Electrostatic Effects on the Stability of Discoidal High-Density Lipoproteins†

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ABSTRACT: High-density lipoproteins (HDL) remove cholesterol from peripheral tissues and thereby help to prevent atherosclerosis. Nascent HDL are discoidal complexes composed of a phospholipid bilayer surrounded by protein α-helices that are thought to form extensive stabilizing interhelical salt bridges. Earlier we showed that HDL stability, which is necessary for HDL functions, is modulated by kinetic barriers. Here we test the role of electrostatic interactions in the kinetic stability by analyzing the effects of salt, pH, and point mutations on model discoidal HDL reconstituted from human apolipoprotein C-1 (apoC-1) and dimyristoyl phosphatidylcholine (DMPC). Circular dichroism, Trp fluorescence, and light scattering data show that molar concentrations of NaCl or Na_2SO_4 increase the apparent melting temperature of apoC-1:DMPC complexes by up to 20 °C and decelerate protein unfolding. Arrhenius analysis shows that 1 M NaCl stabilizes the disks by $\delta\Delta G^* \cong 3.5$ kcal/mol at 37 °C and increases the activation energy of their denaturation and fusion by $\delta E_a \cong \delta\Delta H^* \cong 13$ kcal/mol, indicating that the salt-induced stabilization is enthalpy-driven. Denaturation studies in various solvent conditions (pH 5.7−8.2, 0−40% sucrose, 0−2 M trimethylamine *N*-oxide) suggest that the salt-induced disk stabilization results from ionic screening of unfavorable short-range Coulombic interactions. Thus, the dominant electrostatic interactions in apoC-1:DMPC disks are destabilizing. Comparison of the salt effects on the protein:lipid complexes of various composition reveals an inverse correlation between the lipoprotein stability and the salt-induced stabilization and suggests that short-range electrostatic interactions significantly contribute to lipoprotein stability: the better-optimized these interactions are, the more stable the complex is.

Lipoproteins are macromolecular complexes of proteins and lipids that mediate cholesterol transport and metabolism and are central in the pathogenesis of coronary artery disease. High-density lipoproteins (HDL)[1] remove cholesterol from peripheral tissues to the liver for catabolism via the process termed reverse cholesterol transport. Plasma levels of HDL and its major protein, apolipoprotein A-1 (apoA-1), correlate inversely with the probability of developing atherosclerosis (reviewed in refs *1*−*3*). Nascent HDL form discoidal particles composed of a phospholipid bilayer and proteins that are thought to adopt a "double-belt" helical conformation at the disk circumference (*4*, *5*), thereby conferring lipoprotein stability and solubility (Figure 1). HDL proteins belong to a family of exchangeable apolipoproteins. Their amino acid sequences contain 11-mer tandem repeats that have high propensity to form amphipathic α-helices. Class-A α-helices, which are the major lipid-binding motifs in apolipoproteins, have large apolar faces (30−50% of the surface area) that can bind to the phospholipid surface (*6*). These helices have an unusually high content of charged residues (about 40%) with distinct radial distribution: acidic groups are located in the middle of the polar helical face and basic groups at its edge (Figure 1). This suggests the importance of electrostatic interactions for lipoprotein assembly, structural stability, and functions (*4*, *7*).

Electrostatic protein−protein interactions are implicated in several lipoprotein functions. Most notably, charge complementarity is essential for binding of low-density and very low density lipoproteins (LDL and VLDL) to cell receptors (*8*−*10* and references therein*)*. An array of basic groups in helix-4 of apolipoprotein E (apoE) is critically involved in specific binding of VLDL to the acidic site on LDL receptor (*8*−*10*); in addition, binding of this array to anionic cell-surface heparin sulfate proteoglycans mediates clearance of lipoprotein remnants (*11*, *12*). Specific electrostatic interactions have been proposed to play a role in HDL binding to lipid transfer proteins that mediate lipid exchange among lipoproteins (*13*, *14*), and to lipophilic enzymes such as lecithin:cholesterol acyltransferase (LCAT) whose reaction is central in reverse cholesterol transport (*15*, *16*). Furthermore, cholesterol efflux from tissue cells to HDL, which is the first step in reverse cholesterol transport mediated by the ATP-binding cassette transporter ABCA1, is affected by the charge distribution in helix-10 of apoA-1 (*17*, *18*). This suggests that electrostatic interactions affect the apolipoprotein conformation and/or functional interactions with lipoprotein receptors, lipid transporters, and lipophilic enzymes.

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[1] Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low density lipoprotein; apo, apolipoprotein; DMPC, dimyristoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; LCAT, lecithin:cholesterol acyltransferase; ABCA1, ATP-binding cassette transporter type 1; WT, wild type; CD, circular dichroism; DSC, differential scanning calorimetry; EM, electron microscopy; T-jump, temperature jump.

