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Biochemical and Biophysical Research Communications 317 (2004) 157–161
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The interaction between apolipoprotein serum amyloid A and high-density lipoprotein

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Received 18 February 2004

Abstract

Serum amyloid A (SAA) is a small apolipoprotein that binds to high-density lipoproteins (HDLs) via its N-terminus. The murine isoform SAA2.2 forms a hexamer in solution and the N-terminus is shielded from the solvent. Therefore, it is unclear how the SAA2.2 hexamer might bind HDL. In this study, the binding of SAA2.2 to murine HDL was investigated by glutaraldehyde cross-linking and polyacrylamide gel electrophoresis. The hexamer did not bind HDL significantly at 20 °C. However, at temperatures between 25–30 °C, SAA2.2 became destabilized and its monomeric form bound to HDL. SAA2.2 binding did not significantly replace Apo A-I in HDL particles. At 37–45 °C SAA2.2 binds less to HDL, suggesting that its binding is weak and sensitive to physiological and pathological temperatures, and thereby, potentially modulated, in vivo, by other factors.

Keywords: SAA; HDL; Inflammation; Acute phase; Atherosclerosis; Cholesterol

High-density lipoproteins (HDLs) are serum particles of various sizes and compositions, in which almost half the mass is composed of amphipathic apolipoproteins located on the exterior and the other half of the mass is made of amphipathic or hydrophobic lipids located in the interior [1]. The predominant apolipoprotein (apo) on HDL is apolipoprotein A-I (Apo A-I) [2], and the plasma level of the HDL–apo A-I complex has been shown to correlate inversely with the likelihood of developing atherosclerosis [3,4]. During an inflammatory response, the concentration of the acute phase reactant protein serum amyloid A (SAA) can increase up to 1000 times its basal levels [5], and appears to replace Apo A-I on HDL particles to form acute phase SAA-rich HDL [6]. However, the mechanism by which SAA binds to HDL and replaces Apo A-I is not understood.

We recently discovered that in aqueous solution the murine variant SAA2.2 has a hexameric quaternary structure with a putative central channel [7]. Although a high-resolution structure for SAA2.2 is lacking, limited proteolysis experiments have shown that the N-terminus has partial proteolytic resistance [7], suggesting that this hydrophobic region is structured within the hexamer. Since SAA is predominantly bound to HDL particles in vivo [8], and its N-terminus has been shown to be critical for HDL binding [9,10], it is not clear whether SAA binds to HDL as a hexamer or must dissociate to a monomeric structure with an exposed N-terminus. In this study glutaraldehyde cross-linking (GCL) combined with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was used to address this issue, and the data suggest that HDL binds to monomeric SAA.

Materials and methods

Unless otherwise stated, all chemicals and reagents were obtained from Fisher Scientific (Pittsburgh, PA, USA). SAA-rich murine HDL was purchased from Calbiochem–Novabiochem (La Jolla, CA, USA). Glutaraldehyde was purchased from Sigma Chemicals (St. Louis, MO, USA).

SAA2.2 expression and purification. The murine SAA2.2 cDNA was cloned into a pET21-a(+) vector between the *Nde*I and *Bam*HI sites and transformed into *Escherichia coli* strain BL21 (DE3) pLysS competent cells [11]. SAA2.2 was expressed following a similar procedure to that described by Yamada et al. [12]. First, 6×2 ml of LB broth was

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.03.027

inoculated with a single colony and cells were allowed to grow until an OD_{600} of about 0.7. The cells for the 6 small starter cultures were then combined and added to 2×100 ml LB broth in 500 ml flasks, and grown until an OD_{600} of about 0.7. Cells were centrifuged at 4000 rpm for 10 min, re-suspended in a small volume of LB broth, and divided equally into 6×500 ml LB broth in 2-L flasks. Cells were grown until an OD_{600} of about 1.0. Cells were then centrifuged at 8000 rpm for 8 min, re-suspended in a small volume of LB broth, and divided equally into 6×500 ml LB broth containing 4 mM isopropyl-β-D-thiogalactoside. SAA2.2 expression was induced for 3 h. Cells were harvested by centrifugation at 8000 rpm for 10 min at 4 °C and stored at −20 °C. The composition of the LB broth was 2.5% (w/v) and contained 1% glucose, 500 μg/ml carbenicillin, and 34 μg/ml chloramphenicol, except for the initial culture, which only contained 200 μg/ml carbenicillin. Throughout, cells were grown at 37 °C and shaken at 200 rpm, except during induction (270 rpm). Cells were lysed in 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) buffer (20 mM, pH 8.2) by three consecutive freezing–thawing cycles, and SAA2.2 was purified from the cell extract as previously described [7].

