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Kinetic and Thermodynamic Analyses of Spontaneous Exchange between High-Density Lipoprotein-Bound and Lipid-Free Apolipoprotein A-I

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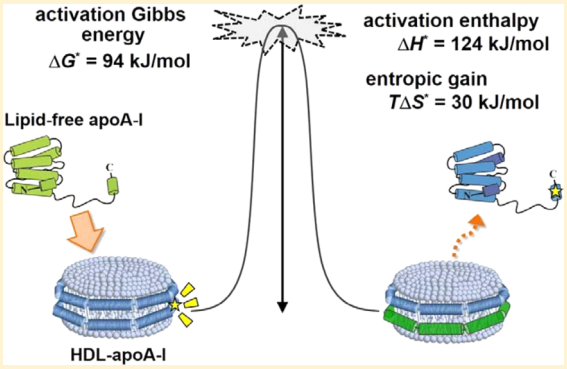
Supporting Information

ABSTRACT: It is thought that apolipoprotein A-I (apoA-I) spontaneously exchanges between high-density lipoprotein (HDL)-bound and lipid-free states, which is relevant to the occurrence of preβ-HDL particles in plasma. To improve our understanding of the mechanistic basis for this phenomenon, we performed kinetic and thermodynamic analyses for apoA-I exchange between discoidal HDL-bound and lipid-free forms using fluorescence-labeled apoA-I variants. Gel filtration experiments demonstrated that addition of excess lipid-free apoA-I to discoidal HDL particles promotes exchange of apoA-I between HDL-associated and lipid-free pools without alteration of the steady-state HDL particle size. Kinetic analysis of time-dependent changes in NBD fluorescence upon the transition of NBD-labeled apoA-I from HDL-bound to lipid-free state indicates that the exchange kinetics are independent of the collision frequency between HDL-bound and lipid-free apoA-I, in which the lipid binding ability of apoA-I affects the rate of association of lipid-free apoA-I with the HDL particles and not the rate of dissociation of HDL-bound apoA-I. Thus, C-terminal truncations or mutations that reduce the lipid binding affinity of apoA-I strongly impair the transition of lipid-free apoA-I to the HDL-bound state. Thermodynamic analysis of the exchange kinetics demonstrated that the apoA-I exchange process is enthalpically unfavorable but entropically favorable. These results explain the thermodynamic basis of the spontaneous exchange reaction of apoA-I associated with HDL particles. The altered exchangeability of dysfunctional apoA-I would affect HDL particle rearrangement, leading to perturbed HDL metabolism.



High-density lipoprotein (HDL) possesses antiatherogenic properties because it can mediate the reverse cholesterol transport pathway in which the principal protein, apolipoprotein A-I (apoA-I), plays a central role.[1−3] Although apoA-I is essentially associated with HDL particles, some 5−10% of the apoA-I in human plasma is present as lipid-poor or lipid-free species that are called preβ-HDL.[4−6] It is thought that the first step in reverse cholesterol transport involves the efflux of cellular lipids to this lipid-poor apoA-I, which is mediated by interactions with ABCA1.[7−9] Thus, the presence of biologically active lipid-poor apoA-I in the arterial wall is essential for cardioprotection,[10,11] and conversely, oxidized apoA-I from human atheroma that has defective cholesterol acceptor activity is associated with increased cardiovascular disease risk.[12,13]

ApoA-I is a 243-residue polypeptide that contains characteristic 11- and 22-residue repeats of amphipathic α-helices.[14] It has been demonstrated that the helical segments are located in the N-terminal domain of the apoA-I molecule spanning residues 1−187[15,16] and folded into a dynamic four-helix bundle.[17−19] The C-terminal region spanning the remainder of the molecule forms an intrinsically disordered domain that mediates high-affinity lipid binding, coupled with α-helix formation by this region.[20,21] The dynamic nature of apoA-I structure and its relative instability presumably explain the facile ability of its helical segments to unfold and refold during HDL formation and maturation.[3]

The apoA-I molecules in HDL particles are also in a highly dynamic state,[22−24] so that apoA-I can dissociate from the HDL surface and exchange between different HDL particles.[25,26] This exchangeable pool is a precursor of lipid-poor apoA-I or preβ-HDL,[4,27,28] and the cycling of apoA-I molecules on and off HDL particles occurs during HDL metabolism. Such dynamic

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association and/or dissociation behaviors of apoA-I on the HDL surface originate from the great flexibility of the apoA-I molecule, which allows it to adopt multiple lipid-bound conformations on HDL particles of different sizes and shapes.[29−32]

Previous studies of site-directed fluorescence-labeled[26] or spin-labeled[33] apoA-I demonstrated that apoA-I indeed spontaneously and rapidly exchanges between HDL-associated and lipid-free pools and that this apoA-I exchangeability is an important aspect of HDL function in cardiovascular disease. In this study, we analyzed the kinetics and thermodynamics of apoA-I exchange between discoidal HDL-bound and lipid-free forms using apoA-I variants labeled with the fluorescence probe 7-nitrobenz-2-oxa-1,3-diazole (NBD). Our results demonstrated that HDL−apoA-I exchange is an enthalpically unfavorable but entropically favorable process in which the transition of lipid-free apoA-I to an HDL-bound state is rate-limiting. Thus, apoA-I variants with impaired lipid binding abilities exhibit reduced exchangeability.

