

Pressure perturbation calorimetry of apolipoproteins in solution and in model lipoproteins

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ABSTRACT

High-density lipoproteins (HDLs) are complexes of lipids and proteins (termed apolipoproteins) that remove cell cholesterol and protect from atherosclerosis. Apolipoproteins contain amphipathic α -helices that have high content ($\geq 1/3$) and distinct distribution of charged and apolar residues, adopt molten globule-like conformations in solution, and bind to lipid surfaces. We report the first pressure perturbation calorimetry (PPC) study of apolipoproteins. In solution, the main HDL protein, apoA-I, shows relatively large volume contraction, $\Delta V_{\text{unf}} = -0.33\%$, and an apparent reduction in thermal expansivity upon unfolding, $\Delta \alpha_{\text{unf}} \leq 0$, which has not been observed in other proteins. We propose that these values are dominated by increased charged residue hydration upon α -helical unfolding, which may result from disruption of multiple salt bridges. At 5°C , apoA-I shows large thermal expansion coefficient, $\alpha(5^\circ) = 15 \cdot 10^{-4} \text{ K}^{-1}$, that rapidly declines upon heating from 5 to 40°C , $\alpha(40^\circ) - \alpha(5^\circ) = -4 \cdot 10^{-4} \text{ K}^{-1}$; apolipoprotein C-I shows similar values of $\alpha(5^\circ)$ and $\alpha(40^\circ)$. These values are larger than in globular proteins. They indicate dominant effect of charged residue hydration, which may modulate functional apolipoprotein interactions with a broad range of their protein and lipid ligands. The first PPC analysis of a protein-lipid complex is reported, which focuses on the chain melting transition in model HDL containing apoA-I or apoC-I, dimyristoyl phosphatidylcholine, and 0–20% cholesterol. The results may provide new insights into volumetric properties of HDL that modulate metabolic lipoprotein remodeling during cholesterol transport.

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Key words: protein hydration; amphipathic class-A α -helix; lipid surface binding proteins; high-density lipoprotein.

INTRODUCTION

High-density lipoproteins (HDL, a.k.a. “good cholesterol”) are complexes containing several proteins (termed exchangeable apolipoproteins) and several hundred lipid molecules. Plasma concentrations of HDL, HDL cholesterol, and the major HDL protein, apolipoprotein A-I (apoA-I), correlate inversely with the incidence of atherosclerosis (Refs. 1–3 and references therein). Cardioprotective effects of HDL and apoA-I result from their central role in reverse cholesterol transport (RCT), which is the essential pathway of cholesterol removal from the body, as well as from their anti-inflammatory and antithrombotic action (for recent reviews, see Refs. 4 and 5). In the first step of RCT, interaction of apoA-I with the plasma membrane, mediated by ATP-binding cassette transporter A1 (ABCA1), generates nascent discoidal HDL.⁶ These small particles ($d \sim 10 \text{ nm}$) are thought to comprise a cholesterol-containing phospholipid bilayer and two molecules of apoA-I that adopt a belt-like α -helical conformation around the particle perimeter,⁷ thereby screening the acyl chains from the solvent and conferring particle stability and solubility (see Fig. 1). At later steps of RCT, apoA-I can dissociate from HDL as a lipid-free or lipid-poor protein that is proposed to provide an important primary acceptor of cell cholesterol.¹¹ Lipid-poor or lipid-free apoA-I can interact with ABCA1 and plasma membrane to form nascent HDL; alternatively, it can bind to existing lipoproteins or undergo catabolism.^{6,11} This work addresses volumetric properties of apoA-I in solution and in discoidal reconstituted HDL (rHDL).

In their lipid-free state in aqueous solution, apoA-I and other exchangeable apolipoproteins adopt a compact conformation with high α -helix content but loosely folded tertiary structure, low thermodynamic stability, large solvent-accessible surface area, high aggregating propensity, affinity for various apolar ligands, and other char-

Abbreviations: ABCA1, ATP-binding cassette transporter A1; apo, apolipoprotein; CD, circular dichroism spectroscopy; DMPC, dimyristoyl PC; DSC, differential scanning calorimetry; EM, electron microscopy; HDL, high-density lipoprotein; MLV, multilamellar vesicle; PC, phosphatidylcholine; PPC, pressure perturbation calorimetry; RCT, reverse cholesterol transport; rHDL, reconstituted HDL; SUV, small unilamellar vesicle.

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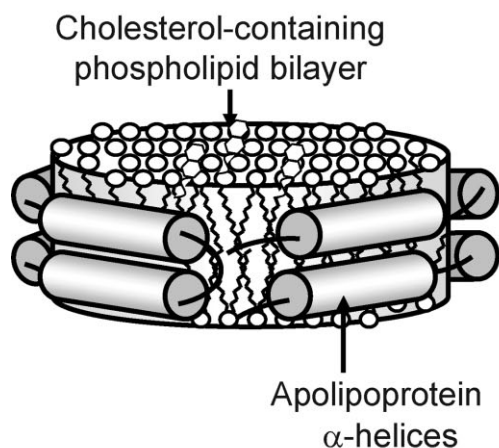
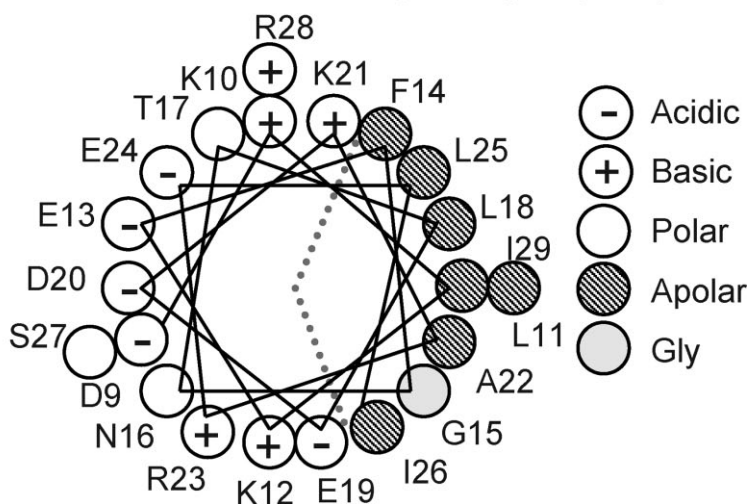
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Model of nascent “discoidal” HDL

