Serum amyloid A forms stable oligomers that disrupt vesicles at lysosomal pH and contribute to the pathogenesis of reactive amyloidosis

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Serum amyloid A (SAA) is an acutely induced plasma protein that functions in innate immunity and lipid homeostasis. SAA is a protein precursor of reactive AA amyloidosis, the major complication of chronic inflammation and one of the most common human systemic amyloid diseases worldwide. Most circulating SAA is protected from proteolysis and misfolding by binding to plasma high-density lipoproteins. However, unbound soluble SAA is intrinsically disordered and is either rapidly degraded or forms amyloid in a lysosome-initiated process. Although acidic pH promotes amyloid fibril formation by this and many other proteins, the molecular underpinnings are unclear. We used an array of spectroscopic, biochemical, and structural methods to uncover that at pH 3.5–4.5, murine SAA1 forms stable soluble oligomers that are maximally folded at pH 4.3 with ∼35% α-helix and are unusually resistant to proteolysis. In solution, these oligomers neither readily convert into mature fibrils nor bind lipid surfaces via their amphipathic α-helices in a manner typical of apolipoproteins. Rather, these oligomers undergo an α-helix to β-sheet conversion catalyzed by lipid vesicles and disrupt these vesicles, suggesting a membranolytic activity. Our results provide an explanation for the lysosomal origin of AA amyloidosis. They suggest that high structural stability and resistance to proteolysis of SAA oligomers at pH 3.5–4.5 help them escape lysosomal degradation, promote SAA accumulation in lysosomes, and ultimately damage cellular membranes and liberate intracellular amyloid. We posit that these soluble prefibrillar oligomers provide a missing link in our understanding of the development of AA amyloidosis.

prefibrillar amyloid oligomers | intrinsically disordered proteins | pH-induced conformational changes | hydrophobic cavities | acute-phase reactant

Significance

Although acidic conditions favor the misfolding of various proteins in amyloid diseases, the molecular underpinnings of this process are unclear. We used an array of spectroscopic, biochemical, and electron microscopic methods to unravel the effects of pH on serum amyloid A, a protein precursor of reactive amyloidosis, the major complication of chronic inflammation and one of the major human systemic amyloid diseases worldwide. We found that at lysosomal pH this protein forms unusually stable proteolysis-resistant soluble oligomers that have solvent-accessible apolar surfaces, disrupt lipid vesicles, and undergo an α-helix to β-sheet transition in the presence of lipids. Such oligomers are likely to escape lysosomal degradation, accumulate in the lysosomes, and disrupt cellular membranes, thereby contributing to the development of amyloid A amyloidosis.

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its transmission in mice. The molecular entity responsible for the AEF activity in AA amyloidosis is unclear and was proposed to comprise degradation-resistant species, including prefibrillar diffusible oligomers or small fibril fragments (23, 25). Here we describe soluble SAA oligomers formed at lysosomal pH in vitro, which have unusually high stability, disrupt lipid vesicles, and hence may contribute to the SAA accumulation in the cells, cell membrane damage, and AA pathogenesis.

Acidic pH, particularly in the lysosomal or endosomal compartment (pH 4.5–5.5), is well-known to favor fibril formation in vivo and in vitro by many protein precursors of major human amyloidoses, including SAA, transthyretin, Ig light chain, Aβ peptide, etc. (see ref. 26 and references therein). In vitro fibril formation by SAA and AA requires acidic conditions (27) that are optimal circa pH 3 (9). Moreover, ex vivo and cell-based immunohistochemical and electron microscopic (EM) studies have clearly established lysosomes as the initial sites of AA fibril formation in macrophages and other cells (26, 28, 29). Besides acidic pH, protein concentration in lysosomes and the activity of acidic proteases probably contribute to the lysosomal origin of fibrillogenesis in this and other amyloid diseases. To elucidate molecular underpinnings for the lysosomal origin of AA fibrillogenesis, here we address the role of acidic pH in SAA folding, aggregation, fibril formation, proteolytic degradation, and interactions with lipid bilayers in vitro.