EXPERIMENTAL PROCEDURES

ApoA-I Proteins. The mutations in apoA-I to introduce R173C (Milano), F229C, F225L/F229A/Y236A, F225L/F229C/Y236A, and L230P mutations or delete E235 (Nichinan) were made using the QuikChange site-directed mutagenesis kit (Stratagene). Wild-type (WT) apoA-I and engineered mutants were expressed in *Escherichia coli* as thioredoxin fusion proteins and isolated and purified as described previously.[18,34] The apoA-I variants progressively lacking different regions along the molecule (Δ1−43, Δ44−65, Δ123−166, Δ191−220, and Δ223−243) have also been described previously.[18,35] Cleavage of the thioredoxin fusion protein with thrombin leaves the target apoA-I with two extra amino acids, Gly and Ser, at the N-terminus. The apoA-I preparations were at least 95% pure as assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. In all experiments, apoA-I variants were freshly dialyzed from 6 M guanidine hydrochloride (GdnHCl) and/or a 1% β-mercaptoethanol solution into the appropriate buffer before use.

Reconstituted HDL Preparation. Reconstituted discoidal HDL was prepared from 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and apoA-I by cholate dialysis and purified by gel filtration chromatography as described previously.[36,37] Spherical HDL was prepared by cosonication of POPC, triolein, and apoA-I.[38] Briefly, POPC (1 mg) and triolein (1 mg) in 2.0 mL of Tris buffer [10 mM Tris, 150 mM NaCl, and 0.02% NaN_3 (pH 7.4)] were initially sonicated using a TOMY ultrasonic disruptor with tapered microtip for 15 s at 27 °C under nitrogen; 2.0 mL of the apoA-I solution (1 mg/mL) was added to the lipid suspension and incubated for 30 min at 37 °C, and then the protein/lipid mixture was sonicated for 1 min at 40 °C three times punctuated by cooling on ice for 30 s. The HDL particles were isolated by sequential ultracentrifugation adjusted over a density range of 1.063−1.21 g/mL in a Beckman Optima TL ultracentrifuge with a TLA-110 rotor at 53000 rpm for 12 h at 4 °C. The average compositions (molar ratio) of discoidal or spherical HDL particles were 1/73 for apoA-I/POPC particles or 1/19/20 for apoA-I/POPC/triolein particles.

Fluorescence Labeling. A 2 mg/mL stock solution of ATTO565 NHS ester (Sigma-Aldrich) or a 10 mM stock solution of IANBD amide (Thermo Fisher Scientific) in DMSO was added to apoA-I so that final molar ratios of probe to protein were 1/1 or 10/1, respectively. The reaction mixtures were incubated at room temperature for 1 h (ATTO565 NHS ester) or overnight (IANBD amide) in the dark. Unreacted ATTO565 NHS ester or IANBD amide was removed by extensive dialysis at 4 °C in Tris buffer (pH 7.4). The degree of labeling was determined using an extinction coefficient for ATTO565 of 1.5×10^5 M^{-1} cm^{-1} at 563 nm or for NBD of 23500 M^{-1} cm^{-1} at 480 nm.

Gel Filtration Chromatography. Lipid-free ATTO565-labeled apoA-I or ATTO565-labeled apoA-I/POPC discs incorporating NBD-PE (0.1 mol % to POPC) were loaded on a Superdex 200 column (60 cm × 1.6 cm) and eluted at a rate of 1 mL/min with Tris buffer (pH 7.4) using a BioLogic DuoFlow system (Bio-Rad). The elution profiles were monitored by either the absorbance at 280 nm or the fluorescences of ATTO565-labeled apoA-I and NBD-PE with excitation and emission wavelengths of 563 and 592 nm and 463 and 536 nm, respectively. The Stokes diameter was determined by comparing the K_{av} value given by the following equation with those of standard proteins with known diameters: $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_0, V_t, and V_e are the void, column, and elution volumes, respectively.

Circular Dichroism (CD) Spectroscopy. Far-UV CD spectra were recorded from 185 to 260 nm at 25 °C using a Jasco J-1500 spectropolarimeter. The apoA-I solutions (50 μg/mL) in Tris buffer (pH 7.4) were subjected to CD measurements in a 2 mm quartz cuvette, and the results were corrected by subtracting the buffer baseline. The α-helical content was derived from the molar ellipticity at 222 nm ($[\theta]_{222}$) using the equation % α-helix = $[(-[\theta]_{222} + 3000)/(36000 + 3000)] \times 100$. For monitoring chemical denaturation, the equilibrium constant of denaturation (K_D) at various concentrations of GdnHCl was calculated from the change in ellipticity values at 222 nm.[39] The Gibbs free energy of denaturation, ΔG_D°, the midpoint of denaturation, $D_{1/2}$, and m value, which reflects the cooperativity of denaturation in the transition region, were determined by the linear equation $\Delta G_D = \Delta G_D^\circ - m[\mathrm{GdnHCl}]$, where $\Delta G_D = -RT \ln K_D$.

