, 7.4 or 8.4	11.7	pentamer
Isolated CRP	Tris/phosphate pH 6.4, 7.4 or 8.4	11.8	pentamer
SAP + MOβDG/Ca^{++}	TN buffer + Ca^{++}	11.5	pentamer
SAP + dAMP/ Ca^{++}	TN buffer + Ca^{++}	10.9	decamer
SAP in whole serum	TE buffer	11.4	decamer
CRP in whole serum	TE buffer	12.2	pentamer

Details of buffer composition in "Materials and Methods" and Results. Experiments were performed in the pairs shown. Differences in absolute elution volume (V_e) values between pairs reflect buffer effects and drift in column performance with time, multiple runs (including whole serum samples) and multiple cleaning cycles. However, within each pair the clear separation between SAP and CRP was invariable except in Tris/phosphate or in the presence of MOβDG and calcium. SAP, serum amyloid P component; CRP, C-reactive protein; FPLC, fast [protein] liquid chromatography; MOβDG, methyl-4,6-O-1-carboxyethylidine-β-D-galacto-pyranoside; TN Buffer, TE buffer,.

any calcium-dependent interactions among the pentraxins and other serum components. Identical results were obtained using serum immunodepleted of SAP to create the gradient and, then, running samples of whole serum spiked with ^{125}I-CRP while SAP was monitored by immunoassay (results not shown). Mouse C3, relative molecular mass (M_r) ~180,000, which was included in the loaded sample, sedimented clearly ahead of both SAP and CRP, compatible with the pentraxins having M_r values of 127,310 and 115,135 respectively. These corresponded to their pentameric configurations and indicated that neither pentraxin could be decameric. When Ficoll gradients were created in solutions of albumin at the physiological concentration of 40 g/l, SAP and CRP from a sample of whole human serum sedimented exactly as they did in gradients formed in whole serum, with the SAP always marginally ahead (Fig. 4). This indicated that the molecular forms of SAP and CRP under these conditions were unaffected by any serum component, other than albumin.

Table 2. Gel filtration of whole human serum on Sephacryl

Medium	Protein	V_e (ml)	Oligomerization State
Sephacryl S-300HR	SAP	255	decamer
Sephacryl S-300HR	CRP	285	pentamer
Sephacryl S-300	SAP	184	decamer
Sephacryl S-300	CRP	212	pentamer

Serum was loaded without dilution and eluted with TE buffer. Identical results were obtained at pH 7.4 and pH 8.0. SAP, serum amyloid P component; CRP, C-reactive protein; Ve, elution volume.

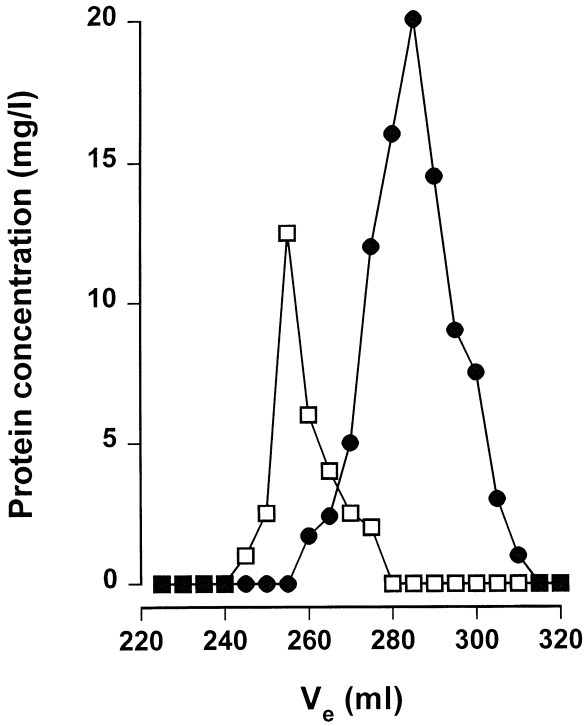

Fig. 2. Conventional gel filtration of whole serum on Sephacryl S-300HR in TE buffer showing the elution profile of SAP (□) and CRP (●). TE buffer, ; SAP, serum amyloid P component; CRP, C-reactive protein; VE, elution volume.