Characterization of HDL particles. The protein composition of HDL particles was determined by reverse phase liquid chromatography/mass spectrometry (LC/MS) in an electrospray ionization (in positively charged ion mode) Agilent 1100 series (Germany) LC/MS instrument. HDL samples were loaded using the autosampler onto an analytic 4.6 mm i.d. C4-RP column (Vydac, CA, USA). An Agilent 1100 binary pump was used to deliver solvents at a flow rate of 0.66 ml/min, and a 5–90% linear gradient (0.1% (v/v) trifluoroacetic acid, 90% (v/v) acetonitrile aqueous solution) was used to elute the proteins. The peaks were monitored at 278 nm and the protein composition was identified by their corresponding mass values. Deconvolution of multiply charged clusters was performed on raw mass spectra using the LC/MS Trap software (Version 4.1).

Glutaraldehyde cross-linking. HDL and SAA2.2 were independently cross-linked or cross-linked to each other by incubating with 0.6% (w/v) glutaraldehyde for a specific amount of time (usually 20 min). The cross-linking reaction was quenched by adding Tris buffer from a concentrated (1 M) stock solution to a final concentration of 0.1 M. The cross-linked HDL and SAA2.2 samples were mixed with an equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer and analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

Results and discussion

Characterization of HDL particles

In vivo, SAA does not appear to bind all HDL particles, but rather, is found specifically associated with the third fraction of HDL in the serum [8]. Therefore, for these studies we used commercially available murine SAA-rich HDL. To characterize these HDL particles, reverse phase high-pressure liquid chromatography (RP/HPLC) and mass spectrometry (MS) were used to confirm the presence of the main expected apolipoprotein components, Apo A-I and SAA. RP/HPLC not only separated the proteins, but also conveniently removed interference from the lipids. From the UV absorbance profile at 278 nm (Fig. 1A), four major peaks were observed, and the molecular mass acquired by MS revealed that three of them are murine SAA1.1, SAA2.1, and Apo A-I (Table 1). The fourth peak, which had a mass of 13,224 Da, was not identified. These data are consistent with the main bands observed by SDS–PAGE

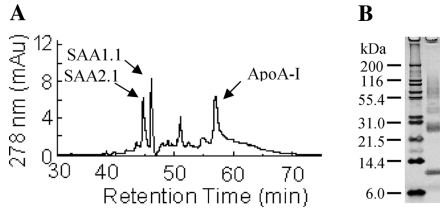

Fig. 1. Analysis of SAA-rich HDL by LC/MS and SDS–PAGE. (A) Reverse phase HPLC chromatogram of HDL (80 μg in 12 mM phosphate buffer (PB), pH 7.4) reveals three major peaks corresponding to SAA1, SAA2, and Apo A-I, as determined by LC/MS (Table 1). (B) SDS–PAGE (10–20% gradient gels) of HDL reveals two major bands, in agreement with the LC/MS data.

(Fig. 1B). The ~45 Da difference in Apo A-I between the experimental and the expected mass could be accounted by naturally occurring polymorphisms of Apo A-I, which have been well documented [13].

Glutaraldehyde cross-linking of HDL and SAA2.2

Glutaraldehyde has been widely used to cross-link oligomeric proteins [14] due to its ability to react with the solvent exposed amino group of lysine side chains. After cross-linking, the oligomeric state of the protein can be conveniently analyzed by SDS–PAGE. We used glutaraldehyde to trap the quaternary structure of the HDL particle. At glutaraldehyde concentrations of 0.7% (v/v) HDL was quantitatively cross-linked, exhibiting a complex of ~150 kDa (Fig. 2A), consistent with the size reported in previous studies [15]. Interestingly, the efficiency of glutaraldehyde cross-linking (GCL) was unaltered even at cross-linking times of 3 h, suggesting that after 20 min all exposed lysine residues were already involved in intra-molecular cross-links within discrete HDL particles. Thus, GCL reactions were usually allowed to proceed for 20 min.