Fluorescence Measurements. Fluorescence measurements were taken with a Hitachi F-4500 fluorescence spectrophotometer in Tris buffer (pH 7.4). The NBD emission fluorescence of proteins at a concentration of 12 μg/mL was recorded from 500 to 650 nm using an excitation wavelength of 480 nm. The kinetics of exchange of apoA-I between HDL-bound and lipid-free states were measured by monitoring the time-dependent decrease in NBD fluorescence intensity for NBD-labeled apoA-I variant, in which the reduction in NBD fluorescence reflects the transformation of NBD-labeled apoA-I from the HDL-bound to the lipid-free state. Discoidal or spherical reconstituted HDL particles were mixed with lipid-free apoA-I at various temperatures to a final molar ratio of 1/2. The relative decrease in NBD fluorescence intensity was calculated according to the equation $(F - F_{min})/(F_0 - F_{min})$, where F and F_0 are fluorescence intensities for NBD-labeled apoA-I in the presence and absence of lipid-free apoA-I, respectively, and F_{min} represents the theoretical fluorescence intensity after complete exchange of NBD-labeled apoA-I that was calculated from fluorescence intensities of NBD-labeled apoA-I in the HDL-bound (F_0) and lipid-free states. The data were analyzed by a one-phase exponential decay curve to estimate the kinetic constant k of the exchange reaction at various temperatures. Thermodynamic parameters were determined from the Eyring equations

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$$\ln\left(\frac{k}{T}\right) = -\frac{\Delta H^*}{R}\frac{1}{T} + \frac{\Delta S^*}{R} + \ln\left(\frac{k_B}{h}\right) \quad (1)$$

$$\Delta G^* = -RT \ln\left(\frac{hk}{k_B T}\right) \quad (2)$$

where k_B and h are the Boltzmann and Planck constants, respectively. The activation enthalpy (ΔH^*) and entropy (ΔS^*) of exchange were obtained from the slope and y-intercept of the linear plot according to eq 1, respectively. The activation Gibbs free energy (ΔG^*) was calculated from k according to eq 2 or from ΔH^* and ΔS^* according to $\Delta G^* = \Delta H^* - T\Delta S^*$.

RESULTS

Characterization of Reconstituted Discoidal HDL. Figure 1A shows a gel filtration elution profile of reconstituted apoA-I/POPC discoidal HDL. Overlapping of peaks of absorbance at 280 nm mainly monitoring apoA-I protein and NBD-PE fluorescence monitoring phospholipid indicated the existence of a homogeneous preparation of discoidal HDL particles (the approximate hydrodynamic diameter was calculated to be 10 nm). Dynamic light scattering measurements also demonstrated a homogeneous distribution of discoidal particles with a diameter of 10 nm (Figure S1 of the Supporting Information). As shown in Figure 1B, CD analysis demonstrated that lipid-free and discoidal apoA-I F229C-NBD have α-helical contents of 40 ± 2 and 86 ± 4%, respectively (Table 1). Although the α-helical content in the lipid-free form is somewhat low, there are no significant differences from previously reported values.[20,29,37] The GdnHCl denaturation curve of discoidal HDL-bound apoA-I was shifted to concentrations of GdnHCl much higher than those seen with lipid-free apoA-I (Figure 1C), indicating a higher degree of structural integrity of disc-bound apoA-I than of the lipid-free form. However, the Gibbs free energy of denaturation (ΔG_D°) that reflects equilibrium thermodynamic stability is similar in disc-bound and lipid-free forms (Table 1), consistent with the notion that HDL particles are stabilized by kinetic factors, not by energy barriers in the thermodynamic equilibrium.[40]

Table 1. α-Helical Contents and Parameters of GdnHCl Denaturation for ApoA-I F229C-NBD in the Lipid-Free State and on POPC Discs at 25 °C

apoA-I F229C-NBD	α-helical content (%)	ΔG_D° (kJ/mol)	m [kJ (mol of apoA-I)$^{-1}$ (mol of GdnHCl)$^{-1}$]	$D_{1/2}$ (M)
lipid-free	40 ± 2	13.0 ± 0.5	13.7 ± 0.5	1.0 ± 0.1
POPC discs	86 ± 4	13.7 ± 0.5	4.0 ± 0.2	3.4 ± 0.3

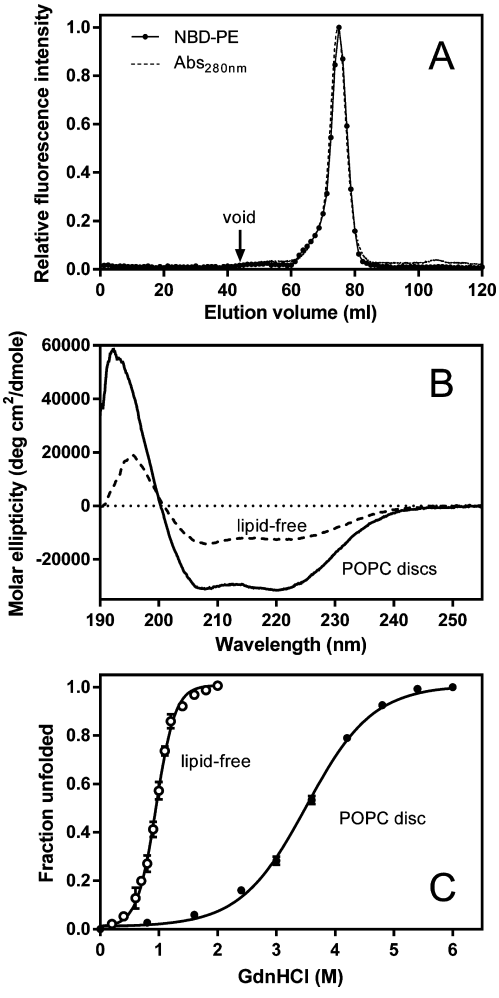

Figure 1. Characterization of apoA-I/POPC discs. (A) Gel filtration profile of apoA-I/POPC discs labeled with 0.1 mol % NBD-PE on a Superdex 200 column monitored by absorbance at 280 nm or NBD fluorescence. (B) Far-UV CD spectra of apoA-I F229C-NBD in the lipid-free state (---) or on POPC discs (—). (C) GdnHCl denaturation curves for lipid-free (○) or POPC disc-bound (●) apoA-I monitored by the change in molar ellipticity at 222 nm.

