Plasma Factors Required for Human Apolipoprotein A-II Dimerization†

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ABSTRACT: Although plasma high-density lipoproteins (HDL) have been implicated in several cardioprotective pathways, the physiologic role of apolipoprotein (apo) A-II, the second most abundant of the HDL proteins, remains ambiguous. Human apo A-II is distinguished from most other species by a single cysteine (Cys6), which forms a disulfide bond with other cysteine-containing apos. In human plasma, nearly all apo A-II occurs as disulfide-linked homodimers of 17.4 kDa. Although dimerization is an important determinant of human apo A-II metabolism, its mechanism and the plasma and/or cellular sites of its dimerization are not known. Using SDS−PAGE and densitometry we investigated the kinetics of apo A-II dimerization and observed a slow ($t_{1/2} = \sim$10 days), second-order process in Tris-buffered saline. In 3 M guanidine hydrochloride, which disrupts apo A-II secondary structure and self-association, the rate was 3-fold slower. In contrast, lipid surfaces that promote apo A-II α-helix formation and lipophilic interaction profoundly enhanced the rate. Reassembled HDL increased the second-order rate constant k_2 by 7500-fold, unilamellar 1-palmitoyl-2-oleoylphosphatidylcholine vesicles increased k_2 850-fold, and physiological concentrations of human serum albumin increased k_2 220-fold. Thus, while dimerization of apo A-II in aqueous buffer is too slow to account for the high fraction of dimer found in plasma, lipids and proteins "catalyze" dimer formation, a process that could occur either intracellularly prior to secretion or in the plasma compartment following secretion. These data suggest that formation of disulfide links within or between polypeptide chains can be controlled, in part, by coexisting lipids and proteins.

The incidence of coronary artery disease in humans is inversely correlated with plasma levels of high-density lipoprotein (HDL)[1]−cholesterol (*1*). HDL has several atheroprotective activities. HDL is the vehicle for reverse cholesterol transport (*2*, *3*), HDL inhibits LDL oxidation (*4*), and HDL is antiinflammatory (*5*). It is difficult to increase levels of HDL by diet or drugs (*1*). Identification of the mechanisms for atheroprotection by HDL could reveal new drug targets. HDL are spherical, heterogeneous particles comprising nearly equal amounts of lipid and protein with a core of neutral lipids surrounded by a surface monolayer of apolipoproteins (apos), cholesterol, and polar lipids, primarily phosphatidylcholine (*6*−*8*). Apos A-I and A-II are the two major HDL proteins. Human plasma contains HDL particles with only apo A-I (LpAI) or both apo A-I and apo A-II (LpAI/AII). The relative amounts of apo A-I and apo A-II determine HDL particle size, lipid composition, and biological activity (*9*−*18*). HDL apos, which are water-soluble and exchangeable (*19*, *20*), are removed as monomers by the renal cubilin receptor (*21*, *22*).

Lipid-free apo A-II is an in vitro acceptor of cholesterol and phospholipid from cells expressing the ABCA-1 transporter to form nascent HDL particles (*23*); in vivo it is more likely that apo A-II promotes reverse cholesterol transport indirectly by displacing apo A-I from mature HDL (*24*). Apo A-II inhibits the rate of HDL remodeling by LCAT, CETP, and hepatic and lipoprotein lipases (*25*−*29*). Apo A-II has some interesting physiological effects that have not been mechanistically linked to its physical properties. Plasma apo A-II levels correlate with free fatty acid and triglyceride levels (*27*, *30*, *31*). Deletion of apo A-II in mice decreases HDL−cholesterol (*17*); however, apo A-II deficiency in humans does not affect lipid and lipoprotein profiles (*32*).

Apo A-II is synthesized by hepatocytes (*33*) as a primary translation product containing leader and pro sequences and a mature peptide of 77 amino acid residues (*34*, *35*). Human and mouse apo A-II have different biological effects (*27*) and amino acid sequences that differ by 45% (*36*). Human apo A-II differs from that of other species by the appearance of Cys6, which forms disulfide-linked homodimers and heterodimers with apos D and E (*37*, *38*). Apo A-II in most other species lacks cysteine and is an obligate monomer (*39*). The presence of two peptide chains in dimeric apo A-II would be expected to make it more lipophilic than the monomer, giving the dimer distinctive physical and physiological properties (*40*). Transfection of mice with dimeric human apo A-II was associated with smaller HDL particles

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[1] Abbreviations: apo, apolipoprotein; apo A-I, A-II, B-100, D, and E, apolipoproteins A-I, A-II, B-100, D, and E, respectively; CETP, cholesteryl ester transfer protein; DTT, dithiothreitol; GuHCl, guanidine hydrochloride; HDL, high-density lipoprotein; HSA, human serum albumin; LCAT, lecithin−cholesterol acyltransferase; LDL, low-density lipoprotein; PC, phosphatidylcholine; PDI, protein disulfide isomerase; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; TBS, Tris-buffered saline; RCM apo A-II, reduced and carboxymethylated apo A-II; rHDL, reassembled HDL; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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and decreased plasma HDL concentrations (*27*, *41*); transfection with a variant of human apo A-II (Cys6 → Ser6) or mouse apo A-II (Asp6) did not affect HDL particle size or plasma concentrations (*42*). Monomeric apo A-II rHDL is a better acceptor of cellular cholesterol from rat Fu5AH hepatoma cells than is dimeric apo A-II rHDL (*40*).