Although the viscous gradients used here, which were formed in undiluted serum or 40 g/l albumin, were not highly resolving and were technically difficult to fractionate cleanly, the overall distribution profiles and the peak fractions were robustly reproducible for all proteins. The broader distributions of SAP and CRP within the gradients, compared with the mouse C3 marker, were compatible with the known molecular asymmetry of the disc-like pentraxins compared to the typical globular C3 molecule. The absence of any high M_r shoulder on the SAP profile and the identical distribution of SAP on gradients formed in serum containing calcium or EDTA, or in pure albumin alone, showed that there was no appreciable binding of SAP to other proteins. These ultracentrifugation observations were, therefore, only compatible with SAP being a pentamer within serum and demonstrate that, under these physiological conditions, it was neither decameric nor complexed with a macromolecular ligand.

Aggregation of SAP in Whole Serum or Albumin Solutions

The calcium-dependent aggregation of isolated pure SAP at the physiological concentration of 30 mg/l was inhibited in a dose-dependent fashion by the presence of human serum albumin, but was only prevented completely by albumin at the physiological concentration of 40 g/l (Fig. 5). Addition of extra calcium, at up to 20 mM final concentration, did not cause any aggregation of SAP at 30 mg/l in either whole human serum or human serum albumin at 40 g/l (results not shown). Addition of SAP to either undiluted whole normal serum, or human serum albumin at 40 g/l, even at grossly supraphysiological concentrations of up to 1 g/l, produced only trace calcium-dependent aggregation. Even when SAP was added to a final concentration of 500 mg/l in serum at a final dilution of 1:3, there was only minimal aggregation (8%); whereas, when the same concentration of SAP was placed in calcium-containing buffer, in the absence of serum or any other protein, there was 100% precipitation.

Discussion

The protomer mass of human SAP is 25,462 D, as predicted by the amino acid and complete, invariant, glycan sequence, and confirmed by electrospray ionization mass spectrometry (ESIMS) measurement (43). An SAP pentamer would, therefore, have an M_r of 127,310 and the decamer would be 254,620. The protomers of human CRP are non-glycosylated and have an M_r of 23,027, as predicted by the amino acid sequence and confirmed by ESIMS measurement (44). Intact native CRP is known from previous gel filtration, ultracentrifuge, X-ray and neutron solution scattering studies to be a pentamer (14,35,45), and its crystal structure confirms the electron microscopic appearance of cyclic pentameric arrangement of the protomers (2,8,10,46,47). The gel filtration V_e observed for native CRP, therefore, corresponds to a molecule with M_r 115,135 and the typical, asymmetric, pentraxin shape. The clear separation of SAP and CRP and smaller V_e for SAP, both on Superose by FPLC and on Sephacryl by conventional chromatography, indicate that SAP is decameric in the buffers used, as we showed previously (14).

