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Structural Stability of Streptococcal Serum Opacity Factor

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Abstract

Streptococcal serum opacity factor (SOF) is a protein that clouds the plasma of multiple mammalian species by disrupting high density lipoprotein (HDL) structure. Intravenous infusion of low dose SOF (4 μ g) into mice reduces their plasma cholesterol concentrations ~40% in 3 h. Here we investigated the effects of pH, ionic strength, temperature, and denaturation with guanidinium chloride (GdmCl) on SOF stability and its reaction vs. HDL. SOF stability was tested by pre-incubation of SOF at various temperatures, pH's, and GdmCl concentrations and measuring the SOF reaction rate at pH 7.4 and 37 °C. SOF retained activity at temperatures up to 58 °C, at pH = 4 to 10, and in 8.5 M GdmCl. The effects of GdmCl, pH, and ionic strength on the SOF reaction rates were also measured. SOF was inactive at GdmCl 1 M; SOF was most active at pH = 5, near its isoelectric point and at an ionic strength of 3 (in NaCl). These data reveal that SOF is a stable protein and suggest that its activity is determined, in part, by the effects of pH and ionic strength on its overall charge relative to that of its reaction target, HDL.

Keywords

High density lipoproteins; protein stability; serum opacity factor; cholesterol

Introduction

Streptococcal serum opacity factor (SOF) is a 100 kDa protein that clouds human serum and plasma via a novel reaction targeting HDL [1]. We have studied the SOF reaction using a recombinant His-tagged SOF (80 kDa) that has had its fibronectin-binding and cell wall anchor domains deleted, and for brevity, we refer to the recombinant protein as SOF because it retains opacifying activity. SOF targets the plasma high density lipoproteins (HDL) [1], which SOF disproportionates into two new lipid species and lipid-free protein [2, 3]. One is a large (~500 nm), cholesteryl ester-rich microemulsion (CERM), which contains nearly all

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of the cholesteryl esters of $\sim 10^5$ HDL particles. The other particle is a small (~ 7 nm) HDL that is protein- and phospholipid-rich. The lipid-free protein is apolipoprotein (apo) AI, the major HDL protein.

The rate-limiting step of the SOF reaction is apo AI desorption from the HDL surface [4–6]. The SOF reaction mechanism was revealed by chemical kinetic studies of its reaction vs. HDL [2] and by cryo electron microscopy [4]; SOF is a fusogen that binds a “host” HDL with a high affinity binding site and a “guest” HDL with a lower affinity delipidation site. SOF displaces apo AI from both the host and guest HDL. During the SOF mediated host-guest association, the cholesteryl ester (CE) flows from the guest to the host and the now delipidated guest is released as a neo HDL. The major protein on the CERM is apo E, a ligand for receptor mediated uptake via multiple hepatic receptors, including LDLR [7, 8]. These findings provoked studies showing that SOF injection into mice reduces their plasma cholesterol $\sim 40\%$ in three hours [9], and according to cell studies, SOF increases hepatic CE uptake by diverting HDL-CE to the LDL receptor [7] and promotes CE metabolism, including bile acid secretion in vitro and in vivo. [8] SOF is potent, showing measurable activity at 10^{-14} M [9]. Given the novelty and potency of the SOF reaction and its cholesterol-lowering effects in mice we studied the stability of SOF and describe our findings here.

Materials and Methods

Materials

HDL were isolated from human plasma by sequential ultracentrifugal flotation at $d = 1.063$ and 1.21 g/mL. A truncated fragment of SOF with opacification activity was purified as described.[10] Tris buffered saline (TBS; 10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM NaN_3) or phosphate buffered saline (PBS; $10\times = 1.37$ M KCl, 0.02 M KH_2PO_4 , 0.08 Na_2HPO_4 .) were used as noted in the text and figure legends.