Results
Effects of pH on the Secondary Structure and Thermal Stability of SAA in Solution. Recombinant murine SAA isoform 1.1 (mSAA1, 103 aa, 11.6 kDa) used in this study is a major amyloidogenic isoform that binds HDL and has 80% sequence similarity to human SAA1. Fig. 1A shows far-UV circular dichroism (CD) spectra of freshly prepared solutions of mSAA1 (termed SAA for brevity) at 25 °C, pH 3.0–7.5, and the α-helical content determined from these spectra. The results were independent of the protein or salt concentration in the range explored (0.02–0.5 mg/mL SAA, 0–150 mM NaCl in 50 mM phosphate buffer). At pH 7.5 in the absence of bound ligands, SAA was predominantly in a random coil conformation characterized by a CD minimum circa 200 nm. At pH 3.0, SAA was also largely unfolded. Surprisingly, at pH 4.3, the CD minimum shifted to ~210 nm and a strong maximum appeared circa 190 nm, indicating a substantially folded structure. This structure was detected at pH 3.5–4.5, which overlaps the lysosomal pH range, with the α-helical content reaching a maximum of 35 ± 5% at pH 4.3. At higher and lower pH, the helical content declined to ~10% (Fig. 1A, Inset). To our knowledge, such a pH-dependent structural transition in SAA has not been previously reported.

The temperature dependence of the secondary structure at various pH was monitored by CD at 210 nm during protein heating and cooling at a constant rate (Fig. 1B). At pH 7.5, SAA was partially α-helical at 5 °C and showed reversible thermal unfolding with a midpoint Tm = 18 °C. At pH 3.0, SAA was also substantially α-helical at 5 °C and unfolded reversibly with a Tm = 18 °C. Unexpectedly, at pH 4.5, the unfolding shifted to much higher temperatures (Tm = 50 °C) and became thermodynamically irreversible, with a large hysteresis in the melting data (Fig. 1B). Such a hysteresis is typical of lipid-bound but not lipid-free apolipoproteins, including SAA (30, 31). Irreversible unfolding of SAA at pH 4.3 was also evident from far-UV CD spectra recorded before and after heating to 80 °C, and the difference spectrum showed an irreversible loss of ordered secondary structure (Fig. 1C, Inset). Further, the heating data of lipid-free SAA at any pH explored showed no scan rate dependence, suggesting the absence of high activation energy (30).

In summary, far-UV CD results in Fig. 1 revealed that lipid-free mSAA1 acquired a partially folded conformation at pH 3.5–4.5. The maximally folded state was observed at pH 4.3 and had 35 ± 5% α-helical content, 50 ± 5% random coil, with the rest forming a β-structure. This structure unfolded on heating circa 50 °C, which is unusually high for lipid-free mSAA. The unfolding was thermodynamically irreversible but did not involve high activation energy. Further studies focused on pH 4.3 wherein the secondary structure was maximal; however, experiments at pH 3.5–4.5 showed similar trends.

Effects of pH on the Hydrophobic Residue Packing and SAA Aggregation in Solution. To monitor pH-dependent aromatic packing, we used intrinsic Trp fluorescence by taking advantage of W18, W29, and W53 in mSAA1. These tryptophans are located in or near the concave apolar face of the SAA monomer that was proposed to bind lipids (32) (see SI Results for details). At pH 3.0 and 7.5, the emission maximum was centered at λmax = 350 nm, indicating exposed Trp (Fig. 2A), which was consistent with the disordered secondary structure observed at these pHs by CD (Fig. 1A). At pH 4.3, a blue shift to λmax = 342 nm indicated a structural reorganization with partial shielding of Trp (Fig. 2A), consistent with more ordered secondary structure observed at pH 4.3 (Fig. 1A).

To probe for solvent-accessible hydrophobic cavities, we used a fluorescent probe 8-anilinonaphthalene-1-sulfonic acid (ANS).
Effects of pH on the intrinsic Trp fluorescence and ANS binding to SAA in solution. (A) Normalized Trp emission spectra showed a pH-dependent shift in $\lambda_{\text{em}}$ (vertical lines). freshly prepared samples contained 0.1 mg/mL SAA in standard buffer. Trp residues in mSAA1.1 (W18, W29, and W53) were excited at $\lambda_{\text{ex}} = 295$ nm. The emission was recorded at 25 °C in all panels. (B) ANS binding to SAA in solution monitored by fluorescence. ANS was excited at $\lambda_{\text{ex}} = 350$ nm. Sample conditions were 40 £M ANS, 10 £M SAA at indicated pH. Emission spectrum of 40 £M ANS in buffer was shown for comparison (dashed line). (C) Förster resonance energy transfer from Trp to ANS. Sample conditions were as in B. Trp was excited at $\lambda_{\text{ex}} = 295$ nm.