However, in the presence of calcium and M*Oβ*DG, the well–characterized calcium-

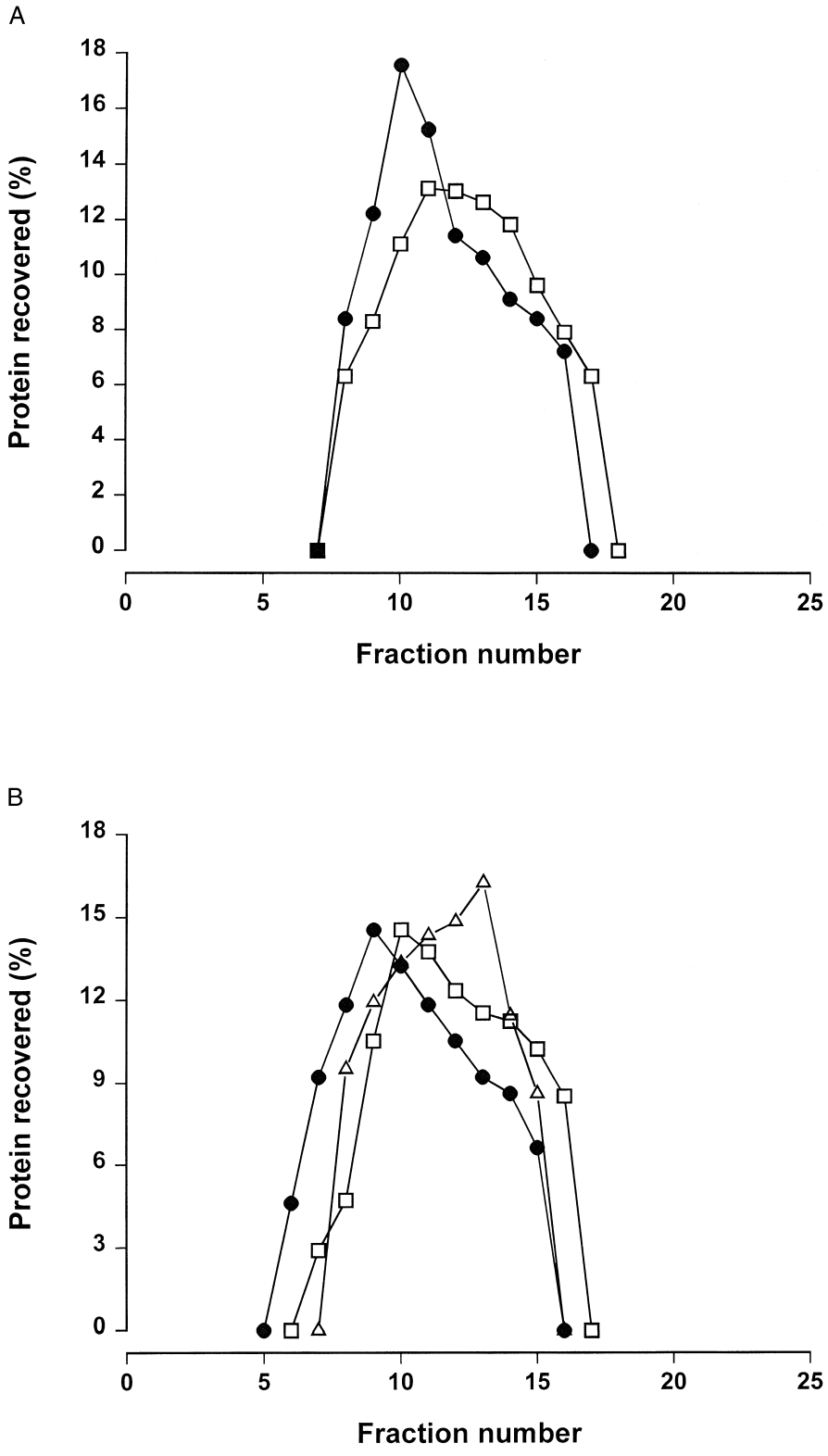

Fig. 3. Ficoll density gradient ultracentrifugation of whole human serum containing added CRP on gradients formed in whole serum, (A) without and (B) with EDTA added to 10 mM final concentration. After centrifugation, the gradient was fractionated from the top, lowest density, fraction 1, to the bottom, highest density, fraction 25. The sedimentation profiles are shown for SAP (□), CRP (●), and mouse C3 (Δ). The latter was added to the sample as a marker of known M_r ~ 180,000. SAP, serum amyloid P component; CRP, C-reactive protein.

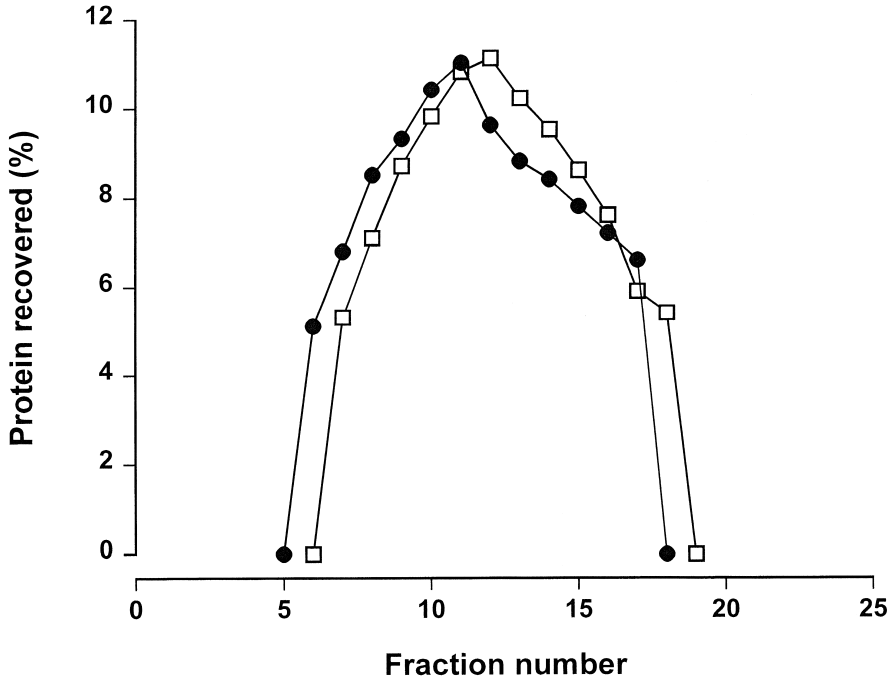

Fig. 4. Ficoll density gradient ultracentrifugation of whole human serum containing added CRP on gradients formed in human serum albumin solution at 40 g/l. After centrifugation, the gradient was fractionated from the top, lowest density, fraction 1, to the bottom, highest density, fraction 25. The sedimentation profiles of SAP (□) and CRP (●) are shown. SAP, serum amyloid P component; CRP, C-reactive protein.