ApoA-I Exchange Monitored by Gel Filtration. We next investigated whether apoA-I can be freely exchanged between discoidal HDL-bound and lipid-free states in the absence of HDL remodeling enzymes.[26] To this end, reconstituted WT apoA-I/POPC discs labeled with NBD-PE were prepared, and the mixture with a 2-fold excess of ATTO565-labeled lipid-free apoA-I was subjected to gel filtration chromatography. Before incubation, both discoidal HDL reported by NBD fluorescence and lipid-free apoA-I reported by ATTO565 fluorescence showed separate single peaks corresponding to hydrodynamic diameters of approximately 10 and 7 nm, respectively (Figure 2A). After incubation for 4 h at 37 °C, a significant amount of ATTO565 fluorescence was detected at the elution volume corresponding to discoidal HDL, indicating that apoA-I exchange occurs without alteration of HDL particle size in the steady state (Figure 2B). In addition, the POPC/apoA-I ratio of the discoidal HDL fraction did not significantly change after incubation (data not shown), consistent with the occurrence of apoA-I exchange rather than apoA-I accumulation. By correcting for the difference in the ATTO565 fluorescence intensity of apoA-I in lipid-free and disc-bound forms (Figure 2B inset), we calculated the amount of ATTO-labeled apoA-I to be distributed in HDL-bound and lipid-free pools at an approximately 1/2 ratio. This finding agrees with the assumption that if all apoA-I species have equal affinities for disc-bound and lipid-free fractions, one-third of ATTO-apoA-I is in the HDL-bound pool and two-thirds in the lipid-free pool at equilibrium. These results demonstrate that the presence of excess lipid-free apoA-I causes efficient displacement of HDL-bound apoA-I.[26,33]

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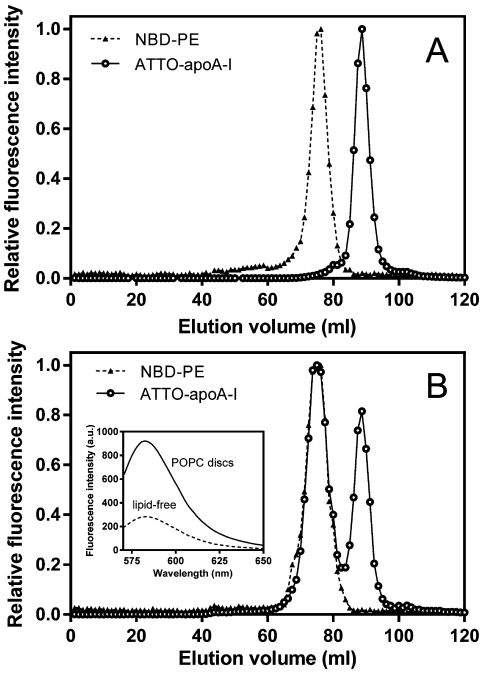

Figure 2. ApoA-I exchange monitored by gel filtration. (A) Gel filtration profiles of lipid-free apoA-I labeled with ATTO565 and apoA-I/POPC discs labeled with NBD-PE. (B) Gel filtration profile of apoA-I/POPC discs after incubation for 4 h with a 2-fold excess of lipid-free ATTO-apoA-I at 37 °C. The inset shows fluorescence emission spectra of ATTO-apoA-I in the lipid-free state (---) or on POPC discs (—).

ApoA-I Exchange Monitored by NBD Fluorescence. To measure the kinetics of the apoA-I exchange reaction between HDL-bound and lipid-free states, we used the apoA-I F229C-NBD variant in which the C-terminal helical region was labeled with an environment-sensitive fluorescent probe NBD. In our earlier studies, pyrene labeling at the same site allowed us to monitor the lipid binding behaviors of the C-terminal domain of apoA-I because of the significant increase in pyrene fluorescence upon binding to lipid particles or HDL.[22,29] In this study, we evaluated the exchange kinetics of NBD-labeled apoA-I between HDL-bound and lipid-free states by monitoring the decrease in NBD fluorescence upon dissociation of NBD-labeled apoA-I from HDL. We note that the Cys replacement and/or fluorescence labeling at F229 has little or no effect on the structure, stability, and lipid interaction of apoA-I.[29]

Figure 3A shows the change in NBD fluorescence emission spectra of apoA-I F229C-NBD/POPC discs upon incubation with a 2-fold excess of lipid-free WT apoA-I at 37 °C. Disc-bound NBD-apoA-I exhibited NBD fluorescence >10-fold greater than that of the lipid-free state, and time-dependent decreases in NBD fluorescence were observed upon dissociation of NBD-labeled apoA-I from discoidal HDL. We note that such decreases in NBD fluorescence are not due to spontaneous dissociation of apoA-I from HDL as opposed to exchange with lipid-free apoA-I because there was virtually no change in NBD fluorescence when the HDL particles were incubated in buffer (Figure 3B). In addition, incubation of apoA-I F229C-NBD/POPC discs with a 2-fold excess of WT apoA-I/POPC discs did not result in a decrease in NBD