ANS alone showed very weak emission with $\lambda_{\text{max}} = 525$ nm, which did not change on addition of SAA at pH 7.5 and changed very little at pH 3, 25 °C (Fig. 2B), indicating lack of ANS binding to SAA at these pHs. This result was consistent with the lack of ordered secondary structure in SAA observed by CD at pH 7.5 and pH 3, 25 °C (Fig. 1A). In contrast, at pH 4.3, the emission greatly increased and showed a 45-nm blue shift to $\lambda_{\text{max}} = 480$ nm, indicating ANS binding (Fig. 2B). Consequently, the ordered secondary structure in SAA detected by CD at pH 4.3 contained hydrophobic cavities large enough to bind ANS. ANS binding to SAA at pH 4.3, but not at pH 3.0 or 7.5, was confirmed in the energy transfer experiments. Trp was excited at $\lambda_{\text{ex}} = 295$ nm, and the ANS emission was recorded at 300–600 nm. No energy transfer from Trp to ANS was detected at either pH 3.0 or pH 7.5. In stark contrast, a nearly complete energy transfer was observed at pH 4.3, indicating ANS binding in close proximity to tryptophans (Fig. 2C).

Together, the results in Figs. 1 and 2 revealed that at pH 4.3, 25 °C, SAA acquired a partially folded structure with high a-helical content, partially buried Trp, and large solvent-accessible hydrophobic cavities. Given the small size of SAA and its high aggregating propensity, such cavities likely formed within protein oligomers.

To test for oligomerization, we performed nondenaturing gel electrophoresis (NDGE) of SAA at pH 3.0–7.5. In this pH range, freshly prepared SAA (1 mg/mL protein) migrated as a single broad band with a hydrodynamic size circa 7.5–8 nm (Fig. 3A), indicating self-association. A potential caveat is that the NDGE–PAGE showed sharp bands corresponding to SAA monomer, dimer, and trimer but no higher order oligomers (Fig. 3B). At pH 4.3, SDS/PAGE showed sharp bands corresponding to SAA monomer, dimer, and trimer but no higher order oligomers (Fig. 3B). At pH 3.0 and 7.5, additional smears corresponding to hexamers (~50 kDa) and higher order oligomers were detected at 0.03–0.06% glutaraldehyde, indicating aggregation. A caveat of glutaraldehyde cross-linking is that it may miss some oligomeric species (e.g., if lysines are protected from cross-linking). Nevertheless, the results in Figs. 1–3 clearly showed that formation of the partially folded stable structure in SAA at pH 4.3 involved compact low-order oligomers and was accompanied by a decrease in nonspecific protein aggregation.

Effects of pH on SAA Interactions with Phospholipids and Their Mutual Remodeling. To test whether the pH-induced structural changes in SAA affected its interactions with lipid bilayers, we analyzed the ability of SAA to remodel multilamellar vesicles (MLVs) of a model zwitterionic phospholipid 1,2-dimyrystoyl-sn-glycero-3-phosphocholine (DMPC; C14:0, C14:0). SAA was previously shown to rapidly clear DMPC MLVs (>100 nm) at pH 7.5 and form SAA–DMPC complexes ~10 nm in size (31, 33). Here, the clearance time course at various pH was monitored by turbidity at 24 °C for 1 h. Rapid clearance was observed at pH 7.5 and 3.0 but not at pH 4.3 (Fig. 4A). Next, SAA was incubated overnight at 24 °C with DMPC MLVs at pH 3.0, 4.3, or 7.5; excess lipid was removed by centrifugation; and the samples were subjected to NDGE. At pH 3.0 and 7.5, SAA formed lipoproteins circa 11–18 nm in size (Fig. 4B), consistent with previous studies at pH 7.5 wherein such lipoproteins were explored in detail (31). In contrast, at pH 4.3, such lipoproteins were not detected and protein migration was consistent with lipid-free SAA (Fig. 4B). Therefore, the ability of SAA to remodel DMPC into lipoproteins was retained at pH 7.5 and pH 3.0 but was abolished at pH 4.3.