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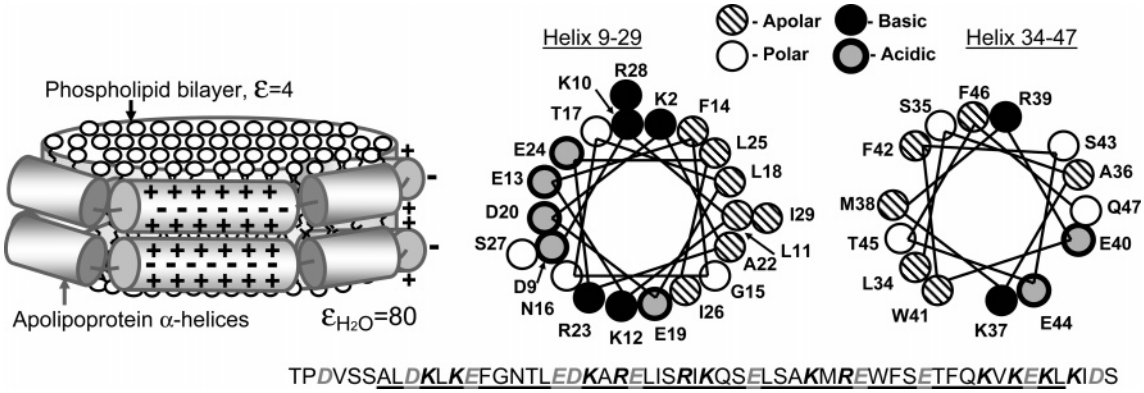

FIGURE 1: Cartoon representation of discoidal HDL and apoC-1. Left: Amphipathic apolipoprotein helices, which are thought to adopt an extended "double-belt" conformation at the particle perimeter (*1*−*5*), interact via the apolar faces with phospholipid acyl chains, thereby screening them from the solvent. Charge distribution in the polar face of class-A α-helices is shown. Right: Amino acid sequence of human apoC-1 (charged residues are in italics, predicted helical structure is underlined) and helix wheel representation of the two amphipathic class-A helices in lipid-free apoC-1 (*49*). The apolar helical faces contact each other in solution but are in contact with lipid on lipoproteins.

Protein−lipid interactions, which are predominantly hydrophobic, may also have a significant electrostatic component. Apolipoprotein absorption to the phospholipid surface may occur via the electrostatic interactions between the protein charged residues and the phospholipid headgroups (*19*). Charge residue distribution is important for protein−lipid insertion and probably accounts for an enhanced lipid-binding affinity of class-A α-helices, because mutations or reversal of charged residues in these helices diminishes their affinity for phospholipids (*20*, *21*). The "snorkel" hypothesis explains it by electrostatic interactions between the phosphatidylcholine headgroups and the basic protein residues (*22*); direct evidence for such snorkeling is provided in the NMR structure of apoC-II (*23*). Thus, electrostatic effects are important for apolipoprotein interactions with both proteins and lipids.

Most acidic and basic groups in apolipoprotein α-helices are located 3−4 positions apart and can form multiple intra- or interhelical salt links that may be structurally and functionally important. For example, salt link switching observed in the crystal structures of apoE isoforms was proposed to account for the defective LDL receptor binding in type-III hyperlipoproteinemia (*24*). In the "double belt" or other related models (*25*−*27*), the antiparallel helices are aligned to maximize their interhelical salt bridges that are thought to stabilize discoidal HDL (*4*, *28*); however, no direct experimental evidence supports such a stabilizing salt bridge formation. In fact, the role of surface salt bridges in macromolecular stability is complex and may be stabilizing or destabilizing depending on the geometry of the charge distribution (*29*−*33*). For example, thermophilic proteins (which have enhanced thermal stability optimized for function at high temperatures) are stabilized by networks of highly optimized surface salt bridges that are facilitated by the high content of charged residues in these proteins (*31*−*36*); the presence of similar salt bridge networks in lipoproteins remains unclear. In this work, we experimentally test the role of electrostatic interactions such as salt bridges in the stability of discoidal HDL.

Earlier studies have addressed the effects of apolipoprotein charged residue mutations on lipid binding and functions (*20*, *21*) and of lipid and protein composition on lipoprotein charge and stability (*37*−*40*). The latter studies have been based on an equilibrium thermodynamic approach. However, our recent analyses of model discoidal HDL (*41*−*43*) and plasma spherical HDL and LDL (*44*, *45*) have revealed that lipoprotein stability is subject to kinetic control. We showed that lipoprotein denaturation involves protein unfolding, dissociation, and fusion of the protein-depleted particles into protein-containing lipid vesicles (*41*, *43*); such a fusion compensates for the decrease in the polar surface and provides a thermodynamically irreversible step in lipoprotein denaturation. In fact, spherical HDL and LDL cannot reconstitute spontaneously; furthermore, model discoidal HDL containing dimyristoyl phosphatidylcholine (DMPC) and exchangeable apolipoproteins may form spontaneously only near the temperature of the DMPC liquid crystal-to-gel transition (T_c = 24 °C) but not at higher temperatures. This accounts for the thermodynamic irreversibility of the heat denaturation of these lipoproteins and precludes equilibrium thermodynamic analysis of their denaturation. Furthermore, we showed that HDL denaturation involves high free energy barriers, $\Delta G^* \cong 17$ kcal/mol, that decelerate protein unfolding and confer particle stability (*41*, *44*).

Here we address for the first time the role of electrostatic interactions in kinetic lipoprotein stability. We use discoidal complexes reconstituted from DMPC and human apolipoprotein C-1 (apoC-1, 57 a.a.) as a model for nascent HDL. ApoC-1 is the smallest exchangeable apolipoprotein that has high sequence homology, structural and functional similarity to larger apolipoproteins, and is a potent activator of LCAT and cholesterol ester transfer protein (*46*, *47* and references therein). ApoC-1 is composed of two class-A α-helices connected by a short linker (*48*, *49*) (Figure 1). It contains 11 acidic and 12 basic residues that can form multiple intra- or interhelical salt bridges. If, as proposed in the double-belt and related models (*2*−*5*, *7*, *25*−*28*), stabilizing salt bridges are the predominant electrostatic interactions on discoidal HDL, ionic screening of these favorable interactions would reduce the lipoprotein stability. Conversely, ionic screening of destabilizing electrostatic interactions would increase the lipoprotein stability. To discriminate between these possibilities, we analyzed the stability of apoC-1:DMPC disks in a broad range of salt concentrations (mM−M) and pH (5.7−8.2). Because charge−charge interactions are most susceptible to screening by salt ions (*36*, *50*), these experiments test the role of Coulombic interactions in lipoprotein stability.