In transgenic mice with either human dimeric apo A-II or mutated monomeric apo A-II (Cys6 → Ser6), ~90% of apo A-II was bound to lipoprotein whereas the remaining ~10% was in an aqueous phase; small apo A-II-only HDL coexisting with normal HDL are likely formed by a physical displacement of apo A-I by high apo A-II concentrations (*42*, *43*). Both dimeric and monomeric reduced and carboxymethylated (RCM) apos A-II displace apo A-I from HDL, giving apo A-II-only HDL; dimeric but not monomeric A-II affects HDL remodeling from larger to smaller particle size (*44*). The majority (>96%) of apo A-II in human plasma occurs as a disulfide-linked homodimer. However, in preliminary experiments with isolated cysteine-containing apolipoproteins (apos D, E, B-100, and A-II), we were unable to observe either homo- or heterodimer formation (*37*). Given the apo A-II plasma residence time of 5.25 days (*45*), our preliminary data suggested that little if any apo A-II would be present as a dimer. Therefore, we have studied the kinetics of apo A-II dimerization with the goal of identifying the molecular basis of the high plasma concentrations of apo A-II dimers.

EXPERIMENTAL PROCEDURES

Reagents and Assays. The standard buffer used in these studies was Tris-buffered saline (TBS) containing 10 mM Tris, 100 mM NaCl, 1 mM EDTA, and 1 mM NaN_3, adjusted to pH 7.4 with HCl. Protein concentrations were determined by modified Lowry assay (*46*) or by absorbance, using extinction coefficients of $\epsilon = 0.67$ at $\lambda = 276$ nm for apo A-II and $\epsilon = 1.1$ at $\lambda = 280$ nm for apo A-I (*47*). Concentrations of phosphatidylcholine (PC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (Avanti Polar Lipids, Inc.) were determined using the phospholipid B assay kit (Wako Chemicals). Reassembled HDL (rHDL) with apo A-I was prepared by the cholate dialysis method (*48*), and rHDL particle size was determined to be 205000 ± 15000 kDa by native (nondenaturing) polyacrylamide gel electorphoresis using 4−20% Tris-HCl gradient ready gels (Bio-Rad) (*49*, *50*). The weight ratio of apo A-I to POPC in the rHDL, 1:2.76, corresponds to a molar ratio of 1:108. Unilamellar POPC vesicles with a particle weight of ~2×10^6 kDa were prepared by sonication and purified by size exclusion chromatography on Sepharose CL-4B (*51*). Human serum albumin (HSA) was from Sigma-Aldrich, St. Louis, MO.

Purification of Apolipoproteins. Human apo A-I and apo A-II were purified as described previously from human plasma HDL (*52*). Stocks of purified apos were stored at −20 °C in 3 M guanidine hydrochloride (GuHCl). Aliquots were dialyzed into TBS to remove GuHCl or used directly for reduction by dithiothreitol (DTT) to prepare the apo A-II monomer.

Preparation of Monomeric Human Apo A-II. Stock human apo A-II (~4 mg/mL in TBS) was a mixture of monomeric and dimeric apo A-II. This mixture was reduced completely to monomeric apo A-II by addition of 20 mM DTT and incubation for 60 min at 37 °C. The reaction mixture (~0.5 mL) was loaded onto a 5 mL bed volume prepacked PD-10/Sephadex G-25 gel filtration column (Amersham-Pharmacia), equilibrated with TBS, and the apo A-II was eluted with TBS to separate the reduced protein from DTT and GuHCl. Aliquots of the protein peak from the gel filtration column were used immediately in the dimerization kinetic studies.

Dimerization Reaction. The kinetics of apo A-II dimerization were based on the rate of disappearance of reduced monomeric apo A-II and the appearance of dimeric apo A-II at 37 °C. Dimerization kinetics were determined in TBS and with the addition of salts (GuHCl, NaCl), lipids (rHDL, POPC unilamellar vesicles), and protein (HSA). The reactions at 37 °C were initiated by the transfer of freshly reduced apo A-II to test tubes containing TBS with and without various salts, lipids, or protein. Aliquots were removed as a function of time, and the dimerization reaction was stopped by addition of 5 mM iodoacetamide for 20 min at ambient temperature (*53*). Aliquots were stored at −20 °C until analysis by SDS−PAGE. Reaction aliquots that contained rHDL or POPC were delipidated by extraction with ethanol−ethyl acetate (1:1 v/v) prior to solubilization in SDS−PAGE sample buffer.

SDS−PAGE. Reaction aliquots were analyzed for the ratio of monomeric to dimeric apo A-II by separation in 18% Tris−glycine Ready Gels (Bio-Rad) under nonreducing conditions. Gels were stained with 0.1% Coomassie Blue R250. Destained gels were photographed with the Kodak electrophoresis documentation and analysis system (EDAS) 290, and the intensity of the apo A-II monomer and dimer bands was quantified using the Kodak 1D image analysis software.

Reaction Kinetics. Zero-order rate constants (k_0) were based on the initial slopes (m) of a line defined by apo A-II monomer concentration against reaction time; second-order rate constants (k_2) were based on the slope of a line defined by 1/(concentration of apo A-II monomer) against reaction time (*54*). The slopes were calculated using the LINEST function in Microsoft EXCEL. In the figures, symbols are the mean and standard error of duplicate or triplicate analyses. Where error bars are not seen, they are smaller than the symbol size.

RESULTS

Analysis of Apo A-II Dimerization by SDS−PAGE. Representative gels used for analysis of apo A-II dimerization kinetics are shown in Figure 1, which shows the data for dimerization in TBS (Figure 1A) and in the presence of unilamellar POPC vesicles (Figure 1B) or rHDL (Figure 1C). The rate of dimerization in aqueous buffer was slow. At a starting concentration of 50 μM reduced monomeric apo A-II, dimeric apo A-II was detectable only after 18 h. Addition of POPC unilamellar vesicles profoundly increased the dimerization rate, so that more than half of the starting monomeric apo A-II was converted to dimeric apo A-II within ~25 min (Figure 1B). Dimerization was even faster in the presence of rHDL, in which case most of the apo A-II was already dimerized within 4 min (Figure 1C).