Next, we explored SAA interactions with a longer chain mono-unsaturated lipid, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC; C16:0, C18:1), which mimics zwitterionic phospholipids in the plasma membrane. To facilitate the bilayer remodeling of this relatively long-chain lipid, POPC was used in the form of small unilamellar vesicles (SUVs, ~25 nm). SAA and POPC SUVs at 1:100 mol:mol protein:lipid were coincubated overnight at 25 °C. NDGE showed that at pH 7.5 and pH 3.0, all SAA comigrated with SUVs, indicating binding. At pH 4.3, no binding was observed and SAA migrated as a free protein (Fig. 5A). Consequently, regardless of the exact nature of the lipid assembly (DMPC MLVs or POPC SUVs), SAA could readily bind...
to a phospholipid surface at pH 7.5 and pH 3.0 but not at pH 4.3. To our knowledge, such pH-dependent apolipoprotein–PC interactions have not been previously reported.

Cross-linking studies showed that, similar to lipid-free SAAs, SAAs in the presence of POPC SUVs formed low-order oligomers (dimers and trimers) and large nonspecific aggregates at pH 3.0 and pH 7.5 (Figs. 3B and S8). However, at pH 4.3 in the presence of POPC, both specific and nonspecific self-association of SAA was greatly diminished and most SAAs either remained monomeric or formed large aggregates (>200 nm) (Fig. S8). These pH-dependent changes in protein oligomerization were accompanied by structural changes in the protein and lipid described below. Previously, we showed that binding to either DMPC or POPC induced α-helical folding in mSAA1 at pH 7.5 (31). Here, we explored the effects of pH on the secondary structure in SAA and its interactions with POPC. SAA was incubated with POPC SUV at 25 °C overnight at either pH 7.5 or 4.3, and far-UV CD spectra were recorded before and after the incubation. As expected, at pH 7.5, binding to POPC induced α-helix folding in SAA, as evidenced by a strong CD maximum observed at 193 nm and minima at 208 nm and 222 nm, which is a signature of an α-helix (Fig. 6A). Surprisingly, at pH 4.3, a very different lipid-induced conformational change was observed: The ∼35% α-helical structure characteristic of free SAA at pH 4.3 was partially lost, and the CD spectrum showed a weaker maximum circa 190 nm and a minimum circa 218 nm, which is a signature of a β-sheet (Fig. 6B). Together, CD spectra in Fig. 6A and B revealed that, in contrast to lipid-induced random coil to α-helix transition observed at pH 7.5, SAA underwent a lipid-induced α-helix to β-sheet transition at pH 4.3. To our knowledge, such a secondary structural transition in apolipoproteins at the lipid surface has not been previously reported.

The kinetics of this unusual transition was explored next. SAA was incubated at 25 °C at either pH 4.3 or pH 7.5, POPC SUVs prepared in the same buffer were added to protein, and the time course of the secondary structural changes was monitored for up to 2 h by CD at 210 nm, θ210(λ) was observed, indicating a slow α-helix to β-sheet conversion in SAA on the lipid surface, which took many minutes to complete (Fig. 6C, gray). Similar pH-dependent trends were observed in SAA on addition of DMPC MLV (Fig. S1).

To test for mutual remodeling of the protein and lipid, we used ANS that avidly bound SAA oligomers at pH 3.5–4.5 (Fig. 2B and C). First, POPC SUVs were equilibrated with ANS for 30 min at 24 °C, and the ANS emission was recorded using λex = 350 nm. At any pH explored, in the presence of POPC SUVs, the emission showed a blue shift and a large increase in intensity, indicating ANS binding to SUVs (Fig. 6D and E and Fig. S2, solid lines). Next, SAA was added at 1:100 mol:mol SAA:POPC at pH 4.3; as a result, ANS emission gradually decreased, reaching a baseline in ~1 h (Fig. 6E, open symbols). In the presence of either SAA alone or POPC SUV alone, ANS emission remained constant; Fig. S2 shows representative data for POPC SUV alone at pH 4.3, indicating that the vesicle–ANS interactions remained invariant at this pH. Hence, loss of ANS binding by both protein and lipid on their coinubation at pH 4.3 indicated their mutual structural remodeling. The protein remodeling by the lipid was also seen by CD showing the α-helix to β-sheet conversion (Fig. 6F). In stark contrast, at pH 7.5, when no β-sheet was induced by the lipid (Fig. 6A), addition of SAA to SUV did not change the ANS emission (Fig. 6D, open symbols). Therefore, the mutual remodeling of the protein and lipid was very different at pH 4.3 and pH 7.5.