MATERIALS AND METHODS

Protein and Lipoprotein Preparation. Human apolipoprotein C-1 (57 amino acids) and its point mutants were synthesized and purified at Quality Control Biochemicals (QCB) as described (*49*). Peptide amino acid composition and purity (97+%) were confirmed by mass spectrometry and HPLC. C-terminal fragment of apoA-1 (residues 198−243) with blocked N- and C-termini, which was synthesized and purified at QCB, was donated by Dr. David Atkinson. Human apolipoprotein A-1 (243 amino acids) was isolated from single-donor human plasma HDL, purified by HPLC to 95% purity, and refolded by dialysis as described (*51*). We used 5 mM TRIS or Na phosphate buffer with apoC-1 (both buffers yielded similar results) and 5 mM Na phosphate buffer with apoA-1 (our data showed that apoA-1 was destabilized by TRIS). Discoidal lipoproteins were obtained by overnight incubation at 24 °C of the protein solution with DMPC suspension in the buffer at pH 7.6; the protein:lipid ratio was 1:4 mg/mg. Alternatively, complexes with DMPC or dipalmitoyl phosphatidylcholine (DPPC) were obtained by cholate dialysis (*52*); DMPC disks obtained by these two methods had similar structure and stability properties. The complexes before (lipoprotein disks) and after heat denaturation (protein-containing lipid vesicles) were visualized by negative staining electron microscopy as described (*41*).

Circular Dichroism (CD) Spectroscopy and Light Scattering. CD data were recorded using an AVIV 215 spectropolarimeter with thermoelectric temperature control as described (*41*, *43*). Briefly, far-UV CD spectra (185−250 nm) and the kinetic and melting data were recorded from buffer solutions containing 20 μg/mL protein concentrations placed in a 5 mm cell. In temperature-jump (T-jump) experiments, the sample temperature was rapidly increased at time $t = 0$ from 25 °C to 65−99 °C, and the time course of the protein unfolding was monitored at 222 nm. In melting experiments, the CD data were recorded at 222 nm upon sample heating and cooling with 1 °C step size and 30−300 s accumulation time per data point, which corresponds to the scan rates of 80−11 K/h. In kinetic and melting experiments, 90° light scattering was monitored simultaneously with the CD signal by using fluorescence accessory. Following the baseline subtraction, the CD data were normalized to protein concentration and expressed as molar residue ellipticity [Θ].

Kinetic analysis of the T-jump data was carried out as described (*41*). Briefly, the data were approximated by single exponentials, $\Theta_{222}(t) = A_0 + A_1 \exp(-t/\tau)$, and the relaxation times $\tau(T)$ were determined at each temperature. The activation energy (enthalpy) $E_a \cong \Delta H^*$ was determined from the slope of the Arrhenius plot, ln τ versus $1/T$. The salt-induced increase in the Gibbs free-energy barrier for the kinetic disk stability at 37 °C, $\delta\Delta G^* = RT\,\delta(\ln \tau)$, was determined by linear extrapolation of the plots $RT \ln \tau$ from the transition range to 37 °C.

Fluorescence Spectroscopy. Measurement of Trp emission spectra was facilitated by the presence of a single Trp38 in apoC-1. The spectra were recorded using a Fluromax-2 spectrofluorimeter with water bath temperature control. Sample conditions were similar to those used in CD experiments. The spectra were recorded from 300 to 540 nm with integration time 0.5 s, excitation wavelength 280 or 296 nm, and 5 nm excitation and emission slit widths. In thermal denaturation studies, the sample was heated from 10 to 99 °C in 5 °C increments and incubated for about 10 min at each temperature, and the spectrum was recorded. The spectra were corrected for buffer baseline, and the peak wavelength was determined.

Differential Scanning Calorimetry (DSC). Excess heat capacity $C_p(T)$ of apoC-1:DMPC disks or DMPC vesicles was measured using a VP-DSC microcalorimeter (MicroCal). Lipid vesicles were prepared by sonication and were used within 48 h. Degassed samples containing 0.2 mg/mL DMPC and/or buffer were heated and cooled at 90 K/h scan rate; $C_p(T)$ data were recorded, corrected for buffer baseline, and normalized to lipid concentration. ORIGIN software was used for the data analysis and display. All spectroscopic and calorimetric experiments in this study were repeated 3−5 times to ensure reproducibility; the representative data are shown in Figures 2−9.