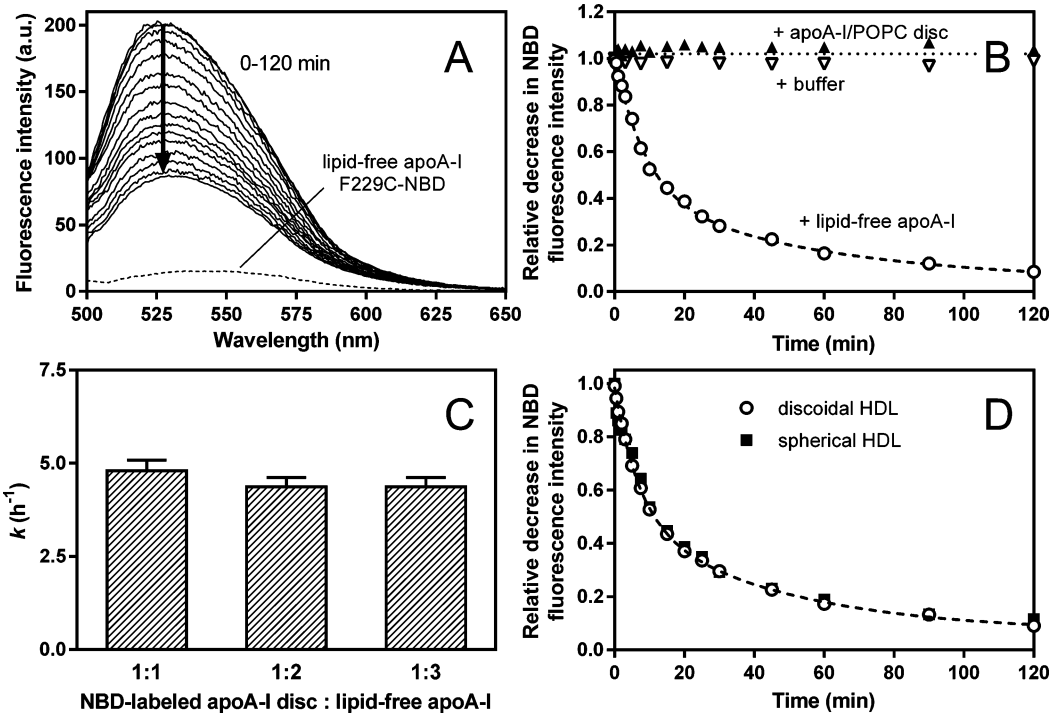

Figure 3. ApoA-I exchange monitored by NBD fluorescence. (A) Change in the NBD fluorescence intensities of apoA-I F229C-NBD/POPC discs upon incubation with a 2-fold excess of lipid-free WT apoA-I at 37 °C. (B) Time courses of the relative decrease in NBD fluorescence intensities of apoA-I F229C-NBD/POPC discs upon exchange with a 2-fold excess of lipid-free WT apoA-I (○) or WT apoA-I/POPC discs (▲) at 37 °C. It should be noted that there was no change in NBD fluorescence upon incubation in buffer (▽). (C) Effect of lipid-free WT apoA-I concentration on apoA-I exchange rates for apoA-I F229C-NBD/POPC discs at 37 °C. (D) Comparison of the NBD fluorescence change for apoA-I F229C-NBD/POPC discs (○) or apoA-I F229C-NBD/POPC/triolein spherical HDL particles (■) upon exchange with a 2-fold excess of lipid-free WT apoA-I at 37 °C.

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fluorescence, despite the occurrence of apoA-I exchange not only between HDL-bound and lipid-free states but also between HDL particles (Figure S2 of the Supporting Information). These results indicate that the change in NBD fluorescence reflects the exchange of NBD-apoA-I between HDL-bound and lipid-free states. The estimated half-time of exchange from Figure 3B (0.17 h) is much shorter than the previously reported result (0.94 h).[26] One possible reason for this discrepancy is that the replacement of Trp with Phe residues used in the previous study may have impacted self-association and lipid interaction behaviors of apoA-I.[41]

As shown in Figure 3C, the rate of apoA-I exchange derived from fitting to a monoexponential decay model does not depend on lipid-free apoA-I concentration, indicating that the frequency of diffusional collisions between disc-bound and lipid-free apoA-I has no influence on the apoA-I exchange kinetics. In addition, the exchange rates of NBD-apoA-I are similar between discoidal and spherical HDL particles (Figure 3D), indicating that the change in particle shape from disc to sphere in HDL remodeling does not affect the apoA-I exchange reaction.

Effects of Deletions or Mutations in ApoA-I on Exchange Kinetics. To investigate which region of the apoA-I molecule is important in terms of its exchange ability, we compared the exchange kinetics of disc-bound apoA-I F229C-NBD upon incubation with lipid-free apoA-I deletion variants lacking different regions along the molecule.[18,35] We note that although the apoA-I conformation in discoidal HDL would be affected by the deletions of different regions, all deletion variants used in this study have an ability to form HDL particles to some extent.[20] As shown in Figure 4A, deletions of N-terminal (residues 1−43 or 44−65) or C-terminal (residues 191−220 or 223−243) regions that have strong lipid binding affinity[20,39] greatly reduced the exchange kinetics of apoA-I, whereas deletion of central residues 123−166 had a relatively small effect. Importantly, deletion of residues 223−243 almost completely inhibited the ability of apoA-I to exchange, indicating that the C-terminal lipid binding region plays a critical role not only in binding to HDL particles[22,24] but also in the ability of apoA-I to exchange between HDL particles.