In previous studies [7] we used GCL to trap the hexameric structure of SAA2.2. Sedimentation velocity analytical ultracentrifugation studies showed that at 25 °C monomeric SAA2.2 is present and in equilibrium with the hexameric protein, suggesting that the integrity of hexameric SAA2.2 is sensitive to physiological temperature [7]. GCL studies at mild temperatures indeed show that hexameric SAA2.2 begins to dissociate to a monomer at temperatures above 20 °C and is fully monomeric by 37 °C (Fig. 2B). This has been confirmed by analytical ultracentrifugation and circular dichroism (unpublished results). These results suggest that unless it is stabilized by other factors, the hexameric structure of SAA2.2 may not be highly populated in vivo.

Monomeric rather than hexameric SAA2.2 binds to HDL particles

GCL of SAA2.2 and SAA-rich HDL mixtures under different conditions allowed us to use this simple method

Table 1
Apolipoprotein composition of SAA-rich HDL determined by LC/MS

Retention time (min)	Assigned protein	Expected mass (Da)	Mass obtained by LC/MS (Da)
44.8	SAA2.1	11,754	11,753
46.2	SAA1.1	11,606	11,605
51.1	Undefined		13,225
57.0	Apo A-I	27,922[a]	27,967

[a] The mass was calculated from the mature protein of the Apo A-I precursor sequence (Accession No. AAH19837) obtained from the NCBI Entrez protein database.

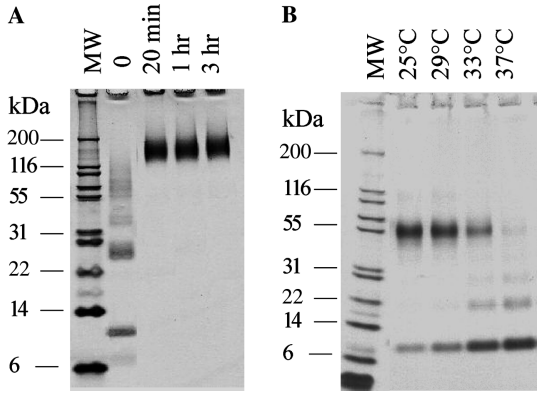

Fig. 2. Cross-linking of HDL and SAA2.2. (A) HDL (40–60 μg in 12 mM PB, pH 7.4) was incubated at 37 °C for 2 h and then cross-linked at the same temperature for 20 min, 1 h, or 3 h by 0.6% (w/v) glutaraldehyde. The cross-linking reaction was quenched by adding Tris buffer. (B) Cross-linking of SAA2.2 (50 μg in 12 mM PB, pH 7.4) at various temperatures. The cross-linked HDL and SAA2.2 samples were analyzed by SDS–PAGE using 10–20% gradient gels.

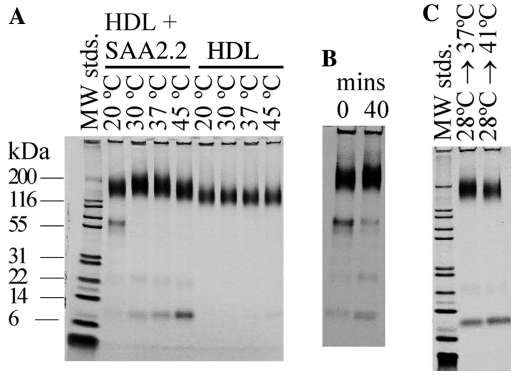

Fig. 3. Cross-linking of HDL and SAA2.2 mixtures. (A) Cross-linking of HDL after incubation with or without SAA2.2 for 30 min at different temperatures. (B) SAA2.2 and HDL were mixed at 25 °C and cross-linked immediately for 60 min or incubated for 40 min before cross-linking for 20 min. (C) SAA2.2 and HDL were mixed and incubated at 28 °C for 30 min, and then at 37 or 41 °C for another 30 min before cross-linking. HDL and SAA2.2 concentration was the same as in Fig. 2.