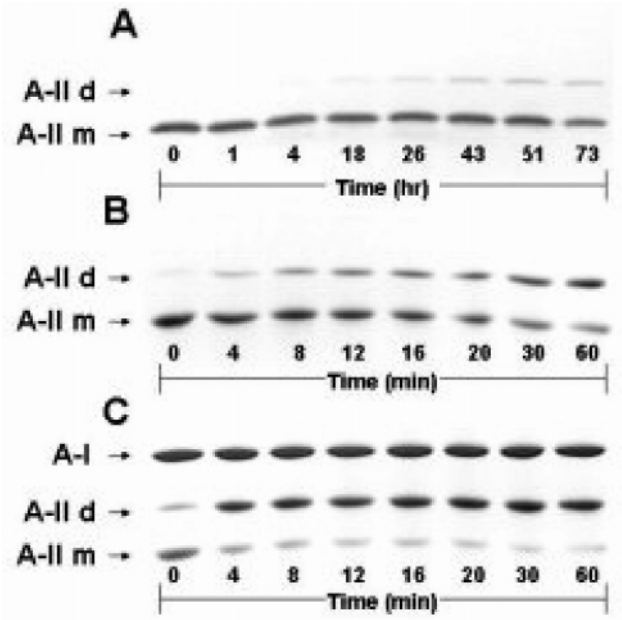

FIGURE 1: SDS−PAGE analysis of apo A-II rates of dimerization. Reactions were initiated as described in Experimental Procedures by the addition of 50 μM reduced monomeric apo A-II to (A) TBS, (B) TBS containing 0.41 μM unilamellar POPC vesicles (0.82 mg/mL POPC), and (C) TBS containing 7.8 μM rHDL vescicles (15 μM apo A-I and 1.2 mg/mL POPC). Aliquots were removed at the times indicated, from 0 to 73 h for (A) and from 0 to 60 min for (B) and (C). Reaction aliquots for (B) and (C) were delipidated prior to SDS−PAGE analysis. Arrows correspond to the mobility of the standard apo A-II monomer (A-II m), dimer (A-II d), and apo A-I (A-I).

Apo A-II Dimerization Rate Is Second Order with Respect to the Apo A-II Monomer Concentration. Assignment of reaction order was based on the best fit of our data for the disappearance of apo A-II as a function of time to kinetic models for zero-, first-, and second-order reactions. Tests of reaction kinetics were conducted for zero-order ([apo A-II] versus t), first-order (ln [apo A-II] versus t), and second-order (1/[apo A-II] versus t) (*54*). Our analysis revealed that apo A-II dimerization was best described by second-order kinetics with respect to the concentration (c) of apo A-II monomer: $\mathrm{d}[\text{apo A-II}] = -k_0\,\mathrm{d}t = k_2[\text{apo A-II}]^2\,\mathrm{d}t$. The zero-order plots for three concentrations of apo A-II are shown in Figure 2A, and the plot of $-k_0 = k_2[\text{apo A-II}]^2$ is shown in Figure 2B. At an initial concentration of 50 μM apo A-II monomer, the approximate concentration of apo A-II in human plasma (*7*), the $t_{1/2}$ for dimerization was about 10 days. Since the normal plasma residence time for apo A-II is 5.25 days (*45*), the slow rate of dimerization would predict that most plasma apo A-II would be monomeric if the reaction occurred in the absence of other cellular or plasma factors. As noted, this is not the case, so it is likely that lipid and/or protein factors within cells or plasma must "catalyze" dimerization.

Effect of GuHCl. We hypothesized that the secondary structure or self-association of apo A-II would affect the rate of dimerization. As protein concentration increases, both monomeric and dimeric apo A-II self-associate with a concurrent increase in apolipoprotein secondary structure (*55*−*57*). The effect of self-association and secondary structure on the rate of dimerization was tested by measuring the rate in the presence of GuHCl, which disrupts secondary

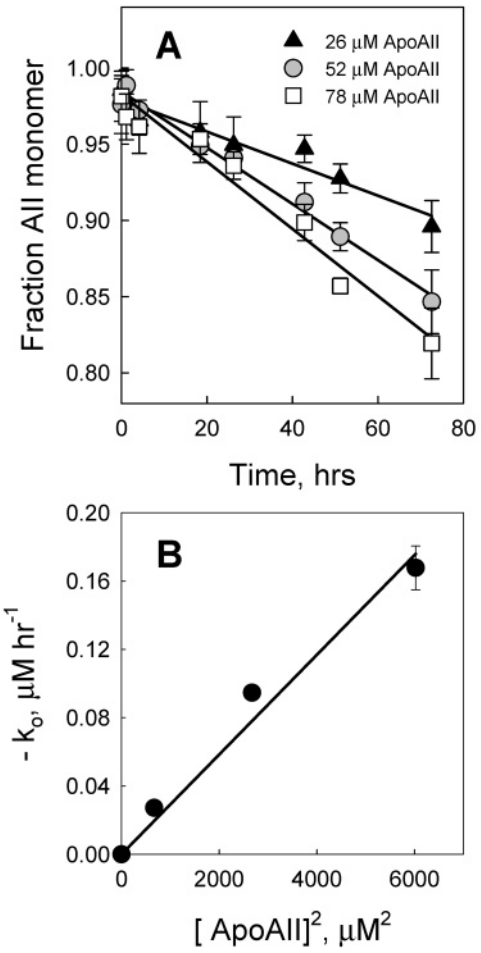

FIGURE 2: Apo A-II dimerization follows second-order kinetics. (A) Zero-order plot of the concentration of monomer vs time at initial apo A-II monomer concentrations of (▲) 26 μM, (●) 52 μM, and (□) 78 μM. Values for the zero-order rate constant k_0 were calculated as the slope of the linear regression fit to the data. (B) A plot of the zero-order rate consant vs initial apo A-II monomer concentration squared is linear, consistent with a second-order rate equation: $\mathrm{d}c = k_0\,\mathrm{d}t = k_2[\text{apo A-II}]^2\,\mathrm{d}t$. Symbols are the mean of duplicate or triplicate analyses; error bars are the standard error of the mean.