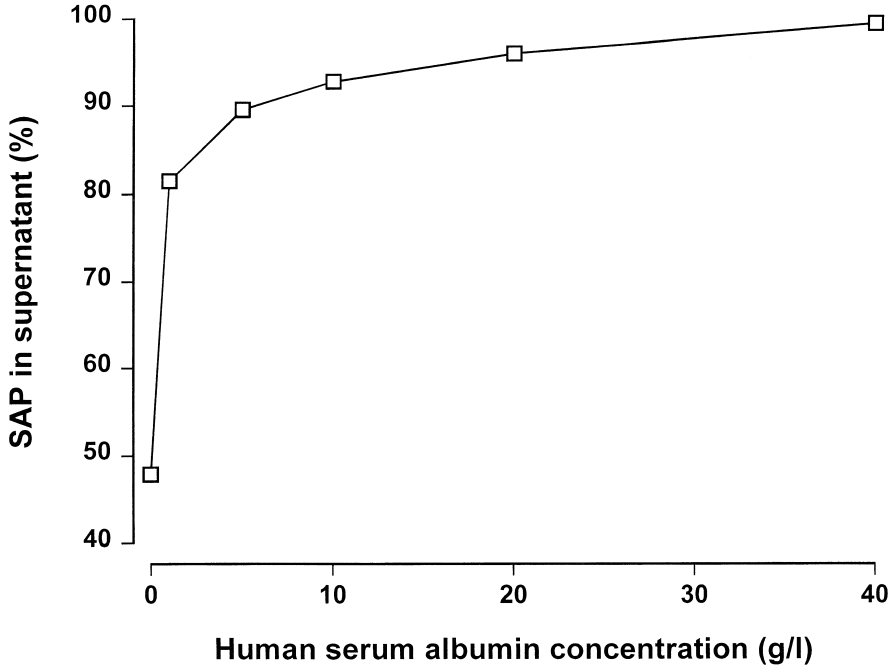

Fig. 5. Effect of human serum albumin concentration on the calcium-dependent autoaggregation of SAP. Isolated pure SAP at a final concentration of 30 mg/l and calcium at a final concentration of 2 mM were incorporated in solutions of albumin at the concentrations shown. The proportion of SAP that then became sufficiently aggregated to be sedimented by centrifugation at 10,000 g for 10 min was measured. SAP, serum amyloid P component.

dependent ligand of SAP (39), human SAP exists as single pentamers. This has been shown by X-ray crystallography (6), and X-ray and solution scattering studies (15). Here we confirm it by gel filtration. The high resolution crystallographic structure of the calcium-dependent complex of SAP with dAMP also revealed SAP pentamers with dAMP molecules held in the calcium-dependent, ligand-binding pocket of each SAP protomer, but base stacking interactions between the nucleotides "cross linked" adjacent pentamers to form decamers (42). Existence of this decamer in solution was confirmed by scattering studies (S. J. Perkins, E. Hohenester, M. B. Pepys, in preparation), and by gel filtration (42; Table 1). It is, thus, clear that in the presence of calcium and low molecular weight ligands, human SAP is prevented from autoaggregating, as it does with calcium alone (28), and exists as single pentamers complexed with ligand, unless there is a secondary interaction between bound ligand molecules, as is the case with dAMP. Interestingly, this result had been suggested earlier, when SAP was found to be pentameric in the low pH, high acetate, high calcium buffer from which it was originally crystallized for X-ray analysis (48), and the calcium-binding ligand pockets of each protomer were occupied by acetate (6).

Solution scattering studies, coupled with the crystal structure of SAP, strongly suggest that the stable SAP decamer seen in calcium-free solution is formed by the stacking face-to-face of the so-called A surfaces of the pentamers, which are those bearing the α-helix of each protomer (15). However, this face of the pentamer has a negative overall charge; whereas, the opposite B face that bears the calcium-dependent ligand binding sites has a positive overall charge. It is, therefore, likely that electrostatic effects make a major contribution to the pentamer-decamer transition. This is consistent with the fact that SAP is pentameric in the presence of ligand and calcium, when binding of two calcium ions and one ligand molecule per protomer markedly alters the charge distribution. It may also explain the current finding that SAP is pentameric in buffer containing a sufficient concentration of phosphate ions, although, it remains decameric in carbonate and triethanolamine buffers at the same ionic strength and pH.