RESULTS

To test the effects of salt on thermal stability of apoC-1:DMPC disks, CD and light scattering melting data were recorded at 222 nm in 0−1 M ionic strength solutions (Figure 2A,B). Addition of 1 M NaCl or 0.33 M Na_2SO_4 at 25 °C had no detectable effect on the disk morphology (EM data not shown) nor on the far-UV CD spectra (and hence the protein helix content) of intact disks. However, it led to a

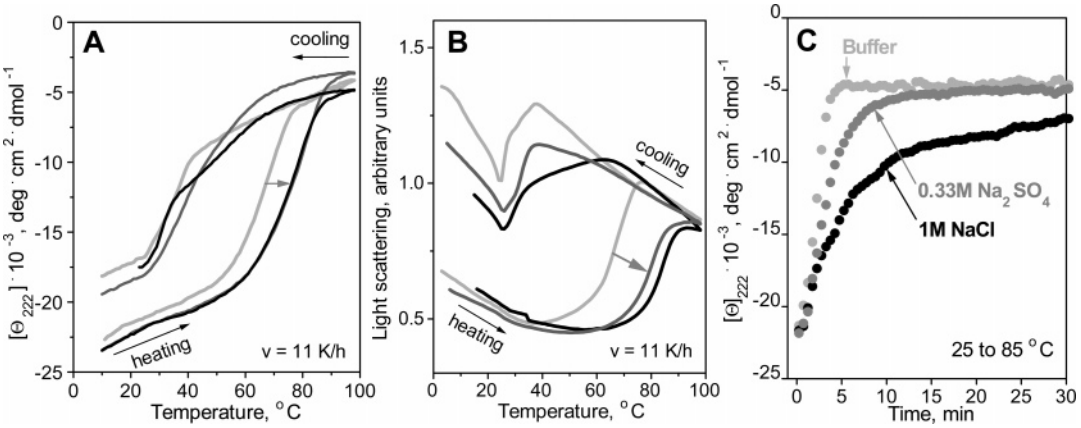

FIGURE 2: Effects of NaCl and Na_2SO_4 on the heat-induced protein unfolding and fusion of apoC-1:DMPC disks monitored by CD spectroscopy. (A) Far-UV CD and (B) light scattering melting curves recorded at 222 nm upon heating and cooling apoC-1:DMPC disks at a rate 11 K/h. (C) Kinetic data $\Theta_{222}(t)$ recorded of apoC-1:DMPC disks following a temperature jump from 25 to 85 °C. Solvent conditions are 5 mM Na phosphate buffer at pH 7.6 in the absence (light gray) and in the presence of 0.33 M Na_2SO_4 (gray) or 1 M Na Cl (black).

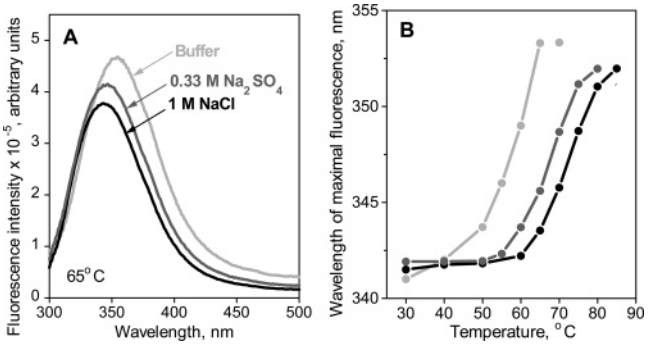

FIGURE 3: Effects of NaCl and Na_2SO_4 on the thermal transition in I29P apoC-1:DMPC disks monitored by intrinsic Trp fluorescence. (A) Trp emission spectra at 65 °C and (B) the melting curves monitored by the wavelength of maximal fluorescence in the absence (light gray) and in the presence of 0.33 M Na_2SO_4 (gray) or 1 M NaCl (black).

large increase in the apparent melting temperature T_m of protein unfolding (monitored by CD, Figure 2A) and particle fusion (monitored by light scattering, Figure 2B), suggesting salt-induced disk stabilization. To test this conclusion, we monitored the time course of protein unfolding on lipoproteins by CD at 222 nm following temperature jumps from 25 to 65−99 °C. Comparison of the kinetic $\Theta_{222}(t)$ data at 85 °C (Figure 2C) and other temperatures clearly shows that addition of salt decelerates protein unfolding and thus increases the kinetic stability of the lipoprotein.

Salt effects on the disk stability were also assessed by intrinsic Trp fluorescence. Emission spectra recorded at 25 °C of apoC-1:DMPC disks in 0−1M ionic strength solution were similar, suggesting that salt has no large effect on the solvent exposure of Trp38; similarly, near-UV CD spectra of the disks did not significantly change upon addition of salt (data not shown). Next, Trp emission spectra were measured at 25−99 °C with 5 °C increments upon sample heating at an approximate rate of 25 K/h. Figure 3 shows the data recorded of mutant I29P apoC-1 complexes with DMPC. In 0−1 M ionic strength solution at 25 °C, the emission peak is centered at 342 nm, indicating largely buried Trp. Temperature increase leads to a red shift indicative of increased solvent exposure of Trp. In the presence of salt the red shift is delayed (Figure 3A) and the fluorescence melting curves are shifted to higher temperatures (Figure 3B). A similar trend is observed in more stable WT apoC-1:DMPC disks (data not shown) that denature at higher temperatures than those containing I29P apoC-1. These results, together with the CD and light scattering data in Figure 2, clearly show that increasing ionic strength from 0 to 1 M stabilizes apoC-1:DMPC disks.