Table 1: Effect of GuHCl on Rate of Apo A-II Dimerization[a]

addition		zero-order plot, c vs t,	second-order plot, $1/c$ vs t,
GuHCl (M)	NaCl (M)	$-k_0$ (μM/h)	k_2 (M^{-1} min^{-1})
0.0	3.0	0.73 ± 0.03	3.31 ± 0.16
0.3	2.7	0.58 ± 0.02	2.73 ± 0.09
1.0	2.0	0.56 ± 0.04	3.22 ± 0.21
3.0	0.0	0.20 ± 0.02	0.51 ± 0.05

[a] The rate of apo A-II dimerization was measured at 37 °C in TBS with the addition of the indicated amounts of GuHCl and/or NaCl to maintain constant ionic strength. The starting apo A-II concentration was 78 μM as a monomer. Zero-order and second-order rate constants were determined as described in Experimental Procedures.

structure and converts self-associated apo A-II to its monomeric form (*58*). To correct for effects due to changes in ionic strength, the total salt concentration was kept at 3 M by addition of NaCl. (Similar results were obtained in the absence of added NaCl; data not shown.) Our results are summarized in Table 1. While lower GuHCl concentrations (0.3 and 1.0 M) had little effect on the rate of apo A-II dimerization, 3 M GuHCl decreased the zero-order rate

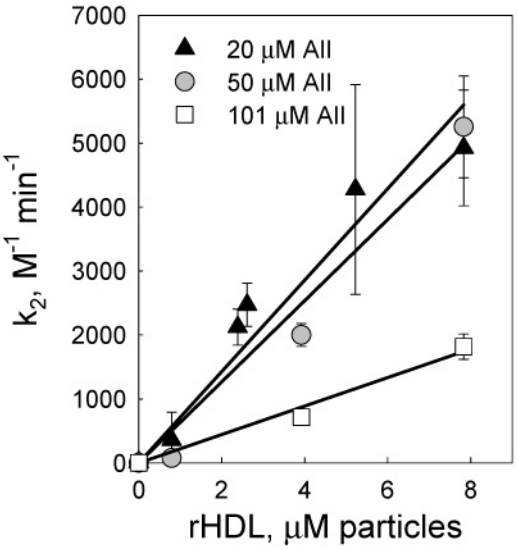

FIGURE 3: rHDL greatly enhances the apo A-II rate of dimerization. Second-order rate constant k_2 vs rHDL concentration at initial concentrations of monomeric apo A-II of (▲) 20 μM, (●) 50 μM, and (□) 101 μM.

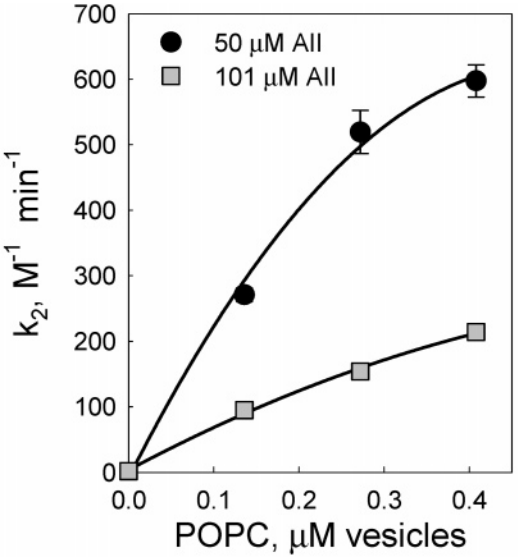

FIGURE 4: POPC unilamellar vesicles enhance the apo A-II rate of dimerization. Second-order rate constant k_2 vs POPC concentration at initial concentrations of monomeric apo A-II of (●) 50 μM and (□) 101 μM.

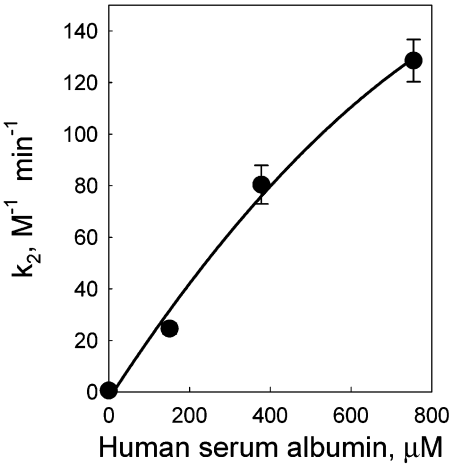

FIGURE 5: Human serum albumin increases the apo A-II rate of dimerization. Second-order rate constant k_2 vs human serum albumin concentration at an initial concentration of monomeric apo A-II of 78 μM. The highest albumin concentration shown corresponds to human plasma levels of albumin.

constant 3.6-fold and the second-order rate constant 6.5-fold.