Class-A α -helical segment (9-29) in apoC-I**Figure 1**

Cartoon representations of a discoidal HDL and of a class-A apolipoprotein α -helix. (A) Nascent discoidal HDL. Cylinders show protein α -helices. (B) Helix wheel diagram showing the N-terminal segment of human apolipoprotein C-I (residues 9–29), which forms class-A amphipathic α -helix that is the major lipid surface binding motif in apolipoproteins.⁸ Dotted lines demarcate polar and apolar helical faces. Apolar face is thought to bind lipid surface, whereas polar face confers particle solubility. Characteristic distribution of charged residues (most of which are located 3–4 positions apart from an oppositely charged group within the helix) allows formation of multiple intra- and interhelical salt bridges that have been proposed to importantly contribute to the stability of the double-belt protein conformation on HDL (Refs. 9 and 10 and references therein).

acteristics of the molten globule-like state^{12–16} (see Material and Methods). This partially folded state has been proposed to facilitate conformational changes in apolipoproteins during their binding to phospholipid surfaces.¹²

Amino acid sequences of apolipoproteins and other related lipid surface binding proteins are composed of 11-mer tandem repeats forming α -helices that are distinctly different from those found in globular or membrane proteins.^{8,17} The major lipid surface binding motif in apolipoproteins is class-A amphipathic α -helix that is characterized by a: (i) large flat well-demarcated apolar face that comprises 30–50% of the total surface area (compared with ~15% in typical globular proteins), (ii) high charge residue content of 30–40% (compared with ~15% in typical globular proteins), and (iii) distinct radial charge distribution, with acidic groups located at the center of the polar face and basic groups near its edges [Fig. 1(B); Table I], which may optimize protein binding to lipid surface

and facilitate formation of multiple intra- or interhelical salt bridges.^{8,10} These characteristics are likely to impart distinct hydration properties to apolipoproteins in solution, which may affect apolipoprotein interactions with other proteins (such as ABCA1) and lipids during RCT. Here, we report the first pressure perturbation calorimetry (PPC) analysis of the hydration properties of two human apolipoproteins, apoA-I (28 kD) and apoC-I (6 kD) (Table I). ApoA-I is the major HDL protein and a key player in RCT. ApoC-I, a minor protein in high- and very low-density lipoproteins, is an important modulator of lipid metabolism; this smallest exchangeable apolipoprotein is a genetic, structural, and functional prototype of larger members of this protein family (Ref. 18 and references therein).

PPC is a relatively new technique developed in conjunction with differential scanning calorimetry (DSC) to determine the volume expansion coefficient, $\alpha_v(T) =$

Table I

Sequence and Structural Properties of Human Apolipoproteins A-I and C-I

Protein	No. of amino acids	No. 11-mer repeats ^a	Charged groups, a.a. (%)	pI	α -Helix content	
					Monomer (%)	Oligomer (%)
apoA-I	243	21	82 (34%)	5.6 ^b	60 ^c	60 ^c
apoC-I	57	4	22 (39%)	6.5 ^b	31 ^c	60 ^c

^aBased on amino acid sequence analysis.⁸

^bBased on isoelectric focusing.^{19,20}

^cEstimated with 5% accuracy based on CD signal at 222 nm.^{12,14}

$1/V \cdot (\partial V / \partial T)_P$ of proteins and other solutes by measuring small heat effects resulting from sample contraction (expansion) upon application (release) of small pressures of about 5 bar.²¹ These heat effects are linked to volume changes in reversible reactions via the Maxwell equation, $(\partial Q / \partial P)_T = -T(\partial V / \partial T)_P = -TV\alpha_v$.^{21,22} In proteins, $\alpha_v(T)$ is dominated by the hydration effects that are distinctly different for charged, polar, and apolar groups. Solvent-exposed apolar residues show negative $\alpha_v(5^\circ\text{C})$ (termed α_5) that increases (i.e., becomes less negative) with temperature, $d\alpha_v(T)/dT > 0$. This is attributed to formation of the bulky hydrogen-bonded water structure around these “structure-making” groups; melting of this structure leads to an increase in $\alpha_v(T)$ at higher temperatures. In contrast, polar and charged “structure-breaking” residues, which promote formation of less structured, more densely packed water, show $\alpha_5 > 0$ and $d\alpha_v(T)/dT < 0$ (Refs. 21–24 and references therein). Thus, in the temperature range from 5 to 40°C, where a typical protein is fully folded, $\alpha_v(T)$ reflects combined effects of solvent-accessible groups on protein hydration. In addition, protein unfolding upon heating may involve volume expansion (or contraction) manifested as a positive (or negative) peak in $\alpha_v(T)$ function. Integration of this peak yields relative volume change upon protein unfolding, $\Delta V_{\text{unf}} = \Delta V/V$, where $\Delta V = V_U - V_F$ is the difference in specific volume V of the unfolded (U) and folded (F) states.²⁵

In contrast to proteins whose volume expansion coefficient is dominated by hydration effects and is relatively small (10^{-4} – 10^{-3}), $\alpha_v(T)$ of phospholipids depends largely on the mechanical properties of the lipid bilayer and may reach 10^{-2} – 10^{-1} at the chain melting transition in phosphatidylcholines (PCs).²⁶ The existing PPC measurements of lipids have been largely focused on volume changes involved in this transition in PC vesicles.^{26–29} To our knowledge, ours is the first application of PPC to protein–lipid complexes. We report PPC data of the complexes reconstituted from apoA-I or apoC-I, dimyristoyl PC (DMPC), and 0–20 mol % cholesterol, which provide a simple model for nascent HDL (whose main constituents are apoA-I, PCs, and up to 20 mol % cholesterol^{30,31}). The results may lead to new insights into apolipoprotein hydration that modulates apolipoprotein interactions with their protein and lipid ligands, and into lipoprotein expansivity that may modulate volume changes during metabolic remodeling of HDL.

MATERIALS AND METHODS

Proteins and lipids

Human apoA-I (243 a. a.) was isolated and purified from plasma HDL of healthy volunteer donors that was collected at the institutional Blood Bank in full compliance with their rules and regulations and with approval

of the institutional review board. The proteins were refolded as described¹² into 10 mM sodium phosphate buffer, pH 7.7, which was the standard buffer used throughout this work. The protein purity, assessed by high-performance liquid chromatography and sodium dodecyl sulfide polyacrylamide gel electrophoresis, was 95+%. Human apoC-I (57 a. a.) with unblocked termini was custom-synthesized by solid-state synthesis and purified to 97%+ purity at 21st Century Biochemicals as described¹⁸; the peptide identity and purity were confirmed by mass spectrometry and high-performance liquid chromatography. The proteins obtained by these methods were well folded, as evidenced by circular dichroism (CD) spectroscopy, and retained their ability to form HDL and activate LCAT (Refs. 12 and 18 and references therein). Lipids, including DMPC and unesterified cholesterol, were 95+% pure from Avanti Polar Lipids. All chemicals were of the highest purity analytical grade.