In fact, EM revealed striking pH-dependent structural changes in lipid vesicles, which were induced by the protein: After 1 h of incubation with SAA at pH 4.3 but not at pH 7.5, most POPC SUVs disappeared and were replaced with elongated curvy features (Fig. 5F). Similar remodeling of DMPC MLVs by SAA was observed at pH 4.3 but not at pH 7.5 (Fig. 6F). In the absence of the protein, no significant vesicle remodeling was observed at these pHs. Consequently, at pH 4.3, SAA drastically disrupted POPC SUVs and DMPC MLVs. Together, the results in Fig. 6 revealed mutual structural remodeling of SAA oligomers and lipid vesicles. At pH 4.3 but not at pH 7.5, this remodeling involved α-helix to β-sheet transition in the protein, a major disruption of lipid vesicles, and a loss of ANS affinity for both protein and lipid. All together, these effects suggest strongly
SAA's potential to disrupt cell membranes at near-lysosomal pH but not in plasma.

**Effects of pH and Lipids on SAA Proteolysis.** To explore the effects of pH on the protein susceptibility to limited proteolysis, SAA was incubated with trypsin at a 1:200 enzyme:substrate weight ratio at 24 °C, and the aliquots taken at various intervals were analyzed by SDS/PAGE. Lipid-free SAA was explored first. At pH 4.3, free SAA was unusually resistant to proteolysis: No fragments were detected even after overnight incubation with trypsin (Fig. 7A). In stark contrast, at either pH 3 or pH 7.5 (Fig. 7B), rapid fragmentation was observed, and most SAA was converted into 8–10 kDa fragments after 5 min. These results were consistent with a well-ordered thermostable secondary structure in lipid-free SAA seen at pH 4.3 vis-à-vis the largely unfolded secondary structure seen at either pH 3 or pH 7.5 at 25 °C (Fig. 1A and B).

Interestingly, the pH dependence of SAA proteolysis was reversed on addition of lipid vesicles. SDS/PAGE clearly showed that in the presence of either DMPC MLVs or POPC SUVs, SAA was cleaved faster at pH 4.3 than at either pH 3 or pH 7.5 (Fig. 7C). This observation suggested that at pH 7.5 and pH 3, SAA binding to the lipid surface, which induced an α-helix to β-sheet conversion (Fig. 6B) and altered protein aggregation (Fig. 5B), greatly decreased SAA protection against proteolysis. In contrast, at pH 4.3, interactions of SAA with the lipid surface, which induced a well-ordered thermostable secondary structure seen at pH 4.3 (Fig. 1A and B).

SAA and its tryptic fragments, which were generated either at pH 7.5 in the absence of lipids (Fig. 7B) or at pH 4.3 in the presence of either DMPC or POPC (Fig. 7C), were subjected to MALDI TOF. The exact mass and, hence, the nature of the protein fragments was similar, showing three major peaks with a molecular mass of 10.31, 9.65, and 7.81 kDa (Fig. 7D). Therefore, similar flexible
sites in SAA were susceptible to proteolysis at various pHs in the absence and in the presence of lipids.

Effects of pH on Amyloid Formation by SAA. To determine the effects of pH on fibril formation, lipid-free SAA (1 mg/mL) was incubated at 37 °C for up to 3 d and was analyzed for microscopic and macroscopic structure using spectroscopic, EM, and biochemical methods. Due to low solubility of SAA at pH ~7, these studies were performed only at an acidic pH; Figs. 8 and 9 show representative results at pH 4.3 and pH 3.0.

First, formation of amyloid-like structure in SAA was monitored for 72 h by using thioflavin T (ThT), a fluorescent dye whose emission increases on binding to amyloid. At pH 3.0, an increase in ThT emission on binding to SAA followed a sigmoidal kinetics (Fig. 8A), which is a signature of the nucleation-growth mechanism characteristic of amyloid formation by many proteins (16, 35). In contrast, at pH 4.3, a larger initial ThT emission was detected that changed little over time. These results suggest that during incubation SAA underwent larger structural changes at pH 3 than at pH 4.3 and that these changes were on pathway to amyloid fibrils at pH 3 but apparently not at pH 4.3.