Effect of rHDL. The kinetics of apo A-II dimerization were studied in the presence of HDL, since it is the plasma carrier of apo A-II and thus interacts with apo A-II in vivo. To model HDL effects, we used rHDL containing only apo A-I; this allowed us to measure the kinetics in the absence of a major experimental confounder, endogenous dimeric apo A-II, while still providing good resolution of apo A-II monomer and dimer and apo A-I (Figure 1C). The ratio of apo A-II to apo A-I in our reaction mixtures was greater than that in human plasma, due to the limitations of detection in our system. However, we tested apo A-I concentrations up to one-third of that found in human plasma (*7*). Addition of apo A-I rHDL at 15 μM apo A-I (7.8 μM rHDL particles) to plasma concentrations of apo A-II (50 μM as monomer) increased the initial zero-order dimerization rate constant 3000-fold and the second-order rate constant 7500-fold (Figure 3). Added rHDL had a significant but lesser effect on k_2 at higher apo A-II concentrations (101 μM). At higher ratios of apo A-II to rHDL, an initial fast dimerization rate was followed by a slower rate, approaching that observed in TBS (data not shown). The extent of dimerization at the end of the initial fast rate corresponded to saturation of the rHDL with apo A-II, consistent with work by others that four molecules of apo A-II monomer displace one molecule of lipid-bound apo A-I (*44*, *59*−*62*). This displacement of apo A-I by apo A-II occurs within minutes (*59*, *62*). In contrast, once bound to rHDL, dimeric apo A-II dissociates slowly (*44*, *62*). This suggests that upon binding rHDL monomeric apo A-II dimerized quickly but, once bound, was not readily displaced by aqueous monomeric apo A-II. The remaining aqueous apo A-II, above the amount needed to saturate the available rHDL, dimerized at a much slower rate.

Effect of Unilamellar POPC Vesicles. rHDL contains both phospholipid and protein, both of which could interact with apo A-II. To determine if a phospholipid surface alone was sufficient to promote apo A-II dimerization, we studied the effect of POPC unilamellar vesicles on the reaction rate. As shown in Figure 4, k_2 increases with increasing POPC concentration with an 850-fold increase in the rate constant at 0.41 μM unilamellar vesicles.

Effect of HSA. The plasma concentration of HSA is ~50 mg/mL. Like HDL, HSA might be expected to interact with apo A-II in vivo since it comprises more than half of the total plasma protein (*63*, *64*). The effect of HSA at 10−50 mg/mL on apo A-II dimerization was tested (Figure 5). At 50 mg/mL, HSA increased the second-order rate constant for apo A-II dimerization 220-fold.

Comparison of rHDL, POPC, and HSA. Of interest is to compare the relative effectiveness of rHDL, POPC unilamellar vesicles, and HSA as catalysts for the apo A-II dimerization reaction. A summary of these data is presented in Table 2. In contrast to the slow apo A-II dimerization rate in TBS, addition of apo A-I−rHDL, at one-third the plasma concentration of apo A-I, decreased the $t_{1/2}$ for dimerization 7000-fold, to less than 2 min. Addition of an equivalent amount of phospholipid as unilamellar vesicles

Table 2: Rate Constants for Dimerization of Apo A-II

apo A-II[a] (μM)	additive[b]		k_2[c] (M^{-1} min^{-1})	normalized rate constant
26			0.77 ± 0.12	1.1
52			0.70 ± 0.04	1.0
78			0.58 ± 0.05	0.8
	rHDL			
	mg/mL POPC	μM particles		
50	0.59	3.9	2000 ± 180	2900
50	1.17	7.8	5260 ± 800	7500
	POPC unilamellar vesicles			
	mg/mL POPC	μM vesicles		
50	0.54	0.27	520 ± 33	740
50	0.82	0.41	598 ± 25	850
	human serum albumin			
	mg/mL	μM		
78	25	378	80 ± 8	140
78	50	755	129 ± 8	220

[a] The apo A-II concentration is the initial concentration of the apo A-II monomer. The human plasma concentration of apo A-II is 0.4 mg/mL or 46 μM as a monomer and 23 μM as a dimer (*7*). [b] Additions to the reaction mixture were rHDL, unilamellar POPC vesicles, and human serum albumin (HSA). Concentrations are given as mg/mL phospholipid and μM lipid particles or vesicles for rHDL and POPC and as mg/mL and μM protein for HSA. [c] Second-order rate constants for conversion of monomeric to dimeric apo A-II were determined in TBS at 37 °C as described in Experimental Procedures. Values are the mean ± SEM.

decreased the $t_{1/2}$ to 20 min, and addition of plasma levels of HSA decreased the $t_{1/2}$ to 120 min. Thus agents present in plasma promote apo A-II dimerization and decrease the $t_{1/2}$ for dimerization to well below the plasma residence time of apo A-II.

Figures 3−5 present data on a molar basis for these agents. However, they differ greatly in size. In particular, the rHDL particles used in these studies had a particle size of 205 ± 15 kDa, compared to 1880 ± 220 kDa for phosphatidylcholine unilamellar vesicles (*51*). Expressed on a molar basis, i.e., fold increase in second-order rate constant per micromolar catalyst, POPC vesicles increase the dimerization rate the most: 2100 fold per micromolar vesicles, compared to 960-fold per micromolar rHDL particles, and 0.29-fold per micromolar HSA. However, comparison of rHDL to POPC on a weight phospholipid basis demonstrates that per milligram POPC, rHDL enhances dimerization about 6-fold more than do POPC unilamellar vesicles (Figure 6).