Lipoprotein reconstitution and characterization

Complexes of apolipoproteins (apoA-I or apoC-I) and lipids (DMPC with and without cholesterol) were prepared by thin film evaporation following established protocols.^{31,32} Briefly, lipids were dissolved in 2 parts chloroform, 1 part methanol, the solvent was evaporated under nitrogen, and the samples were dried overnight under vacuum at 4°C to form lipid films that were dispersed by vortexing in a standard buffer. Multilamellar vesicles (MLVs) prepared by this method were used either as controls in calorimetric experiments or for lipoprotein reconstitution. To form rHDL, protein stock solution was added to the MLV suspension (DMPC to protein weight ratio 4:1) and incubated for 20 h at 24°C (i.e., at the temperature T_c of the chain melting transition in DMPC at which protein-MLV binding and lipoprotein reconstitution is fastest); this was followed by centrifugation to remove uncomplexed lipid. Lipoprotein formation was confirmed by nondenaturing gel electrophoresis and negative staining electron microscopy (EM) under low-dose conditions in a CM12 transmission microscope (Philips Electron Optics) as described.³¹ Biochemical composition of rHDL was determined by using modified Lowry assay for protein, Bartlett assay for phospholipid, and colorimetric analysis for cholesterol. The initial lipid mixture and the lipoproteins reconstituted from this mixture had similar DMPC/cholesterol molar ratios.

Pressure perturbation and DSC

PPC and DSC data of proteins (apoA-I or apoC-I), lipids (MLV containing DMPC and 0–30 mol % cholesterol), and lipoproteins (containing apoA-I or apoC-I, DMPC, and cholesterol that comprised 0–20 mol % of the total

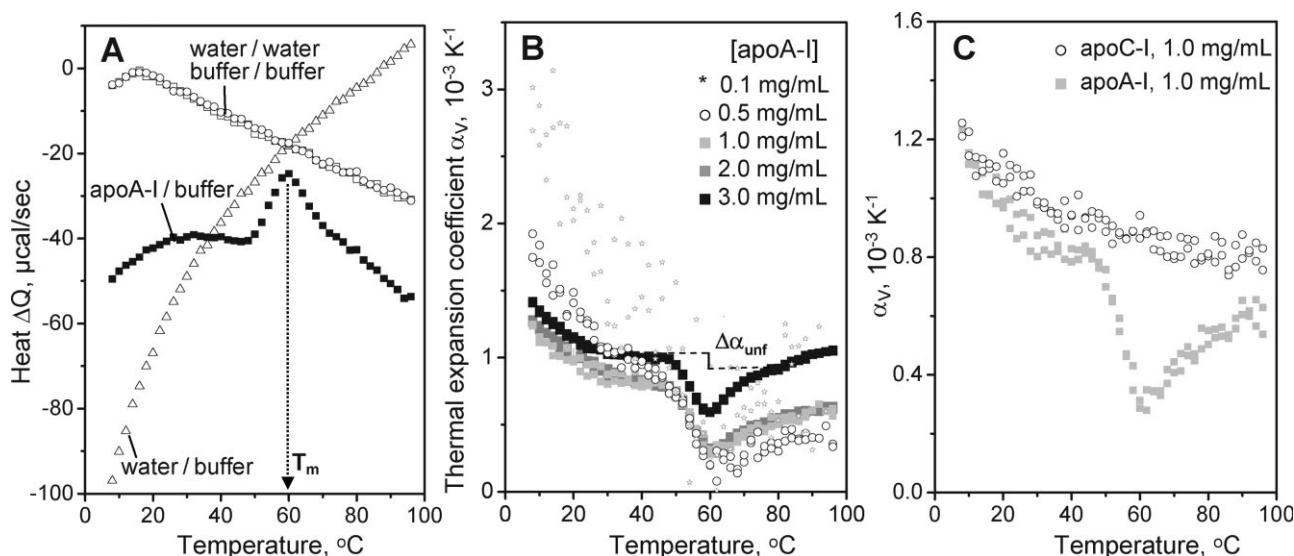


Figure 2

PPC of lipid-free apolipoproteins in aqueous solution. Standard buffer conditions are 10 mM sodium phosphate, pH 7.7; temperature increment is 2°C. (A) PPC data of human apoA-I (3 mg/mL protein) showing heat released/absorbed upon pressure increase/release by 5 bar. Heat absorbed upon pressure release is shown with inverse sign. The $Q(T)$ data recorded upon pressure increase and release closely superimpose (black squares). The peak centered near 60°C reflects unfolding of the secondary structure in apoA-I.¹² Open symbols show water–water, buffer–buffer, and buffer–water baselines. (B) Thermal expansion coefficient $\alpha_v(T)$ of apoA-I (0.1–3.0 mg/mL protein). ApoA-I is fully monomeric up to 0.1 mg/mL protein but self-associates at higher concentrations. Change in volume expansion coefficient upon unfolding, $\Delta\alpha_{unf}$, is illustrated by a vertical line; because of the uncertainty in the choice of the pre- and posttransitional baselines that are extrapolated to the transition range (dashed lines), the value of $\Delta\alpha_{unf}$ may vary from 0.0 to $-2.0 \cdot 10^{-4}$. (C) Thermal expansion coefficient $\alpha_v(T)$ of human apoA-I and apoC-I (1 mg/mL protein).

lipid) were recorded by using VP-DSC microcalorimeter (MicroCal, Northampton, MA) with 0.5 mL cell volume. The sample and reference cells were filled with degassed sample and matching buffer solutions and heated at a constant rate (50°C/h in DSC). The instrument was operated in mid gain mode for proteins and lipoproteins and in high gain mode for lipids. In DSC experiments, a pressurizing cap was used to apply ~ 1.8 bar pressure to the cells to avoid bubbles at high temperatures. Differential heat capacity $C_p(T)$ was recorded, and the buffer–buffer baseline was subtracted from the data. In PPC experiments, a PPC accessory was used that allows application of small (~ 5 bar) pressures to the cells in a programmed regime in the context of a DSC experiment.²⁵ Measurements of the heat effects associated with this pressure allow determination of volume expansion coefficient of the solute on an absolute scale.²¹ Before recording PPC data, the instrument was equilibrated at the starting temperature using auto prescan equilibration mode. Equilibration time and signal slope were determined automatically using VPViewer software. Protein and lipoprotein scans were recorded upon heating from about 5–100°C in mid gain, low noise mode with a 1 s filter time, and a temperature increment of 1–2°C. Scans of DMPC MLV recorded under similar conditions in high gain mode (such as those shown in Fig. 3) did not allow adequate sampling of the narrow gel-to-liquid crystal phase transition in DMPC centered near $T_c \cong 24^\circ\text{C}$. To better characterize this transition, lipid

scans were recorded in a narrow temperature range (from 22 to 26°C) in a constant temperature mode with 0.05°C increment, which corresponds to an approximate heating rate of 0.7°C/h. The data were processed using Origin software.