to gain some structural insight into the binding of SAA to HDL. When SAA2.2 was mixed with HDL at 20 °C for 30 min prior to cross-linking, a significant amount of hexameric SAA2.2 is present in the gel (Fig. 3A), suggesting that hexameric SAA2.2 does not bind to HDL. However, the HDL band is a little broader and migrates at a higher molecular weight (MW) compared to the cross-linked band in the absence of SAA2.2 (Fig. 3A, lanes 6–9), indicating that some SAA2.2 was bound to HDL. At 30 °C, where the SAA2.2 hexamer is highly populated (Fig. 2B) in the absence of HDL, the hexamer band disappears in the presence of HDL and little SAA2.2 monomer is seen (Fig. 3A, lane 3). In addition, the HDL band becomes broader and its MW increases, indicating that SAA2.2 is mostly bound to HDL at 30 °C (Fig. 3A, lane 3). Since hexameric SAA2.2 is unstable (Fig. 2B), it appears that HDL may be interacting with monomeric SAA2.2 that becomes transiently populated due to the hexamer–monomer equilibrium. To probe this idea, SAA2.2 and HDL were mixed at 28 °C and then cross-linked immediately or after 40 min of incubation (Fig. 3B). The diminished SAA2.2 hexamer band seen in the latter case strongly suggests that HDL binds to monomeric SAA2.2 accessible via the hexamer–monomer equilibrium. It is interesting to note the presence of a faint band that runs in between the 31 and 22 MW markers in Figs. 3A (third lane) and B (second lane). Since we usually see this band when SAA2.2 is binding to HDL, it is possible that it may be due to Apo A-I displaced from HDL by SAA2.2. However, one cannot rule out a randomly cross-linked SAA2.2 dimer, which has a similar migration on SDS–PAGE. Thus, it appears that Apo A-1 binds stronger to HDL than SAA, and cannot be easily removed from HDL by the latter.

When SAA2.2 and HDL were incubated and cross-linked at 37 and 45 °C (Fig. 3A, lane 5), the SAA2.2 monomer band became more intense while the HDL band exhibited a lower MW than at 30 °C, suggesting that the SAA2.2 monomer was either released from the HDL or not able to bind at these temperatures. When SAA2.2 and HDL were mixed and incubated for 30 min at 28 °C to ensure binding and the SAA2.2:HDL complex was then incubated at 37 or 41 °C for 30 min before GCL (Fig. 3C), significant amounts of monomeric SAA2.2 were present, suggesting that monomeric SAA2.2 was released at 37 and 41 °C after binding at 28 °C. Also, the slightly higher SAA2.2 monomer band intensity and the lower HDL MW at 41 °C suggest that there was more SAA2.2 monomer dissociated from HDL at this temperature than at 37 °C. In apparent contradiction, when HDL alone was incubated at 37 or 45 °C, and then subjected to GCL at each temperature,

it was found that the structure of HDL was virtually unaffected in this temperature range (Fig. 3A, lanes 8 and 9), with only a slight SAA monomer band present at 45 °C. The reason for almost no dissociation of SAA from HDL in lanes 8 and 9 (Fig. 3A) may be explained by the fact that the commercial HDL used in these studies is not yet saturated, and therefore, may bind stronger to its bound SAA. Alternatively, SAA2.1 and 1.1, which are already bound to the HDL used in this study, may bind to HDL stronger than SAA2.2; however, preliminary studies involving SAA1.1 suggest that this is not the case (unpublished results).

Structural basis for monomeric SAA binding to HDL

Previous studies have established that the N-terminus of SAA is required for its binding to HDL [9,10]. Therefore, the partial proteolytic resistance of the N-terminus of SAA2.2 and its requirement for hexamer formation [7] suggest that this region is buried within the hexamer, and is consistent with our observation here that HDL binds to monomeric SAA2.2. The temperature sensitivity of hexameric SAA2.2 leading to extensive dissociation to the monomer at 37 °C (Fig. 2A) indicates that even if the hexamer is stabilized in the serum by other factors, such as crowding or ligand binding, enough monomeric SAA2.2 is likely to be available for binding HDL.