We next examined the effect of C-terminal mutations that reduce lipid binding affinity on the exchange behavior of apoA-I. Figure 4B shows the exchange kinetics of disc-bound apoA-I F229C-NBD with the lipid-free apoA-I C-terminal variants F225L/F229A/Y236A,[34] L230P,[42] and Nichinan (ΔE235)[43] as well as the Milano (R173C) variant.[44] These C-terminal mutations reduce the lipid binding affinity of apoA-I by decreasing hydrophobicity or disrupting α-helical structure in the C-terminal region. As listed in Table 2, the exchange rates for the apoA-I C-terminal variants were much slower than those of WT apoA-I, and as a result, activation Gibbs free energies of exchange were higher for the C-terminal variants. Importantly, the order of decrease in exchange rates or increase in activation Gibbs free energies (Nichinan < F225L/F229A/Y236A < L230P) agrees with the order of decrease in 1,2-dimyristoylphosphatidylcholine (DMPC) clearance efficiency,[34,42,43] indicating that the lipid binding activity of apoA-I is critical in its exchangeability between HDL-bound and lipid-free states.[26] In agreement with this, the Milano variant that has comparable ability to solubilize DMPC vesicles to WT apoA-I[44] exhibited exchange kinetics similar to those of WT apoA-I (Figure 4B).

To further explore the mechanism of apoA-I exchange, we examined the exchange behavior of apoA-I/POPC discs

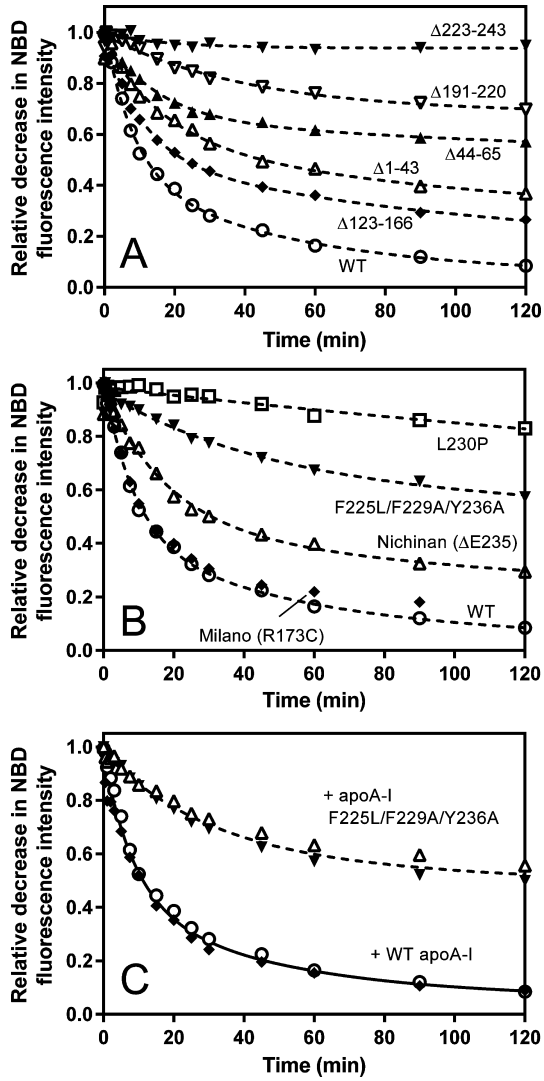

Figure 4. Effect of the apoA-I truncations or mutations on exchange kinetics. (A) Time course of the relative decrease in NBD fluorescence intensities of apoA-I F229C-NBD/POPC discs upon exchange with apoA-I variants lacking different regions along the molecule at 37 °C: apoA-I WT (○), Δ1−43 (△), Δ44−65 (▲), Δ123−166 (◆), Δ191−220 (▽), and Δ223−243 (▼). (B) Time course of the relative decrease in NBD fluorescence intensities of apoA-I F229C-NBD/POPC discs upon exchange with apoA-I variants at 37 °C: WT apoA-I (○), R173C (◆), L230P (□), ΔE235 (△), and F225L/F229A/Y236A (▼). (C) Comparison of apoA-I exchange kinetics between apoA-I F229C-NBD/POPC discs and apoA-I F225L/F229C-NBD/Y236A/POPC discs at 37 °C. ApoA-I F229C-NBD/POPC discs were incubated with a 2-fold excess of lipid-free WT apoA-I (○) and apoA-I F225L/F229A/Y236A (▼), while apoA-I F225L/F229C-NBD/Y236A/POPC discs were incubated with a 2-fold excess of lipid-free WT apoA-I (◆) and apoA-I F225L/F229A/Y236A (△).

composed of the F225L/F229C-NBD/Y236A variant. Despite its reduced lipid binding ability, the apoA-I F225L/F229C-NBD/Y236A variant bound to discoidal HDL exhibited exchange kinetics comparable to those of apoA-I F229C-NBD/POPC discs upon incubation with lipid-free WT apoA-I (solid line in Figure 4C). Thus, alteration of the lipid affinity of the HDL-associated apoA-I has no influence on the kinetics of exchange. In addition, apoA-I F225L/F229C-NBD/Y236A/POPC discs and apoA-I F229C-NBD/POPC discs both showed similar but slower exchange kinetics with the lipid-