Tests of SOF Stability

SOF was pre-incubated under various conditions—pH, ionic strength, and temperature for 30 min. After returning the SOF to standard conditions—TBS and 37°C for ~ 20 – 30 min, SOF activity was determined by measuring the rate of opacification, according to the increased SOF-induced turbidity of HDL measured by right-angle light scattering at 325 nm [2]. The final concentrations of HDL and SOF in the kinetic studies were 0.5 mg/mL and 2 $\mu\text{g/mL}$ respectively. Kinetic parameters were obtained from a fit of the turbidity vs. time data to a growing 2-parameter exponential curve using Sigma Plot 11.2 (Systat Software, Inc.). The SOF reaction was also followed by analyzing the product distribution at the end of the reaction by size exclusion chromatography (SEC) over two Superose HR6 columns (GE HealthCare) in tandem, as described. [2] As assessed by SEC, under the final conditions, none of the additives affected the structure of HDL.

Results

SOF Activity as a Function of Pre-Incubation at Different GdmCl Concentrations

The turbidimetric kinetics of opacification of HDL by SOF was used to determine its stability vs. several perturbants. According to kinetic turbidimetry, SOF maintained its activity vs. HDL and produced the expected products between 0.0 and 0.5 M but not at 1.0 M GdmCl (Figure 1A, B). Although SOF was not active vs. HDL at 1.0 M GdmCl, it was not irreversibly denatured even up to 8.5 M GdmCl. SOF was preincubated with 0 to 8.5 M GdmCl, after which it was diluted to a final GdmCl concentration of ~0.04 M, well within the GdmCl concentration range where SOF is active (as shown in Figure 1). Although SOF retained activity after pre-incubation with up to 8.5 M GdmCl, as the pre-incubation GdmCl concentration increased, the opacification rate increased two-fold plateauing at ~3 M GdmCl (Figure 2A). The SEC profiles of the reaction products were identical, i.e., similar amounts of CERM, neo HDL, and LF apo AI were formed at all GdmCl pre-incubation concentrations (Figure 2B).

SOF Activity as a Function of Pre-Incubation pH

The stability of SOF as a function of pH was determined as above by pre-incubating SOF at various pH's and then returning the SOF to TBS pH 7.4 and measuring the rate of opacification vs. HDL. SOF lost activity during incubations at pH extremes (pH = 3 and 12.5; Figure 3). After SOF pre-incubation at pHs between these extremes, SOF was active with peak activities at pre-incubation pHs of 6 and 10; these peaks were separated by a valley at pH 7 (Figure 3, insert). The vertical line denotes the calculated isoelectric point. Following pre-incubation between pH 3 and 12.5, the SEC profiles of the SOF products are identical (data not shown).

SOF Activity as a Function of pH

The SOF reaction rate was pH-sensitive (Figure 4). The opacification rates were low at pH extremes, pH = 3 and 12, whereas the peak rate at pH 5 was ~10 times faster than that calculated by interpolation for pH 7.4. SEC of the post reaction rates showed that the expected products, CERM, neo HDL and LF apo AI, were formed between pH 5 and 10. At pH 3 LF apo AI formed but neither CERM nor neo HDL were formed.

Thermal Stability—SOF Activity as a Function of Pre-Incubation Temperature

The thermal stability of SOF was determined by pre-incubating SOF at various temperatures for 30 min after which it was returned to 37 °C and its opacification kinetics and product profiles determined as described above. The rate of opacification decreased with pre-incubation temperature with nearly total loss of activity at a pre-incubation temperature range between 58 and 65 °C (Figure 5A, B). Over the same temperature range, the final maximum turbidity ($T_{\max}$) fell to zero at 65 °C (Figure 5A, B). The kinetic data are supported by the SEC data which shows absence of prominent peaks for CERM, neo HDL, and LF apo AI at 65 °C (Figure 5C).

SOF Activity as a Function of Ionic Strength

The effects of ionic strength on the SOF reaction were tested by measuring the kinetics of opacification and the reaction product profiles as a function of NaCl concentration between 0 and 5 M (Figure 6A). These data revealed a maximum in the opacification rate at 3 M NaCl and a gradual decline to ~30% of the maximum rate at 0 and 5 M NaCl. The maximum turbidity was nearly the same at all NaCl concentrations (Figure 6B) and the product profiles were nearly identical for reactions conducted at 0 and 5 M NaCl (Figure 6C).