These conclusions were supported by far-UV CD spectra of SAA at acidic pH recorded before and after 72 h of incubation at 37 °C. At pH 3.0, SAA showed large spectral changes on incubation, indicating a conversion from the predominantly random coil to the β-sheet–rich conformation (Fig. 8B), which is characteristic of amyloid. In contrast, at pH 4.3, little spectral change was detected (Fig. 8C), and the CD spectra remained invariant even after 1 mo of incubation at pH 4.3.

SAA aggregates were visualized by negative stain EM. At pH 3.0, the morphology significantly changed over time, from curvy protofibrils observed in a freshly prepared sample to long nonbranching fibrils ~10 nm in width characteristic of amyloid observed after 72 h at 37 °C (Fig. 8D, Top). In contrast, at pH 4.3, worm-like heterogeneous aggregates and occasional globular structures were observed that showed no major morphological changes after 72 h or longer incubation (Fig. 8D, Bottom). Together, the results in Fig. 8 revealed high temporal structural stability of SAA oligomers at pH 4.3, which contrasts with the gradual structural changes culminating in fibril formation at pH 3.

The microscopic structure in SAA aggregates at pH 3 and pH 4.3 was explored using ANS binding. At pH 3, ANS did not
bind to freshly prepared SAA (Fig. 9A). After 72 h of incubation at pH 3, a blue shift from $\lambda_{max} = 525$ nm to 480 nm and a small increase in the emission intensity was observed, indicating increased ANS binding. In contrast, at pH 4.3, the emission intensity greatly increased and was centered at $\lambda_{max} = 480$ nm in both the freshly prepared and incubated samples, indicating much greater burial of ANS on rapid binding to SAA oligomers at pH 4.3 (Fig. 9A, dark gray). Again, no large spectral changes were observed on prolonged incubation at pH 4.3, supporting high temporal structural stability of SAA oligomers at this pH.

Protein integrity during incubation at 37 °C was analyzed by SDS/PAGE. At pH 3, most soluble protein was hydrolyzed into fragments after 72 h of incubation during which the random coil to $\beta$-sheet transition and fibril formation was observed (Fig. 8B). In contrast, at pH 4.3, most SAA remained intact (Fig. 9B), consistent with the formation of a substantially folded stable conformation at this pH (Fig. 8C).

Finally, to differentiate between soluble oligomers and amyloid fibrils, we used conformation-specific antibodies A11 and OC. A11 binds generic epitopes in prefibrillar protein oligomers, whereas OC recognizes amyloid fibrils formed by various proteins but does not bind to monomers or nonfibrillar aggregates (36). Dot blot analysis showed that at pH 3, freshly prepared SAA did not bind either A11 or OC (Fig. 9C). However, after SAA has been incubated at pH 3 for 72 h, strong binding of both antibodies was observed, suggesting formation of prefibrillar oligomers and mature fibrils. This result was consistent with the EM data showing mature amyloid fibrils formed on SAA incubation at pH 3.0 (Fig. 8D, Upper Right). In stark contrast, SAA incubated at pH 4.3 for up to 72 h showed A11 binding to both freshly prepared and incubated protein, whereas OC did not bind strongly (Fig. 9C). Therefore, at pH 4.3, SAA rapidly formed stable prefibrillar oligomers that did not convert into mature fibrils over time. This result was in excellent agreement with high temporal stability of SAA aggregates observed at pH 4.3 by CD and fluorescence spectroscopy as well as with the worm-like curvilinear rather than fibrillar morphology observed by EM before and after SAA incubation at pH 4.3 (Figs. 8 and 9A).

To test whether the high structural stability of SAA oligomers formed at pH 4.3 was preserved on changes in pH, SAA was dissolved at pH 4.3 and then diluted into buffers at pH 3.0–7.5. Fibril formation, DMPC MLV clearance, and trypsin digestion were performed as described in Fig. S3. The results clearly showed that the protein’s ability to form amyloid fibrils, remodel lipid vesicles, and resist proteolysis was determined by the final pH and was not significantly influenced by the prior exposure to pH 4.3.