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Table 2. Kinetic and Thermodynamic Parameters for Exchange of ApoA-I Variants between HDL-Bound and Lipid-Free States at 37 °C

	k (h^{-1})	ΔG^* (kJ/mol)[a]
WT	4.1 ± 0.3	94 ± 0.2
ΔE235	2.5 ± 0.2	95 ± 0.3
F225L/F229A/Y236A	1.1 ± 0.2	97 ± 0.5
L230P	0.10 ± 0.3	103 ± 4

[a] ΔG^* was calculated from k according to eq 2.

free apoA-I F225L/F229A/Y236A variant (dashed line in Figure 4C). This finding shows that altering the lipid affinity of the lipid-free apoA-I in solution modifies the exchange kinetics. Taken together, these results indicate that the lipid binding ability of apoA-I affects the kinetics of exchange of apoA-I between discoidal HDL-bound and lipid-free states via the association process of lipid-free apoA-I, not by the dissociation of HDL-bound apoA-I.

Thermodynamic Aspects of ApoA-I Exchange. To understand the thermodynamics of apoA-I exchange between HDL-bound and lipid-free states, the kinetics were determined at different temperatures. Figure 5A demonstrates that the exchange kinetics of disc-bound apoA-I F229C-NBD with lipid-free WT apoA-I became faster with an increasing incubation temperature. From the linear relationship between $\ln(k/T)$ and $1/T$ based on the Eyring equation (Figure 5B), activation enthalpy ΔH^* and entropy ΔS^* for apoA-I exchange were obtained, and the values are listed with activation Gibbs free energy ΔG^* in Table 3. These parameters indicate that apoA-I exchange between HDL-bound and lipid-free pools involves a transition state that is enthalpically unfavorable but entropically favorable. That is, the high enthalpy barrier in the transition state is offset by an increase in entropy with apoA-I exchange. Interestingly, such an entropically favorable process was also reported for the exchange of phospholipids between discoidal HDL particles.[45]

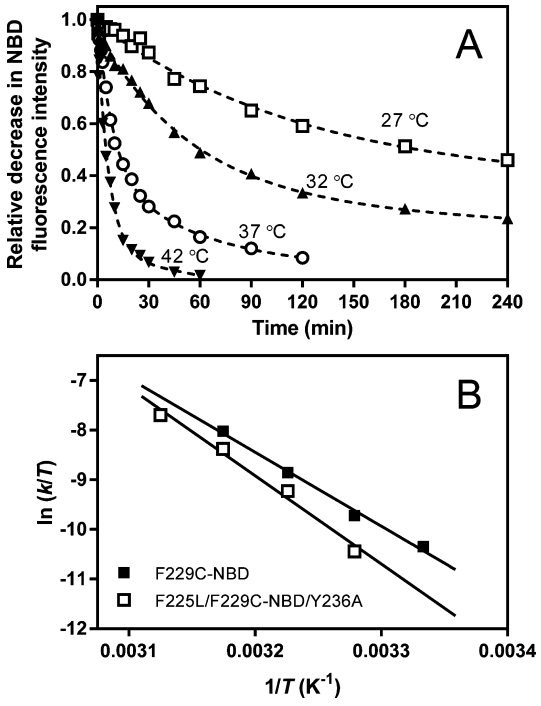

Figure 5. Thermodynamic analysis of apoA-I exchange kinetics. (A) Time course of the relative decrease in NBD fluorescence intensities of apoA-I F229C-NBD/POPC discs upon exchange with lipid-free WT apoA-I at various temperatures. (B) Eyring plots for exchange kinetics of apoA-I-POPC discs of the F229C-NBD (■) and F225L/F229C-NBD/Y236A (□) variants.

Table 3. Thermodynamic Parameters for the Exchange Kinetics of ApoA-I Variants between HDL-Bound and Lipid-Free States at 37 °C

	ΔH^* (kJ/mol)[a]	ΔS^* (J mol^{-1} K^{-1})[a]	ΔG^* (kJ/mol)[b]
F229C-NBD	124 ± 8	94 ± 8	94
F225L/F229C-NBD/Y236A	147 ± 8	167 ± 18	96

[a] ΔH^* and ΔS^* were obtained from the slope and y-intercept of the linear plot, respectively, according to eq 1. [b] ΔG^* was calculated from ΔH^* and ΔS^* according to $\Delta G^* = \Delta H^* - T\Delta S^*$.

Figure 5B and Table 3 also show the Eyring plot and thermodynamic parameters of exchange for the C-terminal variant, apoA-I F225L/F229A/Y236A, obtained from the exchange of apoA-I F225L/F229C-NBD/Y236A/POPC discs with lipid-free apoA-I F225L/F229A/Y236A at various temperatures. As listed in Table 3, this C-terminal mutation significantly increases both the activation enthalpy and entropy, resulting in an increased activation Gibbs free energy and reduced rate of apoA-I exchange. Thus, the impaired exchange ability of the C-terminal variants of apoA-I (Figure 4) appears to arise from the higher activation enthalpy of the transition state.