All experiments in this work were repeated at least three times to ensure reproducibility.

Reversibility of apolipoprotein unfolding

CD spectroscopic studies (using about 0.01–0.2 mg/mL protein) showed that, in contrast to kinetically controlled lipoprotein transitions (Ref. 9 and references therein), thermal unfolding of lipid-free apolipoproteins is independent of the scan rate, is fully reversible upon heating and cooling to about 60°C, and is largely reversible upon heating and cooling to higher temperatures; similarly, DSC data of free apoA-I recorded in consecutive scans were partially or largely reversible (for 1.7 or 0.16 mg/mL protein, respectively^{12,33}). Furthermore, CD and DSC data of apoA-I showed similar melting temperatures T_m and van't Hoff enthalpies ΔH_v at various protein concentration and scan rates used (10–80°C/h). Therefore, despite aggregation of thermally unfolded apolipoproteins, calorimetric data recorded in the transition range are thermodynamically reversible. Consistent with this notion, the PPC data of apoA-I recorded at a heating rate of 12°C/h (not shown) and 24°C/h (see Fig. 2) closely superimposed. Therefore, the Maxwell equation,

which holds for thermodynamically reversible transitions, can be applied to apolipoprotein unfolding.

Partially folded state of apoA-I in solution

Although the molten globular state of lipid-free apoA-I is widely accepted in the field, it has been challenged by Brouillette *et al.*³³ who reported that the calorimetric transition recorded from 0.16 mg/mL of recombinant protein using Nano-DSC II (Calorimetry Sciences Corp.) is well approximated by a three-state model with a single unfolding intermediate, which is at odds with very low-cooperativity unfolding of classic molten globules. Despite this difference in data interpretation as well as in the protein source and the instrument used, CD and DSC data recorded by Brouillette *et al.* agree with our own data on two important points. First, at less than 2 mg/mL apoA-I, the DSC data consistently show near-zero heat capacity increment ΔC_p upon unfolding,^{12,33} indicating no significant changes in solvent exposure of apolar groups upon unfolding and suggesting high degree of solvent exposure of these groups in the folded state of apoA-I. Second, CD and DSC studies consistently show low van't Hoff enthalpy in the range from $\Delta H_v = 32$ –41 kcal/mol.^{12,33} This corresponds to the unfolding of about 31 ± 6 residues in α -helical conformation (assuming the enthalpy of the helix-to-coil transition of 1.1–1.3 kcal/mol per residue³⁴), which is only a fraction of the helical structure lost upon unfolding of apoA-I (that has about 60%, or 146 of its groups in α -helical conformation). The large discrepancy between the measured ΔH_v and the expected calorimetric enthalpy of unfolding of just the helical structure in apoA-I (160–190 kcal/mol) indicates that the cooperatively unfolding unit comprises a relatively small fraction of the apoA-I molecule. These results support the notion that apoA-I in solution forms a partially folded state with largely exposed apolar helical faces and relatively weak interhelical interactions; smaller apolipoproteins, such as apoC-I, are even less well folded in solution.^{13,14} We use this notion to interpret PPC data in this study.

RESULTS AND DISCUSSION

PPC of lipid-free apoA-I in solution

PPC data of lipid-free human apolipoproteins in standard buffer (10 mM sodium phosphate, pH 7.7) were recorded during heating. The exothermic (endothermic) heat response to a pressure increase (release) of 5 bar, $\Delta Q(T)$, was recorded and used to obtain the thermal expansion coefficient, $\alpha_v(T)$. Figure 2(A,B) (black squares) shows $Q(T)$ data recorded of 3 mg/mL apoA-I and the thermal expansion coefficient $\alpha_v(T)$ obtained from these data. The broad negative peak in the $\alpha_v(T)$ function centered near $T_m = 60^\circ\text{C}$ [Fig. 2(B)] reflects

apoA-I unfolding that was observed by DSC and by far-UV CD at these temperatures (Ref. 12 and references therein). This unfolding leads to an apparent reduction in expansivity, $\Delta\alpha_{\text{unf}} = \alpha_U - \alpha_F \leq 0$ [Fig. 2(B), vertical line], which contrasts with $\Delta\alpha_{\text{unf}} > 0$ reported for globular proteins (Table II). Integration of the $\alpha_v(T)$ peak recorded of 3 mg/mL apoA-I yields negative volume change upon unfolding, $\Delta V_{\text{unf}} = \Delta V/V = -0.33\%$. This is comparable or larger than the absolute values reported in PPC studies of globular proteins, $|\Delta V_{\text{unf}}| \leq 0.3\%$ ²¹ (Table II).

In globular proteins, most reported values of ΔV_{unf} are small and negative (Table II), which is attributed to increased hydration of polar groups and internal protein voids upon unfolding.^{23,25} A correlation between ΔV_{unf} and protein stability has been reported for staphylococcal nuclease: increase in protein stability upon mutations caused ΔV_{unf} to become less negative and, eventually, change sign from negative to positive, which was attributed to increased conformational dynamics of the unfolded state at high temperatures.²⁵ Thus, relatively large negative ΔV_{unf} observed in apoA-I in solution (Table II) is consistent with the low thermodynamic stability of this protein, $\Delta G(25^\circ\text{C}) = 2.4$ kcal/mol.¹² Furthermore, hydration of internal voids upon unfolding of the tertiary structure (which is highly hydrated in apoA-I) is unlikely to contribute significantly to ΔV_{unf} . Therefore, the large negative ΔV_{unf} of apoA-I is probably dominated by hydration of the secondary structure, with a possible contribution from the quaternary structure (discussed later).