During ultracentrifugation of serum samples on Ficoll density gradients created in whole serum, there must be sedimentation of proteins throughout the gradient, so that SAP from the loaded serum sample is not within completely unaltered serum. Nevertheless, this seems to be the best approach to estimate the mass of human SAP within a milieu as close to physiological conditions as possible. The results clearly show that SAP exists in serum as single pentamers, that this state is not dependent on calcium, and that SAP remains pentameric when physiological concentrations of albumin alone are present in the gradient, rather than whole serum. Our results demonstrate that, contrary to other claims (30–33), SAP is not complexed with C4-binding protein in serum, nor to fibronectin or any other macromolecular ligand. This is as expected because, as we originally showed, SAP unequivocally does not recognize and bind these other proteins unless pairs of SAP molecules are closely approximated (29). Furthermore, the sedimentation behavior of SAP from serum is unaffected by total depletion of either C4-binding protein or fibronectin from the serum (28). The normal pentameric state of SAP in serum is also not dependent on a low molecular weight ligand. It exists in this form in solutions of purified human serum albumin, is unaffected by the absence of calcium, and no calcium-independent ligand-binding interactions of SAP have been described that occur at physiological ionic strength and pH.

The formation of stable decamers by isolated SAP in the absence of calcium is, thus, an in vitro artefact, resulting from pentamer-pentamer interactions, which are largely electrostatic. The calcium-dependent autoaggregation of isolated SAP, which is completely inhibited by calcium-dependent ligands, including M$O\beta$DG, phosphoethanolamine, dAMP, DNA, and glycosaminoglycans (23,42,49), probably reflects binding by the ligand-binding pocket(s) of one SAP molecule to a structure on another, leading to lattice formation. Analysis of the packing of SAP pentamers in several unrelated crystal forms suggests that Glu167, the side chain of which protrudes from the *N*-terminus of the α-helix on the A face, is the structure responsible (18). Site-directed mutagenesis of Glu167 to Gln or Ser completely abolishes calcium-dependent aggregation; whereas, desialylation and degalactosylation have no effect (50).

The present experiments demonstrate that the absence of autoaggregation of SAP in whole serum, or in physiological albumin con-

centrations, simply results from interference by the large molar excess of albumin (>2000-fold) and other proteins over SAP. Even halving the ambient albumin concentration to 20 g/l leads to some frank precipitation of SAP and, at any albumin level appreciably below the physiological 40 g/l, there must also be substantial nonsedimentable aggregation, sufficient for SAP to display its characteristic binding to C4-binding protein and fibronectin. This presumably explains the observations misinterpreted as evidence for a complex in serum between SAP and C4-binding protein (30–33). At lower albumin concentrations, more extensive overt precipitation of SAP occurs. Increasing the calcium concentration in either whole serum or physiological albumin solutions does not cause any precipitation of SAP, indicating that sequestration of calcium ions by albumin and/or other proteins is not the mechanism by which autoaggregation of SAP is prevented. Also, even very high, supraphysiological concentrations of SAP do not undergo calcium-dependent precipitation when added to whole serum or physiological albumin solution, or even serum diluted 1:3.

Sørensen et al. (16) previously suggested that SAP was a pentamer in whole serum. Although their conclusion was correct, it was based on gel filtration analysis and, as discussed above, fractionation into buffer cannot be informative about the state of SAP in serum. Also, they calibrated their columns with standard globular protein markers and did not include CRP or another known pentameric pentraxin marker protein, despite the established molecular asymmetry of SAP and the anomalous behavior of pentraxins in size-exclusion fractionation procedures. In this study, we show by FPLC gel filtration with all buffers tested, except Tris/phosphate, that SAP, whether loaded in pure form or in whole serum, always elutes well before CRP. This indicates that SAP has a substantially higher M_r than CRP. We obtained the same result with conventional chromatography, using the same medium, eluant and ratio of sample-to-column volume as Sørensen et al. (16). Importantly, although the difference in V_e between SAP and CRP is preserved when whole serum, rather than pure proteins (Table 2), is loaded, the absolute V_e values for both proteins are appreciably higher with serum samples (Table 1), because of the greater viscosity of serum. These factors may explain why Sørensen et al. (16) believed SAP to be pentameric under conditions, in which, as we show here, it is unequivocally in the stable decameric assembly that forms in calcium-free buffers.

Acknowledgments

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