To test the effects of salt on the structure and stability of lipid-free protein, far-UV CD spectra and the melting curves at 222 nm were recorded from 10 to 20 μg/mL apoC-1 solutions of various salt concentrations at pH 7.6. In low-salt solutions, apoC-1 at these concentrations is largely monomeric and has 30% α-helical structure that unfolds upon heating with $T_m \cong 50$ °C (*53*) (Figure 4, open symbols). Increasing protein or salt concentration leads to apoC-1 self-association and concomitant increase in the α-helical structure that unfolds upon heating and cooling from 25 °C (*53*). This results in a small increase in the CD amplitude of lipid-free (but not lipid-bound) apoC-1 observed in 1 M ionic

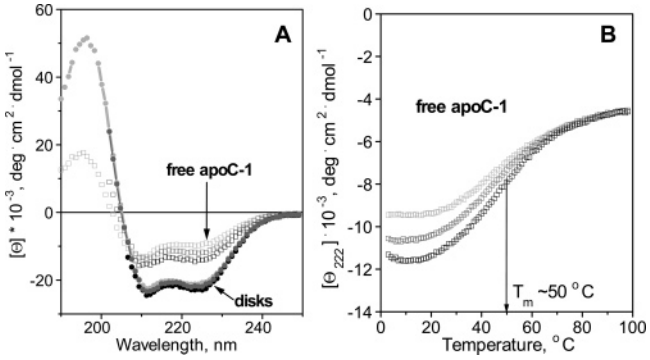

FIGURE 4: Effects of salt on the secondary structure and stability of lipid-free and lipid-bound apoC-1. (A) Far-UV CD spectra of free apoC-1 (open symbols) and apoC-1:DMPC disks (solid lines) and (B) the melting curves $\Theta_{222}(T)$ of free apoC-1 in the absence (light gray) and in the presence of 0.33 M Na_2SO_4 (gray) or 1 M Na Cl (black).

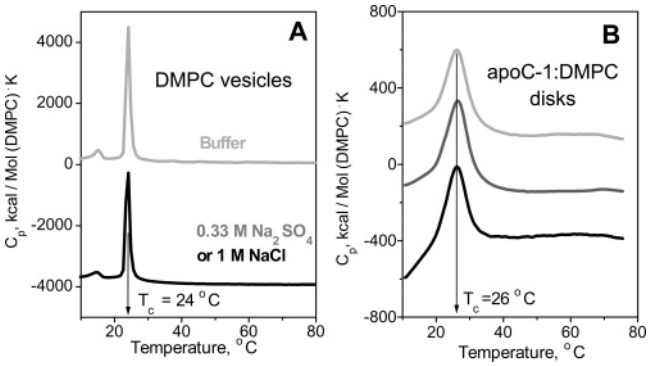

FIGURE 5: Excess heat capacity recorded upon heating DMPC vesicles (A) and apoC-1:DMPC disks (B) in 0−1 M ionic strength solutions. Line coding same as in Figures 2-4. The data are normalized to lipid concentrations and are shifted along the *Y*-axis to avoid overlap.

strength solutions at 25 °C (Figure 4, open symbols). However, the melting temperature and the width of the thermal transition of lipid-free apoC-1 are not significantly affected by salt (Figure 4B), indicating that salt does not stabilize lipid-free protein.

CD spectra in this and earlier studies (*41*) show that the helix content in apoC-1 increases from about 30% in monomeric lipid-free state to 67% upon DMPC binding (Figure 4). To test whether salt may increase the stability of the highly helical protein conformation in the absence of lipid, we monitored apoC-1 unfolding in solutions containing 0−1 M salt and a protein-folding osmolyte trimethylamine *N*-oxide (TMAO). Earlier we showed that 2 M TMAO induces a highly helical conformation in apoC-1 similar to its lipid-bound conformation (*54*). CD melting data of 10−20 μg/mL apoC-1 in 2 M TMAO, 0−1 M NaCl or 0.33 M Na_2SO_4 did not reveal any significant effects of salt on the thermal transition (data not shown). Thus, regardless of the helix content (30−67%), the stability of lipid-free apoC-1 is not significantly affected by salt. Therefore, the salt-induced stabilization of apoC-1:DMPC disks may not result from the effect of salt on the isolated protein moiety.

To test the effect of salt on the lipid moiety, DSC data were recorded during heating at 90 K/h of DMPC vesicles or apoC-1:DMPC disks (Figure 5). The $C_p(T)$ data of the lipid vesicles show a characteristic highly cooperative gel to liquid crystal transition at $T_c = 24$ °C with a pretransition

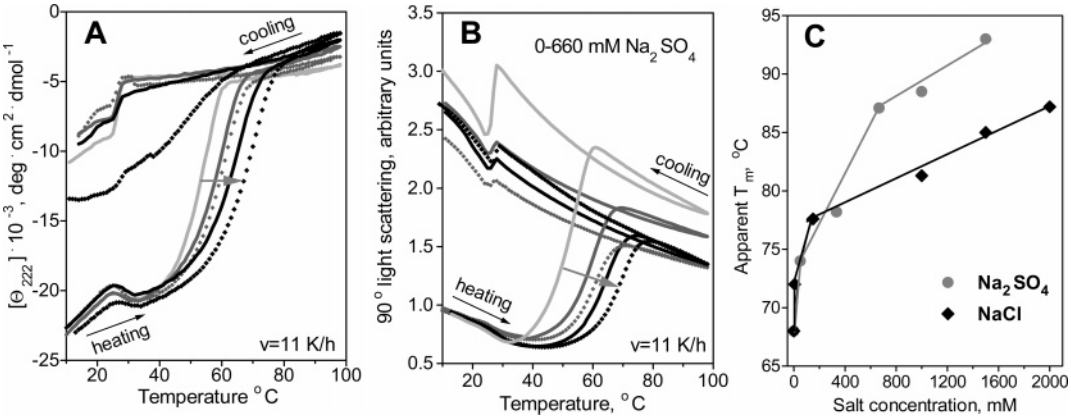

FIGURE 6: Effect of salt concentration on the apparent melting temperature of apoC-1:DMPC disks monitored by CD. Melting curves in 0−660 mM Na_2SO_4 were recorded at 222 nm by CD (A) and light scattering (B) during sample heating and cooling at 11 K/h: 0 M salt (light solid line), 20 mM NaCl (gray solid line), 50 mM NaCl (gray broken line), 330 mM NaCl (black solid line), 660 mM NaCl (black broken line). The data for I29PapoC-1:DMPC disks are shown; the melting curves of more stable WTapoC-1:DMPC disks show a similar trend. The apparent T_m as a function of salt concentration (C) was determined from the CD melting data of WTapoC-1:DMPC disks with accuracy better than 2 °C.

