Catalysis of Covalent Lp(a) Assembly: Evidence for an Extracellular Enzyme Activity that Enhances Disulfide Bond Formation†

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ABSTRACT: The assembly of lipoprotein(a) (Lp(a)) particles occurs via a two-step mechanism in which noncovalent interactions between apolipoprotein(a) (apo(a)) and the apolipoproteinB-100 component of low density lipoprotein precede the formation of a single disulfide bond. Although we have previously demonstrated that the rate constant for the covalent step of Lp(a) assembly can be enhanced by altering the conformational status of apo(a), the resultant rates of covalent Lp(a) particle formation measured in vitro are relatively slow. The large excess of Lp(a) (over apo(a)) observed in vivo can be accounted for by a preferential clearance of apo(a) over Lp(a) and/or a sufficiently high rate of covalent Lp(a) assembly. In the present study, we report that cultured human hepatoma cells secrete an oxidase activity that dramatically enhances the rate of covalent Lp(a) assembly. This activity is likely possessed by a protein because it is heat-sensitive and is retained in the concentrate following ultrafiltration through a 5 kDa cutoff filter. However, a small molecule cofactor for the activity is suggested by the observation that the activity is lost upon dialysis. Plots of Lp(a) assembly rate versus input apo(a) concentration gave rectangular hyperbolae; the reaction displayed an unusual dependence on the concentration of apoB-100, with increasing concentrations of apoB-100 resulting in slower rates of Lp(a) assembly at low concentrations of apo(a), an effect that was alleviated by higher apo(a) concentrations. Interestingly, $V_{max(app)}/K_{m(app)}$ ratios were insensitive to apoB-100 concentration, which is diagnostic of a ping−pong reaction mechanism. In this way, the putative Lp(a) oxidase may be functionally analogous to protein disulfide isomerase, which exhibits a similar mechanism during the catalysis of disulfide bond formation during protein folding, although we have ruled out a role for this enzyme in Lp(a) assembly.

The formation of disulfide bonds is essential for the folding and stability of many proteins that traverse the secretory pathway. A variety of molecular chaperones, including protein disulfide isomerase and heat shock proteins, work in tandem to enhance the rate of disulfide bond formation and protein folding in vivo (*1*). Although pathways that regulate intramolecular disulfide bond formation during protein (re)folding have been extensively examined, there is a paucity of information regarding mechanisms through which intermolecular disulfide linkages are formed. This discrepancy can be explained by the fact that disulfide linkage between two proteins is relatively uncommon. Lipoprotein(a) (Lp(a)[1]), which contains apolipoprotein(a) (apo(a)) that is attached to the apolipoproteinB-100 (apoB-100) component of its low density lipoprotein (LDL)-like moiety via a single disulfide bond, constitutes one example of a covalently linked heterodimer.

Intermolecular disulfide bond formation is more complex than the intramolecular process because it requires a bimolecular interaction that brings two free cysteine residues into close spatial alignment. In the case of Lp(a) particle formation, this is presumably achieved by a specific high affinity ($K_D \sim 20$ nM) noncovalent interaction between apo(a) and LDL that precedes disulfide bond formation (*2*). The process of covalent Lp(a) particle formation is further complicated because it appears to occur separately from the machinery that catalyzes disulfide bond formation within the endoplasmic reticulum. Numerous reports suggest that the two constituent subunits of Lp(a), apo(a), and apoB-100-containing lipoproteins, are independently processed and secreted by liver cells and form covalent Lp(a) particles within the extracellular compartment (*3*−*5*). As such, it has been assumed that covalent Lp(a) particle formation proceeds in a spontaneous (i.e., noncatalyzed) fashion. Consistent with this hypothesis is a recent study from our laboratory that clearly demonstrated that in a purified system the affinity of the noncovalent step (K_D) dictates the efficiency of subsequent disulfide bond formation (*2*). More recently, however, studies from our laboratory showed that the rate of covalent Lp(a) assembly can in fact be regulated by sequences in apo(a), which do not participate in noncovalent interactions with apoB-100 (*6*, *7*). More specifically, the conformational status of apo(a) (i.e., "closed" or "open") modulates the rate of covalent Lp(a) assembly by altering the rate constant for disulfide bond formation but not the affinity of apo(a) for apoB-100. Although the rate constant

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[1] Abbreviations: Lp(a), lipoprotein(a); LDL, low-density lipoprotein; apo(a), apolipoprotein(a); apoB-100, apolipoproteinB-100; ϵ-ACA, ϵ-aminocaproic acid; HBS, HEPES-buffered saline; CM, conditioned medium.

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for covalent Lp(a) assembly can be enhanced by ~6-fold in these in vitro studies, the resultant rates of covalent Lp(a) particle formation are still relatively slow (*8*). The large excess of Lp(a) (over apo(a)) observed in vivo can potentially be accounted for by a preferential clearance of apo(a) over Lp(a) and/or a dramatic enhancement in the rate of covalent Lp(a) assembly. To date, however, neither of these possibilities has been supported experimentally.

In the present study, we provide evidence that disulfide bond formation between apo(a) and apoB-100 is markedly enhanced by putative Lp(a) oxidase activity secreted by cultured human hepatoma (HepG2) cells. These novel data have important implications for understanding the mechanisms that regulate Lp(a) assembly and determine plasma Lp(a) concentrations and offer important insights into the more general process of the catalysis of disulfide bond formation within the extracellular milieu.

EXPERIMENTAL PROCEDURES

Construction and Expression of Recombinant Apo(a) Variants in Mammalian Cells. A 17-kringle (17K)-containing recombinant apo(a) (r-apo(a)) variant as well as a 10-kringle (10K)-containing r-apo(a) variant were constructed in the pRK5 expression plasmid as previously described (*9*, *10*). Human embryonic kidney (HEK) 293 cells stably expressing each of the r-apo(a) species were generated as previously described (*9*).