DISCUSSION

Molecular Mechanism of ApoA-I Exchange. The conformational plasticity and molten globule characteristics of apoA-I[46] allow it to have a flexible and adaptable lipid-bound conformation on different HDL subclasses. Hydrogen−deuterium exchange experiments demonstrated that the multiple α-helices in apoA-I bound to discoidal and spherical HDL particles have stabilities in the range of 3−5 kcal/mol, consistent with the apoA-I molecules being in a highly dynamic state with helical segments unfolding and refolding in seconds.[23,37] On the basis of the two-domain tertiary structure of apoA-I, we previously demonstrated that apoA-I binds to a lipid surface in a two-step manner: α-helices in the C-terminal domain interact with the lipid surface first, followed by the opening of the N-terminal helix bundle to create more helix−lipid interactions.[18,21] Given the reversibility of lipid binding of apoA-I,[22,27,47] apoA-I adopts multiple lipid-bound conformations in which the N-terminal helix bundle is either in or out of contact with lipids.[29,32] Additionally, some of a central part of the apoA-I molecule can be excluded from the lipid surface leaving both the N- and C-terminal helices attached[32,47] because of the lower lipid affinity of α-helices in the central region compared to the that of N- or C-terminal helices.[48] For discoidal HDL, a central apoA-I segment spanning residues 125−158 exhibits bimodal hydrogen−deuterium exchange kinetics, indicating that this segment can adopt an interconverting helix-disordered loop conformation.[37] In agreement with this finding, a study of site-directed Trp fluorescence and spin-labeled electron paramagnetic resonance spectroscopies determined that residues 134−145 in apoA-I are loosely lipid-associated in discoidal HDL particles of different sizes.[30]

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The highly dynamic and flexible conformations of apoA-I on the HDL particles give rise to a labile, easily dissociable pool;[25,26] such apoA-I molecules are precursors of circulating lipid-poor apoA-I or preβ-HDL.[4,28]

In light of the finding that the rate of apoA-I exchange is independent of the collision frequency between HDL-bound and lipid-free forms (Figure 3C), it is plausible that apoA-I exchange occurs in a manner similar to the aqueous diffusion mechanism of cholesterol exchange in which the rate-limiting step is high-energy transference of a hydrophobic cholesterol molecule in a partially desorbed transition state into the aqueous phase.[49] Thus, the conformational transition of apoA-I from a stable HDL-bound helical state into a partially dissociated, disordered state would be rate-limiting for apoA-I exchange. We note that, at first glance, this notion seems to contradict the finding that the reduction of the lipid binding ability of apoA-I affects the rate of association of lipid-free apoA-I with HDL but not the rate of dissociation of HDL-bound apoA-I (Figure 4C). However, because apoA-I does not dissociate spontaneously from discoidal HDL particles without exchange with lipid-free apoA-I, it seems that exchange involves displacement of partially dissociated apoA-I from the HDL particle surface by the lipid-free apoA-I. In support this of idea, deletions of the N- or C-terminal lipid binding regions greatly impair the exchange ability of apoA-I (Figure 4A). In addition, because α-helix formation contributes enthalpically to the energetics of lipid binding of apoA-I,[20,50] it follows that the partially unfolded conformation of HDL-bound apoA-I in the transition state produces the high enthalpy barrier of apoA-I exchange, as shown in this study (Table 3). In this regard, the finding that the exchange rates of NBD-apoA-I are similar between discoidal and spherical HDL particles (Figure 3D) is consistent with helix stabilities and dynamics of the apoA-I molecules being similar on both HDL particles.[23]

Physiological Significance. There has been great interest in recent years in understanding what qualities of HDL are critical for the reverse cholesterol transport pathway.[49] It has been recently demonstrated that cellular cholesterol efflux correlates significantly with the concentration of the ABCA1 substrate lipid-poor apoA-I or preβ-HDL in the serum, rather than simply with the serum HDL cholesterol level.[51] Given that apoA-I molecules in the readily dissociable pool of apoA-I on HDL particles are likely to be precursors of preβ-HDL, the ability of apoA-I to transfer from HDL-bound to lipid-free forms would be a clinically relevant measure of HDL function.[26,33] Indeed, it has been shown that the apoA-I exchangeability of HDL isolated from human subjects, as quantified using electron paramagnetic resonance spectroscopy, is markedly reduced when the subject carries at least one risk factor for cardiovascular disease.[33] In addition, oxidation of apoA-I by myeloperoxidase that occurs in human atheroma was shown to impair the ability of apoA-I to promote ABCA1-mediated cholesterol efflux[52,53] as well as exchange between HDL-bound and lipid-free states.[26] It should be noted that because we are monitoring the exchange rather than net movement of apoA-I, this process does not obviously contribute to increasing the concentration of lipid-free (poor) apoA-I, which is the substrate for ABCA1. However, altered exchangeability would be expected to affect HDL particle rearrangement and metabolism. Thus, it appears that the exchangeability of apoA-I on HDL particles is an important indicator for quantifying HDL functionality.

In conclusion, the kinetic and thermodynamic analyses of apoA-I exchange in this study demonstrate that apoA-I can rapidly and spontaneously exchange between HDL-bound and lipid-free pools through an enthalpically unfavorable but entropically favorable activation process, in which reduced lipid binding affinity impairs the ability of apoA-I to exchange. Because the altered exchangeability of HDL-bound apoA-I would affect the remodeling and rearrangement of HDL particles during metabolism in plasma, our results provide a mechanistic basis for understanding the exchangeable nature of apoA-I lipid binding and associated modulation of its antiatherogenic functions.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

apoA-I, apolipoprotein A-I; CD, circular dichroism; DMPC, 1,2-dimyristoylphosphatidylcholine; GdnHCl, guanidine hydrochloride; HDL, high-density lipoprotein; NBD, 7-nitrobenz-2-oxa-1,3-diazole; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; WT, wild type.

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