at 14 °C; no significant effects of salt on this transition are detected in this (Figure 5A) or other studies (*55*). The lipid phase transition in the disks is shifted to $T_c = 26$ °C and is much broader than in vesicles, reflecting smaller disk size (hence smaller cooperativity unit) (Figure 5B); again, salt has no detectable effect on the lipid phase transition in the disks. In summary, CD melting data of lipid-free apoC-1 (Figure 4) and DSC data reporting on the DMPC phase transition (Figure 5) indicate that salt has no significant effect on the protein stability or on the lipid phase transition. Thus the salt-induced stabilization is specific to apoC-1:DMPC disks and may not result from the salt effects on the isolated protein or lipid moieties.

To test in greater detail the effects of salt on the disk stability, CD and light scattering melting data were recorded in mM−M salt concentrations (Figure 6A,B). WT or mutant apoC-1:DMPC disks were heated and cooled from 10 to 98 °C at a rate of 11 K/h; first derivatives of the CD melting curves were used to determine the apparent melting temperature T_m as a function of salt concentration. A steep increase in T_m is observed at millimolar concentrations, followed by a more gradual increase at 0.2−2.0 M NaCl or Na_2SO_4 (Figure 6C). Thus, disk stabilization is observed in a broad range of salt concentrations, from millimolar to molar.

At millimolar concentrations, the effect of salt on macromolecular stability is mainly due to specific ion binding or ionic screening of electrostatic interactions, while at molar concentrations ionic screening saturates and the hydrophobic effect of salt may become predominant (*36*, *50*, *56* and references therein). The absence of Hoffmeister effects or specific anion or cation binding to apoC-1:DMPC complexes is suggested by the observation that similar ionic strength solutions of various salts (NaCl, KCl, Na_2HPO_4, Na_2SO_4) have comparable effects on the disk stability (data not shown for KCl and Na_2HPO_4). To test whether the salt effects on the disk stability are due to electrostatic or hydrophobic interactions, we analyzed the disk denaturation in 40% sucrose solution, which has comparable water activity to 0.6 M NaCl solution but has no large effect on the electrostatic interactions. Sucrose had no detectable effect on the melting CD data (not shown). However, similar data recorded at an

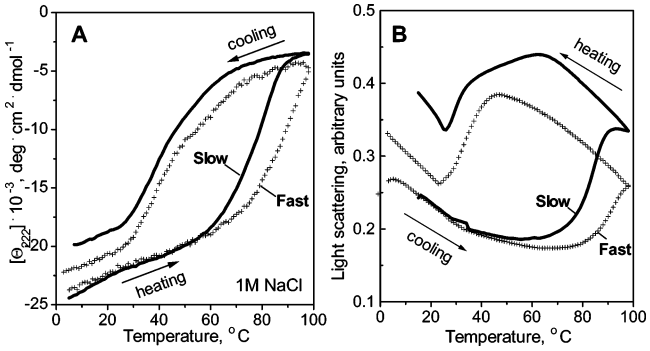

FIGURE 7: Scan rate effects on the thermal transition in apoC-1:DMPC disks. CD (A) and 90° light scattering melting curves (B) were monitored simultaneously at 222 nm from a sample containing 1 M NaCl during heating and cooling at a rate of 80 K/h (+++) and 11 K/h (−).

11 K/h scan rate in 0.6 mM NaCl showed a large increase in the disk stability, as indicated by an increase in the apparent T_m from 68 °C in 0 M NaCl to about 80 °C in 0.6 M NaCl (Figure 6C). Consequently, the stabilization of apoC-1:DMPC disks at these salt concentrations may not result from the hydrophobic effect of salt and hence must originate from ionic screening of the unfavorable charge−charge interactions.

To test the role of short- versus long-range Coulombic interactions in the disk stability, the net charge on the disks was altered by changing the pH from 5.7 to 8.2 and thereby changing the net charge on apoC-1 (pI = 6.5) from positive to negative, or by mutating charged residue Arg23 to Pro. CD melting data (not shown) revealed no detectable effects of pH or R23P substitution on the disk stability or on the salt-induced stabilization. Thus, changes in the net charge (and hence in the long-range Coulombic interactions) do not significantly alter the disk stability. Consequently, the salt-induced disk stabilization must result from ionic screening of short-range electrostatic interactions.