In summary, our fibrillation studies at acidic pH, including secondary structural analysis, ThT and ANS binding, negative-stain EM, and antibody binding, revealed distinctly different structural and aggregation properties of lipid-free SAA at pH 3.0 and pH 4.3 (Figs. 8 and 9). At pH 3.0, SAA gradually formed amyloid in a process involving a sigmoidal increase in ThT fluorescence; this process culminated in formation of classic amyloid fibrils 10 nm in width that bound fibril-specific OC antibody. This process involved a gradual random coil to $\beta$-sheet conversion (Fig. 8B) rendering SAA labile to proteolysis (Fig. 9B). In contrast, at pH 4.3, SAA rapidly formed soluble prefibrillar oligomers that bound A11 but not OC, showed unusual temporal structural stability in lipid-free state in solution, had a substantial $\alpha$-helical content, were protected from proteolysis, and had solvent-accessible hydrophobic cavities large enough to bind ANS (Figs. 8 and 9). Such compact soluble prefibrillar oligomers are particularly relevant to amyloid disease because of their potential to perturb cell membranes, which is suggested by the vesicle disruption (Fig. 6F), as well as their resistance to proteolytic degradation at near-lysosomal pH (Fig. 74), which may facilitate lysosomal accumulation of SAA.

**Discussion**

**SAA Forms a Potentially Cytotoxic Species at pH 3.5–4.5.** This study reports a compact soluble oligomer formed by lipid-free SAA at pH 3.5–4.5. This oligomer is maximally folded at pH 4.3, with 35% $\alpha$-helix at 25 °C, and unfolds irreversibly circa 50 °C, thus showing an unusually high thermostability for free SAA (Fig. 1). In the absence of lipids, this oligomer is remarkably resistant to proteolysis (Fig. 7A), and its secondary structure remains invariant for days on incubation at pH 4.3, 37 °C (Fig. 8C). Further, in contrast to pH 3.0 or pH 7.5, lipid-free SAA at pH 4.3 does not form large nonspecific aggregates (Fig. 3B). Rather, it forms low-order preamyloid oligomers that bind the diagnostic dye ThT and the A11 antibody but do not readily convert into mature fibrils (Figs. 8 and 9). These oligomers contain large solvent-accessible hydrophobic cavities that bind ANS (Fig. 2). The hydrodynamic size of these oligomers is circa 7.5–8 nm, consistent with the partially folded SAA hexamers, although larger species such as octamers and decamers cannot be excluded; however, glutaraldehyde cross-linking suggests that dimers and trimers are the key building blocks (Fig. 3B). Unlike similar-size mSAA1 oligomers described in previous detailed studies (24), the pH 4.3 oligomers are distinct in their high structural stability (Fig. 1B) and affinity for A11 seen in the freshly solved structure (Fig. 9C). Such stable soluble preamyloid oligomers of SAA, which may represent a potential lysosomal storage form of SAA, have not been previously reported.

An important property of these oligomers is their aberrant interactions with lipid bilayers. Despite its 35% $\alpha$-helical content, SAA at pH 4.3 does not bind to phospholipid vesicles via its amphipathic $\alpha$-helices and does not form lipoprotein particles in a manner typical of apolipoproteins (Figs. 4 and 5A). Instead, SAA undergoes a gradual lipid-induced $\alpha$-helix to $\beta$-sheet transition (Fig. 6B and C), which increases protein’s susceptibility to proteolysis (Fig. 7C). The slow rate of this transition suggests that energy barriers must be overcome to reorganize the helical structure in oligomeric SAA and convert it into a $\beta$-sheet (Fig. 6).
B and C). Importantly, this transition involves mutual structural remodeling of the protein and lipid, including disruption of phospholipid vesicles (Fig. 6F). We hypothesize that such an α-helix to β-sheet conversion in soluble oligomeric SAA and the concomitant disruption of lipid bilayers may be an early step in the cytotoxic pathway of protein misfolding in amyloid. Because no cells are viable in the pH range of 3.5–4.5 where the compact SAA oligomers were stable, this hypothesis could not be tested directly in cytotoxicity studies; similarly, the effects of pH on toxicity of other amyloidogenic proteins such as amylin cannot be determined directly in cell cultures (37). However, strong credibility to our idea is lent by the observation that SAA at pH 4.3 disrupts POPC SUVs and DMPC MLVs (Fig. 6F) and, hence, has a high potential to perturb cell membranes.