DISCUSSION

In human plasma, nearly all apo A-II occurs as disulfide-linked dimers of which the majority are homodimers of apo A-II; a small fraction of apo A-II forms heterodimers with other cysteine-containing apolipoproteins with only a minor amount of apo A-II being present as monomer (*37*). In the absence of lipid and protein surfaces, apo A-II dimerization followed second-order kinetics with respect to apo A-II concentration (Figure 2). This reaction order is consistent with a bimolecular reaction in which the rate-limiting step involves the collision of the cysteine −SH group on one apo A-II monomer with that of a cysteine on another monomer according to eq 1. With an apo A-II plasma concentration

$$2(\text{apo A-II}) \rightarrow (\text{apo A-II})_2 \quad (1)$$

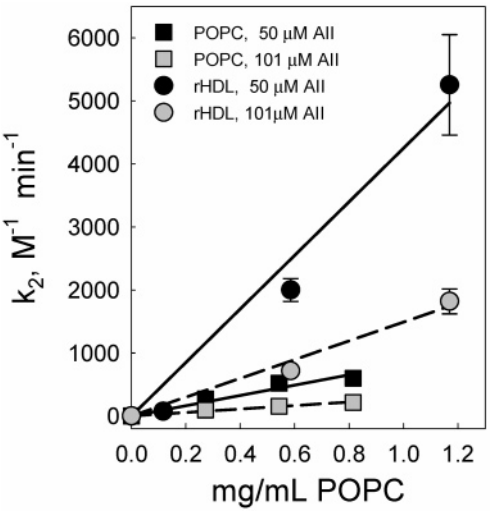

FIGURE 6: Comparison of rHDL and POPC unilamellar vesicle catalysis of the apo A-II rate of dimerization. Second-order rate constant k_2 vs mg/mL POPC in either rHDL (●, ○) or POPC unilamellar vesicles (■, □) at initial concentrations of monomeric apo A-II of 50 μM (●, ■) and 101 μM (○, □). Whole human plasma contains about 2.2 mg/mL phospholipid, with about 0.85 mg/mL in HDL (*7*).

of 5 × 10^{-5} M, the rate for this process ($rate_{DIM}$) can be described by eq 2, which is the integrated form for the

$$\text{rate}_{DIM} = k_{DIM}[\text{apo A-II}]^2 = k_{DIM}[5 \times 10^{-5}\,\text{M}]^2 = k_{DIM} \times 2.5 \times 10^{-9}\,\text{M}^2 \quad (2)$$

kinetics of a second-order reaction. An upper limit on the rate of reaction can be obtained from the diffusion-controlled rate constant [k_{dif} = 6 × 10^9 M^{-1} s^{-1} (*65*)] estimated from the modified Debye equation (*66*, *67*), as in eq 3. If apo

$$\text{rate}_{DIM} = 6 \times 10^9\,\text{M}^{-1}\,\text{s}^{-1} \times 2.5 \times 10^{-9}\,\text{M}^2 = 15\,\text{M}\,\text{s}^{-1} \quad (3)$$

A-II dimerization were diffusion-controlled, at plasma concentrations most apo A-II would be dimeric, as observed. However, in TBS the observed dimerization rates are slow compared to diffusion-controlled rates, too slow to give the high fraction of dimeric apo A-II observed in human plasma, suggesting that steric factors, which frequently limit the rates of bimolecular reactions, are involved. In a diffusion-controlled reaction, each collision between reactants gives a product. In TBS, there must be a large fraction of unproductive collisions between apo A-II monomers.

Mature apo A-II contains 77 amino acid residues of which only one is cysteine. If one assumes to a first approximation that there is an equal probability of each amino acid in apo A-II colliding with another apo A-II, then the probability that this collision is through a cysteine residue is 1/77 = 0.013. The probability that the point of contact is a cysteine residue on the second apo A-II molecule is also 1/77 so that the total probability for a cysteine-cysteine collision is $(0.013)^2 = \sim 1.7 \times 10^{-4}$. Combining this steric factor with eq 3 gives

$$\text{rate}_{DIM} = 1.7 \times 10^{-4} \times 15\,\text{M}^1\,\text{s}^{-1} = 2.4 \times 10^{-3}\,\text{M}^1\,\text{s}^{-1} \quad (4)$$

The rate calculated by eq 4 is still fast compared to the observed dimerization rate in TBS (Table 2):

$$\text{observed rate}_{DIM} = k_2[\text{apo A-II}]^2 = 0.70\ M^{-1}\ min^{-1} \times (5 \times 10^{-5}\ M)^2 = 2.9 \times 10^{-9}\ M\ s^{-1}$$

so that there remain significant other factors that limit the reaction rate in aqueous buffer, including a high free energy of activation or transient exposure of free −SH groups.

The observed second-order rate constant k_2 for apo A-II dimerization, 0.012 M^{-1} s^{-1}, is similar in magnitude to those in published reports on the kinetics of disulfide bond formation. For example, second-order rate constants for reaction of albumin and homocysteine to form albumin−S−S−homocysteine are in the range of 0.045−0.26 M^{-1} s^{-1} (*68*). In another study of disulfide bond formation, the second-order rate for the uncatalyzed reaction of a model peptide disulfide with glutathione was k_2 = 0.33 M^{-1} s^{-1} (*69*). In this case, protein disulfide isomerase (PDI) catalysis increased the second-order reaction by 400−6000-fold. For comparison, rHDL increased apo A-II dimerization by 2900−7500-fold (Table 2). It is striking that binding of apo A-II monomer to the lipid surface of rHDL facilitates disulfide bond formation to the same extent as does catalysis by PDI.