To analyze the kinetics of the disk denaturation, CD and light scattering melting data were recorded upon sample heating and cooling at 11−80 K/h scan rates; Figure 7 shows such data recorded in 1 M NaCl. Large low-temperature shifts are observed in the heating curves recorded at slow rates, indicating a slow thermodynamically irreversible

transition with high activation energy (*57*). The kinetics of this transition in high-salt solution was analyzed in T-jump CD experiments (Figure 8A) and was compared with similar data recorded in 0 M salt (Figures 2C, 8B). The T-jump data at 85 °C (Figure 2C) and other temperatures show slower unfolding in high-salt solution, which translates into significant shifts in the Arrhenius plots. At 80 °C, these shifts correspond to salt-induced disk stabilization by $\delta\Delta G^* = RT\,\delta(\ln \tau) = 0.8 \pm 0.2$ kcal/mol in 0.33 M Na_2SO_4 and by $\delta\Delta G^* = 1.5 \pm 0.2$ kcal/mol in 1 M NaCl (Figure 8B); the accuracy of these estimates reflects the fitting errors and the deviations among the data sets recorded in different experiments. Linear extrapolation of the plots suggests that at 37 °C the salt-induced disk stabilization increases to about $\delta\Delta G^* = 2.3 \pm 0.5$ kcal/mol in 0.33 M Na_2SO_4 and $\delta\Delta G^* = 3.5 \pm 0.5$ kcal/mol in 1 M NaCl (double arrows in Figure 8B). This is substantial compared to the Gibbs free-energy barrier $\Delta G^* \cong 17$ kcal/mol that determines the disk stability in low-salt solution at near-physiological temperatures (*41*). Furthermore, the slopes of the Arrhenius plots indicate a significant increase in the activation energy (enthalpy) $E_a \cong \Delta H^*$ of the disk denaturation upon addition of salt, from $E_a = 25 \pm 5$ kcal/mol in the absence of salt to $E_a = 35 \pm 7$ kcal/mol in 0.33 M Na_2SO_4 and $E_a = 38 \pm 7$ kcal/mol in 1 M NaCl. Consequently, the salt-induced stabilization of apoC-1:DMPC disks is an enthalpic effect.

To test the effects of salt on other apolipoprotein−lipid complexes, we carried out thermal denaturation studies of discoidal lipoproteins containing various proteins, including selected apoC-1 mutants as well as human apoA-1 and its C-terminal (198−243) fragment that forms the primary lipid binding site. The details of these studies will be reported elsewhere; here we summarize the effects of salt on these complexes. Compared with WT apoC-1, I29P apoC-1 forms DMPC disks of reduced stability and G15A,M38L apoC-1 forms disks of increased stability. The CD melting data of these disks recorded at a rate of 11 K/h in 0−1 M NaCl show that the complexes with I29P or WT apoC-1 are significantly stabilized by salt while those with G15A,M38L apoC-1 show a much smaller stabilization (Figure 9A).

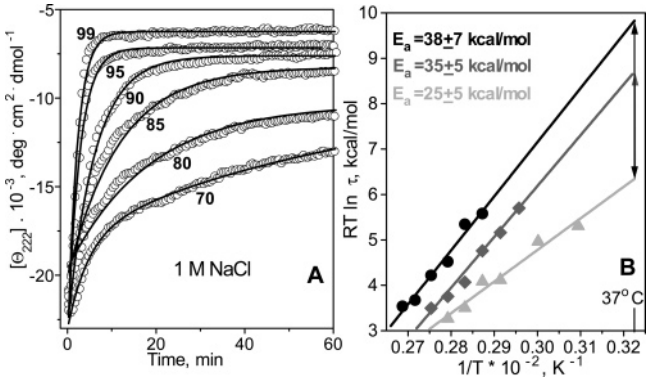

FIGURE 8: Effects of salt on the thermal unfolding kinetics of apoC-1:DMPC disks. (A) Kinetic T-jump data recorded by CD following a rapid temperature increase at $t = 0$ from 25 °C to higher temperatures (indicated on the lines). Solid lines show single-exponential fitting of the $\Theta_{222}(t)$ data. The data were recorded in 1 M NaCl; similar data in the absence of salt were reported earlier (*41*). (B) Arrhenius plots in the absence (light gray) and in the presence of 0.33 M Na_2SO_4 (gray) or 1 M NaCl (black). Salt-induced increase in the disk stability at 37 °C is indicated by double arrows.

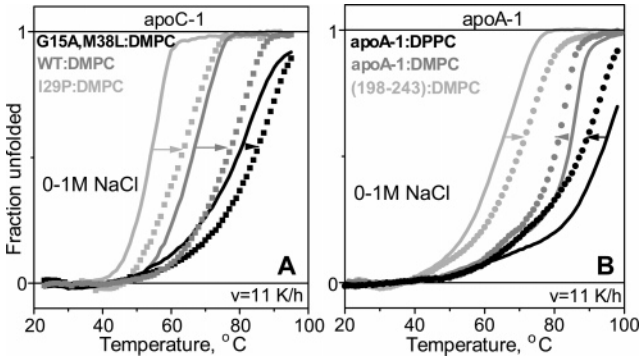

FIGURE 9: Effects of salt on thermal denaturation of discoidal lipoproteins with various composition. CD data $\Theta_{222}(T)$ were recorded at 11 K/h heating rate in the buffer containing 0 M salt (solid line) and 1 M NaCl (broken line) from the disks containing (A) DMPC and I29P apoC-1 (light gray), WT apoC-1 (gray), or G15A,L38M apoC-1 (black); (B) DMPC disks containing C-terminal (198−243) fragment of apoA-1 (light gray) or full-size apoA-1 (gray), and DPPC disks with apoA-1 (black).