Significant differences between the volumetric properties of apoA-I and globular proteins are also observed in the pretransitional temperature range [Fig. 2(B), Table II]. At 5°C , apoA-I shows $\alpha_5 = 15 \cdot 10^{-4} \text{ K}^{-1}$, larger than that reported for globular proteins ($\alpha_5 = 6$ – $10 \cdot 10^{-4} \text{ K}^{-1}$) (Table II). Also, the change in $\alpha_v(T)$ upon heating from 5 to 40°C , $\alpha_{5-40} = \alpha_v(40^\circ\text{C}) - \alpha_v(5^\circ\text{C})$, is $\alpha_{5-40} = -4 \cdot 10^{-4} \text{ K}^{-1}$ for apoA-I, larger than that in globular proteins ($\alpha_{5-40} = -0.9$ to $-3.5 \cdot 10^{-4} \text{ K}^{-1}$)²¹ (Table II). Observation of large positive α_5 and large negative α_{5-40} is consistent with the correlation between α_5 and α_{5-40} reported for globular proteins: the larger the fraction of polar and, particularly, charged groups, the larger is α_5 and the more negative is α_{5-40} .²¹ Thus, high content of charged residues in apoA-I (34%, nearly twice as much as in typical globular proteins) may explain the behavior of its $\alpha_v(T)$ function at 5 – 40°C .

Effects of protein concentration on the $\alpha_v(T)$ function of apoA-I

Protein self-association at mg/mL concentrations that are used in calorimetric studies is a potential caveat in these studies. To optimize the signal-to-noise ratio in the PPC data and thereby increase accuracy in determining

Table II

Volumetric Parameters of apoA-I in Solution Obtained by PPC: Comparison with Globular Proteins

Protein	pH	α_5 (K ⁻¹)	α_{5-40} (K ⁻¹)	α_{40} (K ⁻¹)	$\Delta\alpha_{\text{unf}}$ (K ⁻¹)	ΔV_{unf} (%)
Chymotrypsin ^a	2.4	6.3×10^{-4}	-0.9×10^{-4}	5.4×10^{-4}	1.1×10^{-4}	0.23
Pepsinogen ^a	6.4	6.8×10^{-4}	-1.2×10^{-4}	5.6×10^{-4}	1.0×10^{-4}	0.00
HEW lysozyme ^a	2.5	7.1×10^{-4}	-1.7×10^{-4}	5.4×10^{-4}	1.4×10^{-4}	-0.08
Trypsin inhibitor ^a	4.0	7.2×10^{-4}	-1.9×10^{-4}	5.3×10^{-4}	0.5×10^{-4}	-0.06
Ribonuclease A ^a	5.5	8.2×10^{-4}	-2.2×10^{-4}	6.0×10^{-4}	1.5×10^{-4}	-0.29
T4 Lysozyme ^a	3.6	9.4×10^{-4}	-2.6×10^{-4}	6.8×10^{-4}	0.5×10^{-4}	-0.23
SNase ^b	—	10.0×10^{-4}	-3.5×10^{-4}	6.5×10^{-4}	1.2×10^{-4}	-0.15
ApoA-I ^c	7.7	15.0×10^{-4}	-4.0×10^{-4}	11.0×10^{-4}	-1.0×10^{-4}	-0.33

The accuracy in determining volume expansion coefficient $\alpha_v(T)$ at 5 and at 40°C, α_5 and α_{40} , and their difference, $\alpha_{5-40} = \alpha_{40} - \alpha_5$, is 10–15% and is determined by the reproducibility of the PPC data. Uncertainty in the determination of the pre- and posttransitional baselines in the PPC data limits the accuracy in determination of the relative volume change upon unfolding, $\Delta V_{\text{unf}} = (V_U - V_F)/V$, to about 30%; for the same reason, the change in expansivity, $\Delta\alpha_{\text{unf}} = \alpha_U - \alpha_F$, may range from about 0.0 to -2.0×10^{-4} depending on the data set and the baseline choice.

^aFrom Ref. 21.

^bFrom Ref. 35.

^cBased on the PPC data recorded of 3 mg/mL apoA-I [Fig. 2(B), black squares].

volumetric parameters of apoA-I, we used a low-salt protein solution of 3 mg/mL concentration [Fig. 2(A,B), black squares]; under these conditions, folded apoA-I is significantly self-associated. In fact, apoA-I self-association under similar solvent conditions and room temperature starts above 0.1 mg/mL protein.^{12,33,36} Even though the signal-to-noise ratio in the PPC data of fully monomeric apoA-I recorded at 0.1 mg/mL protein was prohibitively low, the $\alpha_v(T)$ data recorded of 0.5–2.0 mg/mL apoA-I were more amenable to interpretation [Fig. 2(B)]. Compared to these data, the $\alpha_v(T)$ function recorded of 3.0 mg/mL apoA-I had similar shape but was shifted to higher values. The largest difference was observed in the posttransitional temperature range and was probably caused by aggregation of the thermally unfolded apoA-I, which was also detected under similar conditions by DSC.¹² Importantly, $\alpha_v(T)$ functions recorded from 0.5 to 3.0 mg/mL apoA-I had similar shapes in the protein unfolding range (50–70°C), suggesting that ΔV_{unf} is not greatly affected by changes in the protein self-association degree. Therefore, ΔV_{unf} of apoA-I probably does not have a large contribution from hydration of the quaternary structure. We conclude that the large negative ΔV_{unf} is dominated by increased hydration of polar and, particularly, charged groups upon unfolding of the secondary structure in apoA-I. This increased hydration may result, in part, from the disruption of numerous putative salt bridges in the apolipoprotein α -helices.

Similar shapes of the PPC data observed at various apoA-I concentrations [Fig. 2(B)] also suggest that $\Delta\alpha_{\text{unf}} \leq 0$ is an intrinsic property of the apoA-I molecule and not a result of its self-association. In contrast, PPC studies of other proteins report $\Delta\alpha_{\text{unf}} > 0$ (Table II). The basis for this observation should become clear once the molecular determinants for $\Delta\alpha_{\text{unf}}$ are better understood. One possibility is that, since for apolar groups $\Delta\alpha(T)/dT > 0$ and for charged/polar groups $\Delta\alpha(T)/dT < 0$, negative $\Delta\alpha_{\text{unf}}$ reflects the dominant effects of charged residue hydration upon apoA-I unfolding. This notion is consistent

with the DSC observation of a near-zero heat capacity increment ΔC_p upon apoA-I unfolding, indicating that this unfolding leads to little additional solvent exposure of apolar groups.¹² In summary, $\Delta\alpha_{\text{unf}} \leq 0$ suggested by our PPC data of apoA-I may reflect dominant effects of increased charged residue hydration upon unfolding, which may result from disruption of numerous putative salt bridges in class-A α -helices.