The secondary and tertiary structures of apo A-II could be important determinants of the rate of dimerization. Although apolipoproteins undergo concentration-dependent oligomerization, these effects are probably not important considerations in the analysis of our experiments. At least two studies have measured self-association of other single-chain forms of apo A-II. Rhesus monkey apo A-II, in which Ser6 replaces Cys6, forms significant amounts of oligomers only at concentrations higher than used in this study (*55*). Reduced and carboxymethylated human apo A-II (RCM apo A-II) forms oligomers more readily, with about 50% self-association at 50 μM (*57*). GuHCl shifts this equilibrium to the dissociated form. In our study with native human apo A-II monomer, similar rates of dimerization were observed at 0, 0.3, and 1.0 M GuHCl (Table 1), indicating that little if any apo A-II was self-associated in the reaction mixture, since these concentrations of GuHCl are sufficient to effect apo A-II monomer formation from self-associated dimers and tetramers (*57*). It seems that RCM apo A-II self-associates at lower concentrations than does the rhesus monkey or native human apo A-II monomer.

However, the apo A-II secondary structure has a significant effect on the rate of dimerization. Under our experimental conditions, apo A-II is monomeric and according to circular dichroic studies contains ∼35% α-helical structure (*70*, *71*). In 3 M GuHCl apo A-II converts to a fully extended random coil, and k_2 for dimerization is decreased 6.5-fold (Table 1). The slower rate of dimerization may be attributed to the increased surface area of the protein in its extended form. In folded apo A-II, the exposed cysteine represents a greater fraction of the surface residues than in the fully extended protein, thereby increasing the probability that a collision with another apo A-II will involve the cysteine SH groups. Also, in the partially folded form, the amphipathic helices, likely helix 3 (*71*, *72*), may undergo sticky collisions, thereby forming transiently self-associated apo A-II molecules that have a greater probability of forming a disulfide bond.

Given the greater enhancement of dimerization by rHDL than by POPC unilamellar vesicles, which contain no protein, we examined the effects of added protein. Albumin was selected for this test because of its abundance in plasma (*64*). Although physiological concentrations of HSA enhanced the reaction in a robust way (∼200-fold, Figure 5), this effect was considerably smaller than that of rHDL and POPC unilamellar vesicles (Table 2). Although this finding suggests that apo A-II interacts with HSA, the in vivo contribution of HSA to catalysis of dimerization is likely to be small compared to the much faster rates of dimerization catalyzed by lipid-containing particles. However, apo A-II dimerization in the endoplasmic reticulum−Golgi pathway may occur in the presence of other not yet identified proteins that provide sites for binding apo A-II monomers to favor dimerization.

The most dramatic effect on the rate of apo A-II dimerization was elicited by rHDL and POPC unilamellar vesicles, both of which have phospholipid surfaces. These particles increased the rate of apo A-II dimerization by 2−3 orders of magnitude. Like other apos, apo A-II spontaneously associates with particles having lipid surfaces (*70*), and this fact provides the key to the probable mechanism for the "catalysis" of apo A-II dimerization. Under conditions where the bulk concentration of apo A-II might be 50 μM, association with rHDL or POPC unilamellar vesicles has the effect of concentrating the apo A-II to a smaller surface, giving rise to a very high local concentration. Up to eight apo A-II monomers can bind one rHDL particle (J. B. Massey, unpublished data; ref *40*). With a density of 0.95 g/mL, for an rHDL concentration of 3.9 μM, the POPC weight of 0.59 g will have a volume of 0.62 mL. If the association of apo A-II with rHDL is maximal, then 3.9 μM rHDL binds 8 × 3.9 = 31.2 μM apo A-II. This gives a local concentration of 31.2 μmol/0.00062 L = ∼50 mM, a 1000-fold increase in the local concentration of apo A-II. On the basis of eq 2, the rate would be increased by a factor of $1000^2 = \sim 1 \times 10^6$. This increase is far greater than that observed for several likely reasons. First, the diffusion of an apo A-II molecule on a surface that is much more viscous than that of water will greatly reduce the number of collisions. According to measurements by pyrene excimer fluorescence or fluorescence depolarization the internal viscosity of micelles and lipid membranes is on the order of 200 times that of water (*73*, *74*). Second, steric factors, though not known, are likely to be different from those for apo A-II in water. Third, in rHDL, apo A-I could interact with apo A-II to impede its diffusion toward another apo A-II molecule (*75*). Last, self-association of apo A-II molecules on the surface of rHDL or POPC unilamellar vesicles may reduce the number of productive collisions.

Catalysis by POPC unilamellar vesicles and rHDL was compared on the basis of both particle concentration and lipid concentration. When compared on the basis of particle concentration, POPC vesicles were a better "catalyst" than rHDL particles (Figures 3 and 4). This may be due to their much larger size: about 25 nm in diameter compared to 9 nm for our rHDL preparation (*76*, *77*). Thus, the surface area of a POPC vesicle is greater than that of rHDL and on a particle basis provides more sites for interaction with apo A-II. However, when compared on a weight basis, rHDL is the more efficient catalyst (Figure 6). Binding of apo A-II to rHDL is rapid, with displacement of apo A-I occurring in