Furthermore, DMPC disks with the C-terminal (198−243) fragment of apoA-1, which are relatively unstable in low-salt solution, are significantly stabilized in 1 M NaCl. Interestingly, more stable disks composed of DMPC and full-size apoA-1 show a small salt-induced destabilization; an even larger destabilization is observed in highly thermostable apoA-1:DPPC disks (Figure 9B). Taken together, our results suggest that the stabilizing effects of salt depend on the protein and lipid composition and correlate inversely with the lipoprotein thermostability. Relatively unstable lipoproteins (such as DMPC complexes with WT or I29P apoC-1 or with the C-terminal fragment of apoA-1) are strongly stabilized by salt; lipoproteins with moderate intrinsic stability show only small effects of salt that may be stabilizing (G15A,M38L apoC-1:DMPC) or destabilizing (apoA-1:DMPC); finally, highly thermostable lipoproteins, such as apoA-1:DPPC disks, are significantly destabilized by salt. Thus, salt stabilizes relatively unstable lipoproteins but destabilizes thermostable lipoproteins.

DISCUSSION

Our results show that apoC-1:DMPC complexes are stabilized by salt in a broad range of concentrations, from millimolar to molar. This effect is enthalpy-driven, as indicated by the salt-induced increase in the Arrhenius activation energy of the disk denaturation (Figure 8B); this is the first time we observe an increase in E_a induced by changes in the solvent conditions. The absence of the disk stabilization in 40% sucrose solution (which has water activity equivalent to about 0.6 M NaCl) suggests that the salt-induced stabilization of the apoC-1:DMPC disks cannot be accounted for solely by the hydrophobic effect. This is further supported by the observation that the effects of different salts on the disk stability show no clear correlation with the cation or anion position in the Hoffmeister series; for example, Na^+ and K^+ or sulfate and phosphate have comparable effects on the disk stability (data not shown). Furthermore, the observation that some lipoproteins (such as apoC-1:DMPC) are stabilized while others (apoA-1:DPPC) are destabilized by salt is difficult to explain solely by the hydrophobic effect. Taken together, these results suggest that the hydrophobic effect can contribute to the salt-

induced disk stabilization, yet it cannot account for the observed effects of salt on the disk stability. Therefore, ionic screening must play a key role in these effects.

The stability of apoC-1:DMPC disk remains invariant at pH 5.7−8.2 and/or as a result of the R23P charge residue mutation; consequently, the changes in the net charge by 1−2 units (which affect the long-range electrostatic interactions (r > 12 Å) such as repulsion between the disks or between the protein molecules on the disk) may not play a large role in the disk stabilization. Therefore, the stabilizing effect of salt on apoC-1:DMPC disks must be due to ionic screening of local electrostatic interactions. Since the stability of lipid-free apoC-1 is not significantly affected by salt (Figure 4B), these interactions are significant on the disks but not in lipid-free protein. Furthermore, since charge−charge interactions are most susceptible to ionic screening (*36*, *50*) and since all charges in apoC-1:DMPC disks are located on the protein, salt-induced disk stabilization results mainly from ionic screening of the unfavorable short-range interactions (r < 10 Å) among the protein charged groups. The most likely candidate for such interactions is the repulsion between the stretches of basic groups that form the interface between the polar and apolar faces in class-A α-helices. In lipoprotein disks, basic residues are confined to the disk circumference and are located in the low-dielectric environment at the lipid−water interface (Figure 1), which amplifies their repulsion. Other electrostatic interactions, such as surface salt bridges with suboptimal geometry, may also be destabilizing (*29*−*33*). Ionic screening of these unfavorable interactions leads to a large salt-induced stabilization observed in apoC-1:DMPC disks. In contrast, ionic screening of the buried salt bridges (which are highly favorable) is expected to be destabilizing; therefore, relatively unstable lipoproteins such as apoC-1:DMPC disks may not have a major contribution from the buried salt bridges to their stability.

The salt effects reported here reveal that the predominant electrostatic interactions on discoidal HDL may be favorable or unfavorable depending on the disk composition. Thus, the notion of interhelical salt bridges that confer specificity and stability to the protein conformation on HDL disks (*4*, *7*, *25*−*28*) should be expanded to include disks such as apoC-1:DMPC that have predominantly unfavorable electrostatic interactions. Such unfavorable interactions lead to relatively low disk stability that can be greatly enhanced by ionic screening. Similar arguments apply to other relatively unstable lipoproteins that are significantly stabilized by salt, such as DMPC complexes with Pro-containing apoC-1 mutants or with the C-terminal (198−243) fragment of apoA-1. In more stable disks, such as G15A,M38L apoC-1:DMPC or apoA-1:DMPC, the effects of salt are relatively small, suggesting that the ionic screening on favorable and unfavorable electrostatic interactions and the hydrophobic effect of salt nearly cancel out. Finally, in highly thermostable lipoproteins such as apoA-1:DPPC disks, favorable electrostatic interactions dominate, leading to high disk stability that is significantly reduced by salt. A similar correlation between the effects of salt and thermostability was predicted and observed in the cold shock protein family: the mesophilic protein was stabilized by salt while its more stable thermophilic and hyperthermophilic analogues were progressively destabilized (*36*). This correlation between the salt sensitivity and thermostability was verified in other series of thermophilic homologues and was proposed to be a general phenomenon that may result from interplay between the long- and short-range electrostatic interactions (*36*). Our results suggest that this phenomenon is not limited to protein thermophiles; it extends to mesophilic macromolecular complexes that are stabilized by kinetic barriers, such as HDL disks, and does not necessarily involve long-range electrostatic interactions. Taken together, our results suggest that short-range Coulombic protein−protein interactions provide an important contribution to the stability of discoidal HDL: the better optimized these interactions are, the higher the lipoprotein stability is.

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