The requirement of an exposed SAA N-terminus for HDL binding can be rationalized based on the presence of three consecutive canonical heptad segments within the first 23 residues of SAA2.2 that are likely to form an amphipathic α-helix (Fig. 4). While this hydrophobic region is buried within the hexamer, it appears to be highly exposed within the monomer, and is presumably the reason for the low solubility of SAA in aqueous solution and its high tendency towards aggregation [12]. It is very common for apolipoproteins to have primary structures capable of forming amphipathic α-helices, and extensive research on apolipoproteins, especially on Apo A-I, has shown that their ability to form an amphipathic α-helix is the structural feature that allows them to associate with lipid particles [1,16].

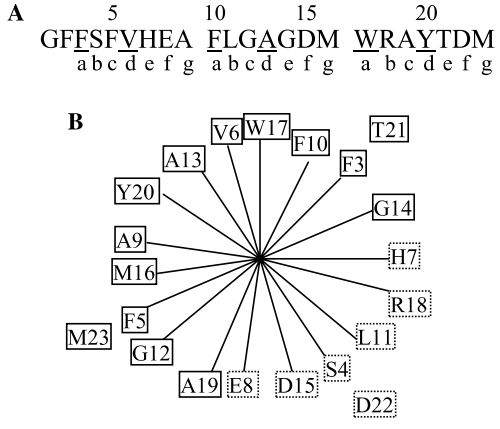

Fig. 4. Model of putative amphipathic α-helix in the N-terminus of SAA2.2. (A) The N-terminal 23-a.a. residue sequence of SAA2.2 suggests that three consecutive canonical heptad α-helices could be formed and are denoted by lowercase letters (a–g). (B) The residues of the putative α-helices are arranged in a wheel. The solid and dashed boxes designate the hydrophobic and hydrophilic faces of this long α-helix, respectively.

Implications concerning SAA function

It has been demonstrated that during chronic inflammation, the concentration of SAA in the serum and the load of SAA on HDL particles increase [6]. Apo A-I is commonly believed to be the "good" apolipoprotein on HDL in terms of reducing the risk of atherosclerosis [3,4]. In recent years it has been established that atherosclerosis is intimately associated with inflammation [17]. While the physiological function of SAA, a major acute phase reactant, is still largely undetermined, long-lasting high SAA expression is expected to decrease the proportion of Apo A-I bound to HDL, and consequently, may alter HDL's anti-atherosclerotic properties [2,18]. However, the decrease of Apo A-1:HDL complex may be due to SAA binding to the limited number of sites on HDL rather than SAA displacement of Apo A-1 from HDL particles. In addition, the high expression level (up to 1 mg/ml) [5] of SAA during the acute phase reaction suggests that there might be a significant proportion of SAA not bound to HDL. It has been documented that SAA exists in vivo not only in the predominant HDL-bound form, but also in lipid-free form [19]. Furthermore, it is interesting that even-though monomeric SAA is stabilized when bound to HDL, it may begin to dissociate at physiological and fever-like temperatures, especially when the HDL may be saturated with SAA (Fig. 3). Our data support the concept that HDL-bound and lipid-free forms of SAA may coexist, consistent with the SAA distribution found in human serum [8]. Therefore, the many functions that have been proposed for SAA [19] may be partly due to SAA's ability to exist and function in more than one oligomeric form, and it is possible that the increased SAA expression during inflammation may serve, among other possibilities, to both stabilize the hexameric structure of HDL-free SAA and to allow the formation of SAA-rich HDL particles. However, chronic inflammation leading to persistent high levels of SAA can cause SAA to deposit into amyloid fibrils, leading to the disease of reactive amyloidosis [20]. We have observed that long incubation of lipid-free SAA2.2 at 37 °C is sufficient to form amyloid fibrils in vitro (unpublished results). Thus, the discrimination of different SAA isoforms to form amyloid fibrils in vivo may be at least partially based on their relative capability of forming

oligomers of high α-helical content, and the affinity of monomeric SAA to HDL. Further structural and functional studies will hopefully allow these hypotheses to be tested and continue to unravel the various functions of this very intriguing protein.

Acknowledgments

Our thanks to Dr. Dmitri Zagorevski for Mass Spectrometry analyses and the Department of Chemistry and Chemical Biology for support of the Mass Spectrometry facility. The electrospray ionization mass spectrometer was obtained through NSF Grants CHE-0091892. This work was supported by a grant from the American Heart Association to W.C.

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