Discussion

The recombinant SOF used in this study contains 806 amino acid residues and one cysteine residue and has a calculated pI of 6.2.[11] Using the loss of opacification activity as a marker of irreversible denaturation, our studies examined the effects of physicochemical perturbation of SOF by a chaotrope (GdmCl), pH, temperature, and ionic strength, each of which can induce protein unfolding or changes in net charge in ways that would be expected to affect activity. SOF was unreactive in 1 M GdmCl (Figure 1). Given that the rate-limiting step in the SOF reaction is the release of lipid-free apo AI, displacement of apo AI from HDL by GdmCl could underlie the loss of activity. However, apo AI displacement only occurs at much higher concentrations, 2.8 M. [12] Alternatively, GdmCl could interfere with hydrophobic SOF-HDL association.

One remarkable observation was the reversibility of the unfolding of SOF by GdmCl concentrations as high as 8.5 M (Figure 2). This occurred despite the observation that SOF is inactive in 1 M GdmCl (Figure 1). Moreover, pre-incubation of SOF in high concentrations of GdmCl potentiated the reaction kinetics; this effect was small, ~2-fold, and could be due to the disruption of inactive SOF oligomers.

Tests of SOF activity as a function of pre-incubation pH revealed a near total loss of activity at pH extremes where one might expect hydrolysis. This is likely due to irreversible misfolding rather than proteolysis since SDS PAGE revealed no fragments after incubation with the various perturbants (Supplementary Figure 1). However, over the pH mid-range, 4–10 the activity varied only slightly and showed a ~40% decrease between pH 6 and 7. This is close to the calculated isoelectric point and may be due to self-association of some SOF into inactive oligomers or aggregates. The optimal pH range for SOF kinetics vs. HDL was narrower with the activity being greater than 50% of maximum only between 4.5 and 6, an effect that closely correlated with the pI of 6.2. The SOF reaction was fastest at pH = 5 where it would have a positive charge. The pIs of apo AI and apo AII, the major proteins of the SOF target, HDL, are respectively 5.2 and 5.0. Thus, at this pH HDL likely carries little or no net charge which would reduce the electrostatic interactions between SOF and HDL to ~zero. Others have shown that the pH of maximal activity correlates with the pH of maximal stability on a large collection of proteins and speculated that such a correlation allowed the macromolecules to tolerate small pH fluctuations that inevitably occur in vivo [13]. Moreover they showed that pH-optimum of activity and stability do not correlate with the isoelectric point, pI, or with the optimal temperature for activity.

Tests of the pre-incubation temperature-dependence of SOF kinetics showed a linear loss of activity between 25 and 58 °C with irreversible loss of activity at 65 °C (Figure 5). This loss of activity is in the same range in which many common globular proteins, including albumin, are irreversibly denatured [14] but at a lower temperature range than that of lysozyme [15]. Thus, the thermal stability of SOF is not distinct from those of many other globular proteins. Despite its novel reaction mechanism, its stability vs. most perturbants is not unusual except for its reversible unfolding in GdmCl, an effect that deserves additional study.

SOF is a novel protein with an unprecedented activity. Based on both cell and animal studies, SOF appears to have potential as a therapeutic for promoting reverse cholesterol transport, the clearance of excess cholesterol from atherosclerotic plaques. In order to design SOF based therapeutics, it is necessary to elucidate the structure and function of the SOF reaction mechanism. We have proposed a model in which SOF is a heterodivalent fusogen that binds a “host” HDL via its high affinity docking site while displacing apo AI thereby exposing the lipid core; a lower affinity binding site sequesters a “guest” HDL while displacing its apo AI and forming a continuous tethered stalk through which guest lipids transfer to and coalesce with those of the host HDL while a delipidated guest HDL is extruded.[2] These reactions are repeated until all of the neutral lipid in ~200,000 HDL particles is transferred to a new large (500 nm) cholesteryl ester-rich microemulsion that underlies the clouding of SOF-infected plasma. X-ray crystal structure experiments are planned in order to validate this model. These studies of SOF stability will provide valuable information to facilitate the X-ray crystal structure studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

SOF	Serum opacity factor
HDL	high density lipoprotein
CERM	cholesteryl ester-rich microemulsion
GdmCl	guanidine hydrochloride
LDLR	low density lipoprotein receptor

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Figure 1.