In the pretransitional temperature range, $\alpha_v(T)$ data recorded of 1.0–3.0 mg/mL apoA-I largely overlapped; hence, the values of α_5 and α_{5-40} were similar (listed in Table II). However, at 0.5 mg/mL protein, these values increased to $\alpha_5 = 17 \cdot 10^{-4} \text{ K}^{-1}$ and $\alpha_{5-40} = -8 \cdot 10^{-4} \text{ K}^{-1}$, and at 0.1 mg/mL protein, further increase was apparent [Fig. 2(B)]. These effects may result from the temperature-induced changes in the self-association degree of the folded protein. Similarly, increased absolute values of α_5 and α_{5-40} upon reduction in protein concentration were observed in ribonuclease and were attributed to changes in the degree of protein self-association.²³ We conclude that α_5 and α_{5-40} of monomeric apoA-I (at 0.1 mg/mL protein) are comparable or larger than those listed in Table II (at 3 mg/mL protein). Therefore, large positive α_5 and large negative α_{5-40} reflect inherent properties of the apoA-I molecule and are not a result of its self-association.

PPC data of lipid-free apoA-I and apoC-I compared

To test whether relatively large α_5 and α_{5-40} observed in apoA-I are characteristic of other exchangeable apolipoproteins, we compared the $\alpha_v(T)$ functions of human apoA-I and apoC-I. Figure 2(C) shows PPC data of these proteins recorded under identical conditions using 1 mg/mL protein in standard buffer. In contrast to apoA-I, apoC-I demonstrates no cooperative changes in $\alpha_v(T)$ upon thermal unfolding. This is not surprising, given the broad low-cooperativity thermal unfolding of this small

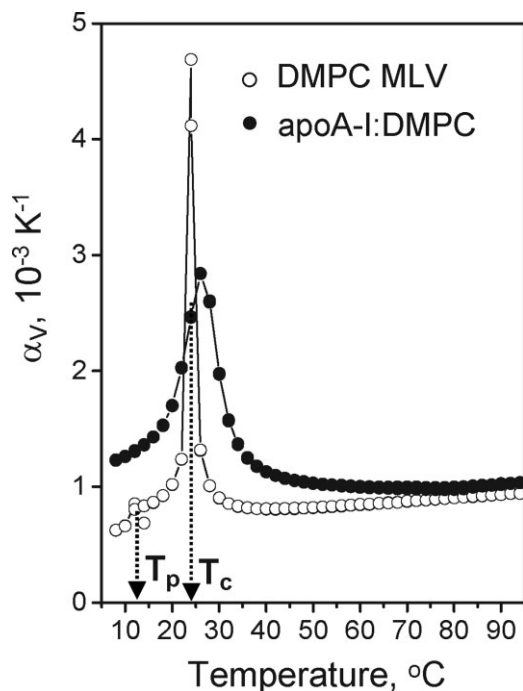


Figure 3

PPC data of DMPC MLV and apoA-I:DMPC disks. Suspensions of DMPC MLV (12 mg/mL lipid) and of discoidal apoA-I:DMPC complexes (3 mg/mL protein, 12 mg/mL lipid) in standard buffer were used. The temperatures T_p of the pretransition (observed at 14°C in MLV) and T_c of the main lipid phase transition (observed at 24°C in MLV and at 26°C in discoidal rHDL) are shown.

polypeptide.¹⁴ Furthermore, apoC-I self-association, which starts above 0.01 mg/mL protein concentration, induces formation of additional helical structure observed by CD; the α -helix content in self-associated apoC-I is maximal near 25°C and decreases upon heating due to monomer unfolding and upon cooling due to oligomer dissociation below 25°C.¹⁴ Because of their low cooperativity, these high- and low-temperature unfolding transitions in apoC-I are not detected as peaks in the $\alpha_v(T)$ function. The effects of these transitions on the PPC data of apoC-I are probably relatively small, because the observed $\alpha_v(T)$ functions of apoA-I and apoC-I nearly overlap at 5–40°C, leading to similar values of α_5 and α_{5-40} for these proteins [Fig. 2(C)]. Consequently, the unusually large positive α_5 and large negative α_{5-40} are characteristic of not only apoA-I but also apoC-I and, possibly, other exchangeable apolipoproteins.

PPC data of apolipoproteins and globular proteins compared

Large positive α_5 and large negative α_{5-40} observed in apoA-I and apoC-I are probably dominated by hydration of charged residues that comprise about 1/3 of all groups in these proteins (Table I). High α -helical content may

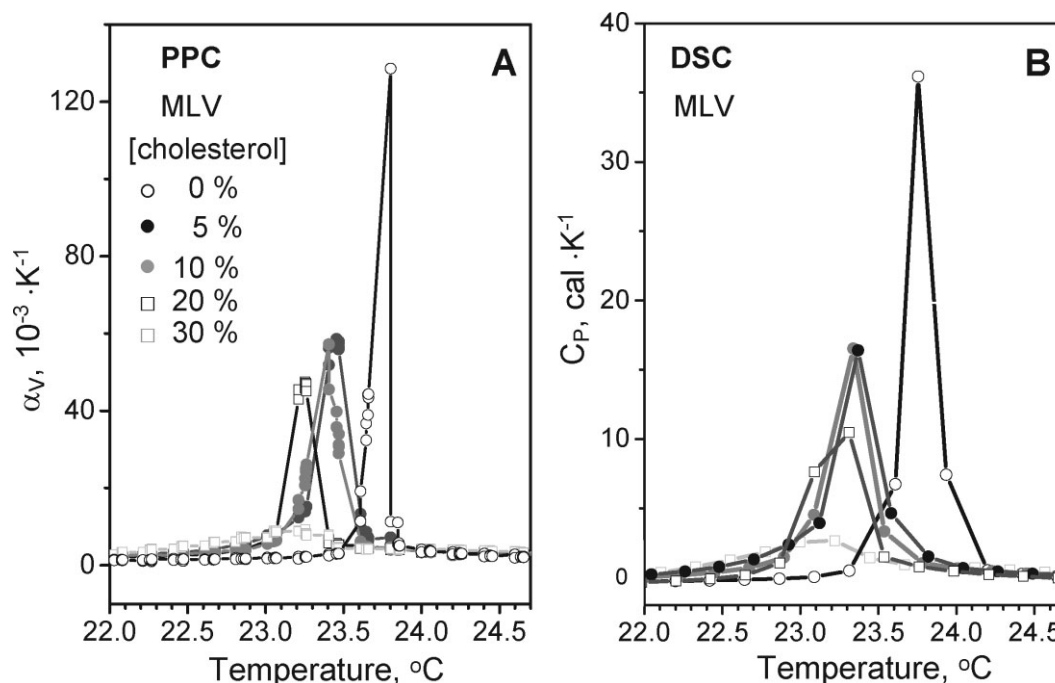
also contribute to the large values of α_5 and α_{5-40} , as suggested by a correlation between these values and the helical content reported in a PPC study of model peptides²⁴; the peptides with the greatest α -helix content showed most positive α_5 and most negative α_{5-40} .²⁴ Furthermore, in globular proteins α_{5-40} is roughly proportional to the solvent-accessible surface area,²¹ which is relatively large in apolipoproteins. In summary, large positive α_5 and large negative α_{5-40} of apolipoproteins in solution probably result from their high charged residue content, with a contribution from the high helical content and large solvent-accessible surface area.