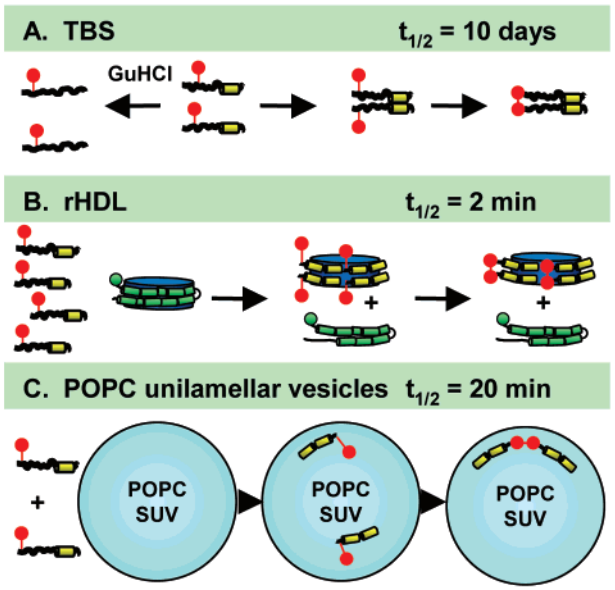

FIGURE 7: Scheme for dimerization of apo A-II. (A) Reaction in TBS. (B) Reaction catalyzed by apo A-I rHDL. (C) Reaction catalyzed by POPC unilamellar vesicles (POPC SUV). The relative sizes of rHDL and POPC SUV are drawn approximately to scale. Apo A-II (yellow) and apo A-I (green) structures are simplified cartoons depicting extended, helical, and globular domains of the aqueous and lipid-bound apos (*71*, *72*, *79*, *81*, *82*). Cys6 in apo A-II is represented by red balls, with the disulfide link in apo A-II homodimers shown by linked red balls. One lipid-bound apo A-I molecule is displaced by four apo A-II monomers or two apo A-II dimers (*59*−*62*). A-I is drawn on rHDL with a hairpin turn, and apo A-II dimer is drawn with disulfide-linked monomers in parallel configuration, but other conformations are also consistent with available data (*72*, *78*, *82*).

less than 1 min (*44*, *59*). Once bound, two factors could make rHDL the more efficient catalyst for apo A-II dimerization. The first factor is the location of apo A-II in rHDL. According to current models of discoidal HDL, the apos are confined to a "belt" around the circumference of the particle (*78*); confinement of apo A-II to this belt region would have an additional concentrating effect on apo A-II and increase the probability of productive collisions leading to dimerization. In contrast, within POPC vesicles two monomers could be distributed to locations that are remote from each other, and the time to diffuse to a reactive configuration would be much longer. Since interaction of lipoproteins with spherical phospholipid vesicles is thought to entail burial of the hydrophobic face of amphipathic helixes below the charged surface, into contact with the acyl chains, diffusion of apo A-II across the surface of POPC vesicles could be impeded. A second factor is the influence of a curved surface on rHDL; the curvature of the apo A-II peptide backbone conforms well to the curvature along the belt of the phospholipid disk (*72*). This interaction might better stabilize a productive configuration of apo A-II monomers that leads to dimerization. Support for this hypothesis requires additional studies of the spatial arrangement of apo A-II on rHDL (J. B. Massey et al., work in progress), similar to those reported for apo A-I (*78*, *79*).

In Figure 7 we have summarized our model for the covalent dimerization of apo A-II in aqueous solution, in the presence of rHDL, and in the presence of POPC unilamellar vesicles. In GuHCl, monomers of apo A-II are random coils and form covalent dimers only when collisions between monomers provide contact between cysteine residues on a pair of reacting monomers (Figure 7A). The number of productive collisions is much lower than that predicted on the basis of collision theory of diffusion-controlled reactions because of steric requirements. In TBS, monomers of apo A-II are partially folded and presumably unassociated. Nevertheless, the rate of association is more than three times faster than that observed in GuHCl. As discussed, this difference may be due to the small amount of secondary structure in helix 3 of the apo A-II monomers; helices on a pair of apo A-II monomers could form a "sticky" collision that keeps cysteine residues adjacent to each other longer and thereby increases the probability of forming a disulfide bond.

In the presence of rHDL, the initial and distinguishing step is the association of apo A-II monomers with rHDL (Figure 7B). Although data for the rate of association of apo A-II monomers are not available, indications are that, like apo A-I and dimeric apo A-II, it should occur on the time scale of minutes or less (*44*). Binding of apo A-II displaces apo A-I from HDL and rHDL with a stoichiometry of four molecules of apo A-II monomer displacing one molecule of apo A-I (*24*, *59*−*62*). Whereas binding to rHDL would increase the local concentration of apo A-II monomers, confinement to the circumference of the disk provides an additional concentrating effect so that dimerization is very fast, though still not diffusion controlled because of steric limitations. The mechanism for dimerization in the presence of POPC unilamellar vesicles is similar except that apo A-II monomers are not confined to one region of the particle (Figure 7C). As a consequence, the local concentration of apo A-II monomers is slightly lower, and the dimerization rate is slower.

Although the physiological site of apo A-II dimerization is unknown, it is now clear that, to account for the high plasma fraction of dimeric apo A-II, lipids and/or protein surfaces must participate in the process. It remains to be determined if human apo A-II is secreted by hepatocytes as a monomer or disulfide-linked dimer. Whereas our data show that plasma factors catalyze dimerization, there may be similar lipid and protein surfaces that chaperone the process in the endoplasmic reticulum. One of these could be protein disulfide isomerase, which catalyzes disulfide bond formation in many proteins as they pass through the endoplasmic reticulum (*80*). As noted, enhancement of apo A-II disulfide bond formation by rHDL was comparable in magnitude to that reported for PDI (*69*). Thus, we have identified a unique mechanism for the catalysis of disulfide bonds that may be applicable to other lipophilic cysteine-containing proteins. One conspicuous candidate is apo B-100, a highly lipophilic protein that contains a disulfide cluster in the amino terminus. Future studies of cellular processing of apos B-100 and A-II and other proteins destined to form disulfide links could provide new information on mechanisms controlling the production of secretion-competent proteins and lipoproteins.

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