SOF Reaction Kinetics in GdmCl. Kinetic data were collected as described in Methods at various GdmCl concentrations as labeled. **a.** The plots are the original data (—) and the fit of the data to a rising-exponential function (—). The rates were $(7.3 \pm 0.03) \times 10^{-2}$ and $(7.8 \pm 0.04) \times 10^{-2} \text{ min}^{-1}$ at 0.0 and 0.5 GdmCl respectively. Respective relative T_{max} were 2.6 ± 0.03 and 1.6 ± 0.04 . **b.** SEC of reaction products as labeled; the elution profile at 1.0 M GdmCl is identical to that of HDL, the starting material (bottom panel). Lipid free = LF.

Figure 2.

Effects of GdmCl on SOF Stability. HDL (0.1 mL, 15 mg/mL) was diluted with TBS (2.8 mL), transferred to a thermostatted fluorescence cuvette (37 °C) in the spectrofluorimeter and incubated for 10 min. SOF (18 µg in 13 µL) was mixed with 87 µL of various GdmCl solutions with their concentrations adjusted to give the desired final GdmCl concentration when diluted to 100 µL. After 30 min, the SOF solution was diluted into the HDL solution for a total of 3 mL and the intensity of the right angle light scattering followed with time. Rate constants (k) were determined as described in Figure 1. **a.** Chemical kinetics; original data (—); fit of the data (—). **b.** SEC of SOF reaction products as labeled. After dilution of the highest final GdmCl concentration, i.e., 8.5 M GdmCl, the GdmCl concentration was reduced to 0.3 M, well within the concentration range shown in Figure 1 to retain SOF activity. For the GdmCl tests at lower concentrations, the final concentration was proportionately lower. The filled curve is the SEC for untreated HDL.

Figure 3.

Effects of pH on SOF Stability. SOF (18 μg in 13 μL) was added to TBS (87 μL) solutions at different pHs and incubated for 30 min at room temperature. The SOF solutions were diluted into a cuvette containing a pre-warmed (37 $^{\circ}\text{C}$) solution (2.9 mL of 10 X PBS with enough HDL to give a final concentration of 0.5 mg/mL. After 10 min 100 μL SOF (18 μg) was added to the HDL and the rate constants (k) determined as in Figure 1. Original data (—); fit of the data (—).

Figure 4. Effects of pH on SOF Reaction Kinetics and Products. Solutions of HDL were prepared and adjusted to each pH by titration after which they were transferred to a cuvette and pre-incubated at 37 °C for 10 min. The reaction was initiated by the addition of SOF as described in Figure 2 legend. **a.** SOF reaction kinetics at various pH values as labeled. **b.** Plot of the relationship between SOF reaction kinetic constant and the pH of the reaction; the horizontal bar is the range of calculated values for the pI of SOF using several online algorithms, e.g. <http://isoelectric.ovh.org>. **c.** SEC of the final reaction products.

Figure 5. SOF Thermal Stability—Effect of Pre-Incubation Temperature on SOF Reaction Kinetics and Products. SOF (18 μg in 13 μL + 2.9 mL TBS) were incubated at 24, 30, 37, 44, 51, 58, 65, 72, 80 $^{\circ}\text{C}$ for 30 min. The solutions were cooled on wet ice for 5 min, transferred to fluorescence cuvettes, and equilibrated at 37 $^{\circ}\text{C}$ for ten minutes in the cell compartment of the fluorimeter after which the reaction was initiated by the addition of HDL (~1.5 mg in 100 μL), also pre-incubated at 37 $^{\circ}\text{C}$. **a.** Kinetic curves for the SOF reaction at various temperatures as labeled. **b.** Kinetic constants and maximum turbidity taken from an analysis of the data in Panel a. **c.** SEC of the SOF reaction products obtained from Panel a.

Figure 6.

Effects of Ionic Strength on the SOF Reaction. SOF was added to HDL in various concentrations of NaCl up to 5 M and equilibrated at 37 °C in a cuvette in the cell compartment of the fluorimeter for 10 min; final concentration of HDL and SOF were 0.5 mg/mL and 6 µg/mL respectively. **a.** Kinetic curves for the SOF reaction at various NaCl concentrations as labeled. **b.** Kinetic constants and maximum turbidity taken from an analysis of the data in Panel (a). **c.** SEC of the SOF reaction products obtained at 0 and 5 M NaCl; profiles of the reaction products at other NaCl concentrations (not shown) were similar.