Despite rapid decline in $\alpha_v(T)$ of apoA-I and apoC-I upon heating from 5 to 40°C, $\alpha_v(T)$ at 40°C remains relatively high [$\alpha_{40} = 8\text{--}11 \cdot 10^{-4} \text{ K}^{-1}$ depending on the apolipoprotein concentration, Fig. 2(B,C)] when compared with globular proteins ($\alpha_{40} = 5.4\text{--}6.8 \cdot 10^{-4} \text{ K}^{-1}$) (Table II). Thus, at near-physiologic temperatures, apolipoproteins in solution have higher expansivity than globular proteins. This effect, which is probably dominated by hydration of charged groups, may contribute to the structural plasticity of apolipoproteins, which is necessary for their functional interactions with other proteins and lipids (Refs. 12 and 16 and references therein).

PPC of apoA-I:DMPC complexes: comparison with DMPC MLV

Reconstituted apoA-I:DMPC complexes were used to record the first PPC data of a lipoprotein. The complexes were heated from 5 to 98°C, and the PPC data were recorded with 2°C increment; the results were compared with similar PPC data recorded of DMPC MLV (see Fig. 3).

Hydrated PCs form various mesomorphic phases, such as liquid-ordered (l_o) and liquid-disordered (l_d) phase; transition among these phases can be induced by changes in temperature, pressure, lipid composition, and so forth (26,37,38 and references therein). DMPC undergoes an ordered (rippled) to l_d phase transition at $T_c \cong 24^\circ\text{C}$ preceded by gel-to-ripple pretransition at $T_p \cong 14^\circ\text{C}$, which have been well characterized by DSC and, more recently, by PPC (26,27 and references therein). Consistent with these studies, our PPC data of DMPC MLV show the main transition at 24°C and the pretransition at 14°C (Fig. 3, open symbols). PPC data of apoA-I:DMPC complexes show only the main transition that has increased $T_c \cong 26^\circ\text{C}$ and reduced cooperativity, as evident from the increased width and decreased amplitude of the $\alpha_v(T)$ peak (Fig. 3, black circles). This is consistent with the DSC data of apoA-I:DMPC disks and reflects relatively small disk size ($d \cong 11 \text{ nm}$ when compared with $d > 100 \text{ nm}$ in MLV), the fact that only a fraction of lipid molecules that are not in direct contact with the protein undergo the phase transition, and the lack of interbilayer interactions in the disks (39 and references therein).

**Figure 4**

Effects of cholesterol on the chain melting transition in DMPC monitored by PPC and DSC. Freshly prepared MLVs of DMPC (3 mg/mL in standard buffer) containing 0–30 mol % cholesterol were used. Temperature increment in PPC was 0.05°C; heating rate in DSC was 50°C/h. Thermal expansion coefficient was recorded by PPC (A) and heat capacity was recorded by DSC (B). Molar concentrations of cholesterol are indicated in the figure.

In addition to the lipid phase transition, DSC data of apoA-I:DMPC disks recorded at 90°C/h heating rate show a thermodynamically irreversible transition at the apparent $T_m \cong 90^{\circ}C$ (T_m is lower at slower heating rates); this transition reflects protein unfolding and dissociation accompanied by disk fusion into vesicles.^{32,40} PPC data of apoA-I:DMPC disks do not show this high-temperature transition (Fig. 3, black circles), implying that the volume changes in this transition are too small to be detected by PPC.

Effects of cholesterol on the chain melting transition in DMPC vesicles and disks

Cholesterol is an essential structural and functional constituent of HDL, and plasma level of HDL cholesterol is an important negative predictor of atherosclerosis (1–3 and references therein). In nascent HDL, free (unesterified) cholesterol reportedly comprises 5–20 mol % of the total lipid.^{6,30,31} Cholesterol incorporation into rHDL, such as apoA-I:DMPC complexes, leads to formation of larger particles (Refs. 31 and 41 and references therein). Here, we used PPC and DSC to assess the effects of variations in cholesterol concentration on the chain melting transition in apoC-I:DMPC complexes. The results were

compared with the PPC data recorded of DMPC MLV containing 0–30 mol % cholesterol content (Figs. 4 and 5).

Cholesterol in PC bilayers induces l_o state with properties intermediate between the gel (ordered chains) and l_d (fluid chains) (Ref. 28 and references therein). Cholesterol diminishes the difference between the two phases and shifts the equilibrium toward the l_d phase, thereby slightly reducing the temperature T_c of the main transition; above 30 mol % cholesterol, the difference between the two phases disappears (Refs. 42 and 43 and references therein). PPC and DSC data of DMPC MLV in Figure 4, which were recorded upon sample heating from 22 to 25°C, are consistent with this notion. These data show that increasing cholesterol content from 0 to 20 mol % in DMPC MLV leads to a reduction in T_c by 0.5°C, from 23.75 to 23.25°C, and diminishes the cooperativity of the transition, as indicated by the peak broadening. At 30 mol % cholesterol, the difference between the two phases disappears and the transition is barely detectable (light squares).