To our knowledge, such unusual pH-dependent interactions with lipids have not been previously reported for SAA or any other HDL proteins. In fact, the accepted concept is that lipid surface binding by apolipoproteins stabilizes their amphipathic α-helices and protects them from proteolytic degradation and misfolding (see refs. 33, 34 and references therein). SAA interactions with PCs at pH 4.3 provide an exception from this general rule.

Other structural details of the SAA oligomers are presented in SI Results. They provide evidence for partial folding of SAA helices at pH 4.3 and HDL residual α-structures at pH 3.5–4.5 and propose the titration of acidic residues as a possible driving force for the pH-dependent structural changes in lipase-free SAA.

Potential Relevance to Amyloid Disease. Studies of other intrinsically disordered amyloidogenic proteins and peptides, such as Alzheimer’s Aβ peptide and synuclein, have postulated that an α-helix to β-sheet conversion in partially folded soluble protein oligomers can be an early step in amyloid formation (see refs. 38, 39 and references therein). Furthermore, compact soluble polymorphic low-order oligomers of various amyloidogenic proteins, including SAA (21–24), are thought to comprise the pathogenic species that can disrupt cell membranes and/or mediate prion-like transmission (7, 8, 13–15, 40). Notably, studies of soluble low-order oligomeric forms of Aβ have revealed a direct correlation between the ANS binding and cytotoxicity (9), whereas studies of a model amyloidogenic protein HypT reported that “structural flexibility and hydrophobic exposure are primary determinants of the ability of oligomeric assemblies to cause cellular dysfunction” (13). We hypothesize that small soluble oligomers of SAA, such as those formed circa pH 4.3, which bind ANS, disrupt phospholipid bilayers, and convert from an α-helix to β-sheet on interacting with these bilayers, may be involved in the pathogenesis of AA amyloidosis. We speculate that such SAA oligomers can disrupt cellular membranes and liberate the amyloid into the extracellular space.

Although in vivo formation of such SAA oligomers remains to be established, their observation in vitro only at pH 3.5–4.5 suggests limited sites for their possible formation in the body. Such oligomers may be stable in the gastric tract, which might contribute to oral transmission of AA amyloidosis in animals (25). Besides the gastric tract, pH 3.5–4.5 can be found in vivo only in urine or in lysosomes. A fraction of SAA that is filtered in the kidney may be reabsorbed via the receptor-mediated endocytosis, followed by acidification of endocytic vesicles and their fusion with lysosomes (41). Because kidney is the primary site of AA deposition, this pathway of SAA exposure to lysosomal pH in the kidney may contribute to renal AA amyloidosis. Moreover, SAA internalization in lysosomes of various cells, including macrophages that are associated with tissue amyloid (21, 42), and the lysosomal origin of AA fibrillogenesis is well established (26, 28, 29). Our finding of unusual SAA oligomers at pH 3.5–4.5 helps provide molecular basis for the lysosomal origin of AA fibrillogenesis. We posit that high structural stability of SAA oligomers at lysosomal pH and their resistance to proteolysis help them escape lysosomal degradation and accumulate in lysosomes, reaching high SAA concentrations that favor amyloid fibril formation. Further, the membranolytic potential of SAA oligomers helps this amyloid to escape from the cells, ultimately leading to massive deposition of extracellular amyloid in AA disease. Moreover, SAA oligomers that disrupt lipid bilayers may potentially contribute to the cytotoxic effects in AA amyloidosis. We posit that such soluble prefibrillar oligomers, which are stable at pH 3.5–4.5 but not in plasma, provide a missing link in our understanding of AA amyloid formation.

Materials and Methods
Recombinant murine SAA1 (mSAA1, 103 aa, 11.6 kDa) was expressed in Escherichia coli and purified to 95% purity as described previously (9). All other materials, which were commercially obtained as described in SI Materials and Methods, were of highest available purity. Methods of sample preparation and analysis by using an array of biophysical and biochemical techniques are described in SI Materials and Methods.

Note Added in Proof. While this paper was in press, a cell-based study came out demonstrating that mSAA1 accumulation in lysosomes leads to membrane disruption, lysosomal leakage, and cell death, while the preformed amyloid is deposited outside the cells and seeds the fibrillation of extracellular SAA (43). The current paper provides the biophysical basis for these cell-based studies that are in excellent agreement with our proposed mechanism.

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