PPC data of lipoproteins reconstituted from apoC-I, DMPC (1:4 protein/PC weight ratio), and cholesterol that comprised 0–20 mol % of the total lipid are shown in Figure 5. Negative staining EM and nondenaturing gel electrophoresis of these lipoproteins showed that the disk diameter in the major population of particles increases

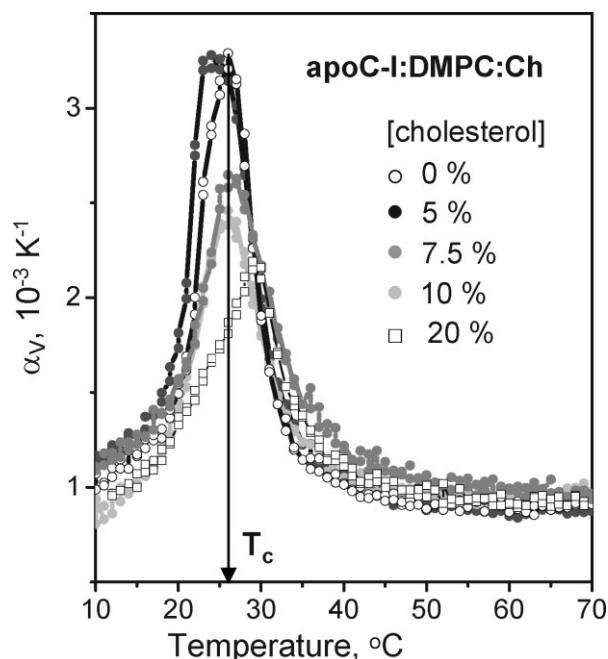


Figure 5

Thermal expansion coefficient of lipoprotein complexes containing apoC-I, DMPC, and various amounts of cholesterol. The concentrations used are 0.2 mg/mL protein, 0.8 mg/mL DMPC in standard buffer; molar concentrations of cholesterol are indicated in the figure.

from about 10–17 nm upon increasing cholesterol content from 0 to 10 mol %; from 15 mol % cholesterol onward, small unilamellar vesicles (SUV) with diameters $d > 22$ nm are formed.³¹ For lipoproteins containing no cholesterol, the PPC data in Figure 5 (open circles) were consistent with the DSC data⁴⁰ and showed a chain melting transition centered at $T_c = 26^\circ\text{C}$. Because of the peak broadening in lipoproteins (width at half-height $\Delta T_{1/2} = 7^\circ\text{C}$, open circles in Fig. 5) compared with MLV [$\Delta T_{1/2} = 0.1\text{--}0.3^\circ\text{C}$, Fig. 4(A)], the accuracy in T_c determination for lipoproteins was reduced to about $\pm 1^\circ\text{C}$ (when compared with $\pm 0.05^\circ\text{C}$ for MLV). Within this error margin, the PPC data of lipoproteins containing 0, 7.5%, and 10 mol % cholesterol showed similar values of T_c and $\Delta T_{1/2}$ (open, gray, and light gray circles in Fig. 5). These PPC data also showed reduced peak height and, hence, reduced volume change of the transition upon increasing cholesterol content. This is not surprising, given that cholesterol reduces the difference between the two PC phases and decreases the void volume in the PC bilayer.⁴³ An apparent exception from this trend was observed in the disks containing 5 mol % cholesterol (Fig. 5, black circles). PPC data of these disks showed no reduction in the transition amplitude and a marginally significant reduction in T_c to 24.5°C when compared with cholesterol-free disks ($T_c = 26^\circ\text{C} \pm 1^\circ\text{C}$); the origin

of this apparent anomaly is under investigation. Finally, at 20 mol % cholesterol, the SUV that formed under these conditions showed further reduction in the amplitude of the PPC transition that was shifted to $T_c = 29^\circ\text{C} \pm 1^\circ\text{C}$ (Fig. 5, open squares). Consistent with these PPC data, the DSC data (not shown) also showed a large reduction in the transition amplitude and an increase in T_c from 26 to 29°C upon increasing cholesterol content from 0 to 20 mol %. Taken together, the results in Figures 3 and 5 illustrate the utility of PPC for the analysis of volumetric properties of model discoidal and, potentially, mature spherical lipoproteins.

SUMMARY

This is the first attempt to use PPC for the analysis of lipid surface binding proteins and their complexes with lipids. The results reveal distinct differences in the volumetric parameters of apolipoproteins and globular proteins. First, lipid-free human apolipoproteins A-I and C-I in solution at ambient temperatures have a large positive thermal expansion coefficient $\alpha_v(T)$ that rapidly declines upon heating from 5 to 40°C [Fig. 2(B,C)], a behavior characteristic of charged/polar protein hydration.^{21,23} Therefore, despite high content of apolar residues ($\sim 30\%$ in apoA-I and apoC-I) and their substantial solvent exposure in the loosely folded solution conformation, apolipoprotein hydration is dominated by charged residues that comprise $\geq 1/3$ of all residues in this protein family. This emphasizes the importance of electrostatic interactions involving charged groups in apolipoproteins and their partners in RCT. Charge–charge interactions have been proposed to play essential roles in apolipoprotein interactions with LCAT, ABCA1, phospholipid and cholesterol transfer proteins, lipoprotein receptors, cell surface heparan sulfate proteoglycans, and so forth (Ref. 9 and references therein); they are also likely to contribute to apolipoprotein interactions with a broad range of other HDL-associated proteins that are involved in immune response and other HDL functions (Ref. 5 and references therein). Furthermore, apolipoprotein adsorption to phospholipid surface (e.g., during formation or nascent HDL or during apolipoprotein exchange among lipoproteins) has been proposed to occur via the electrostatic interactions between apolipoprotein charged residues and the phospholipid headgroups.⁴⁴ Hydration of apolipoprotein charges is expected to importantly affect these functional protein–protein and protein–lipid interactions.

Second, our PPC results showed that apoA-I unfolding involves a relatively large negative volume change and an apparent expansivity increment $\Delta\alpha_{\text{unf}} \leq 0$ [Fig. 2(B)], which contrasts with $\Delta\alpha_{\text{unf}} > 0$ observed in globular proteins (Table II). This suggests dominant effect of increased hydration of charged groups upon α -helical unfolding in apoA-I and may reflect disruption of

numerous salt bridges in class-A apolipoprotein α -helices.

Finally, our results suggest that PPC holds promise for the analysis of volumetric properties of lipoproteins (Figs. 3 and 5). Future PPC studies of these properties may prove useful for understanding volume changes involved in lipid core phase transition in spherical lipoproteins (that occurs at near-physiologic temperatures and may importantly affect lipoprotein structure and function⁴⁵), as well as in apolipoprotein adsorption/desorption to/from lipoprotein surface in response to changes in surface pressure,⁴⁶ insertion of cholesterol and cholesterol-processing proteins into this surface, fusion of spherical lipoproteins (reviewed in Ref. 47), and other aspects of metabolic lipoprotein remodeling during cholesterol transport.

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