Transient Transfection of HepG2 Cells and Assessment of Covalent Lp(a) Particle Formation. HepG2 cells, grown to 70% confluence in 100 mm dishes, were transfected with 5 μg of a plasmid encoding 10K r-apo(a) using FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's protocol. After 8 h, the cells were collected, reseeded onto a 6-well plate (Costar), and allowed to recover overnight. At this time, the cells were washed with Cys-/Met-DMEM (ICN) and incubated with this medium for 1 h at 37 °C. The medium was replaced with 1 mL of fresh Cys-/Met-DMEM containing 300 μCi Tran^{35}S label (ICN), and radiolabeled apo(a) and apoB-100 were allowed to accumulate and form covalent Lp(a) particles in the conditioned medium (CM) for different times (1−8 h). At the indicated times, the medium was collected, and further covalent Lp(a) particle formation was inhibited by the addition of 100 mM ϵ-aminocaproic acid (ϵ-ACA). Following a preclearing step (2 h at 4 °C) with Protein A-Sepharose (Amersham Life Sciences) to remove fibronectin, 80% of each sample was immunoprecipitated using 1 μg of a sheep anti-apo(a) polyclonal antibody (Ab5-33; generously provided by Hugh Hoogendorn, Affinity Biologicals Inc., Hamilton, Ontario, Canada), whereas the remaining 20% was immunoprecipitated with 1 μg of a sheep anti-human apoB-100 antibody (Serotec). Lp(a) complexes were separated from either uncomplexed apo(a) or apoB-100 by SDS−PAGE on 4% polyacrylamide gels under nonreducing conditions, and the bands were quantified by densitometry as previously described (*7*). Because covalent Lp(a) complexes contain both radiolabeled apo(a) and apoB-100, the densitometric signal for the apo(a) and apoB-100 components within the Lp(a) band was determined by correcting for the number of Cys/Met residues in either apo(a) or apoB-100 compared to those in Lp(a). The rates of apo(a) secretion, apoB-100 secretion, and Lp(a) assembly were calculated at each 2 h time interval by dividing either the total amount of apo(a) or apoB-100 secreted or the amount of Lp(a) formed by 2 h. All of the densitometric values were standardized to the total cell protein (mg) determined by Bradford assay of the cell lysates at each time point.

Metabolic Labeling with ^{35}S-Cys/Met and Purification of 10K and 17K r-apo(a). HEK 293 cells, stably expressing 10K and 17K r-apo(a), were grown to confluence in 100 mm plates. HEK 293 cells (10 plates for each r-apo(a) variant) were incubated in 6 mL Cys-/Met-DMEM (Invitrogen) for 1 h at 37 °C. At this time, the medium was replaced with 6 mL of fresh Cys-/Met-DMEM containing 300 μCi Tran^{35}S label, and the labeled proteins were allowed to accumulate in the CM for 24 h. The CM was collected, pooled, supplemented with PMSF (1 mM) and clarified by low speed centrifugation (5 min at 1000*g*). ^{35}S-labeled 10K and 17K r-apo(a) were purified from the CM by lysine-Sepharose chromatography as previously described (*9*, *10*). Following purification, the proteins were extensively dialyzed against HEPES-buffered saline (HBS; 20 mM HEPES, 150 mM NaCl at pH 7.4), and the protein concentrations were determined by measuring the absorbance at 280 nm (corrected for Rayleigh light scattering) using previously determined extinction coefficients (*9*).

Purification of LDL. LDL, in the 1.02 g·mL^{-1} < d < 1.063 g·mL^{-1} density fraction, was purified from the plasma of a normolipidemic donor as previously described (*11*). The protein concentration was determined by a modified Bradford assay using bovine serum albumin (BSA; Sigma) as a standard. Purified LDL was stored at 4 °C for no longer than 4 days prior to use. ApoB-100-containing lipoproteins, in the d < 1.063 g·mL^{-1} density fraction, were purified from the CM of HepG2 cells using an identical procedure.

Preparation of the Conditioned Medium from HepG2 Cells. HepG2 cells were cultured in the presence of minimal essential medium (MEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Sigma). Cells, grown to confluence in 6-well plates, were washed with serum-free MEM (3 × 2 mL/well) in order to remove residual serum proteins and then incubated with serum-free MEM (1 mL/well). Secreted proteins were allowed to accumulate in the CM for 24 h at 37 °C. At this time, the CM was collected, pooled and briefly clarified by low speed centrifugation (5 min at 1000*g*). This material will be hereafter referred to as HepG2-CM. The protein concentration was determined using a modified Bradford assay with BSA as the standard. It is important to note that because the concentration of the putative Lp(a) oxidase in the HepG2-CM is not known the activity present within two HepG2-CM preparations cannot be compared directly. As such, experiments in which the concentrations of apo(a), apoB-100, and HepG2-CM were varied were all conducted with the same preparation. The results obtained are representative of at least three independent experiments using different HepG2-CM preparations.

In one experiment, the HepG2-CM was centrifuged through a 5 kDa-cutoff Centricon (Millipore), and the filtrate (<5 kDa) and concentrate (> 5kDa) were collected until the sample was concentrated 2-fold. In another experiment, the HepG2-CM was boiled for 10 min, centrifuged at 14 000*g* for 10 min, and the supernatant was assayed for activity. In another experiment, the HepG2-CM was dialyzed (5 kDa

cutoff) against 4L of HBS for 2 h at 4 °C. In a further experiment, covalent Lp(a) assembly assays (see below) were conducted in the presence of HepG2 cells in the presence of either freshly added MEM or MEM that had been preincubated on the cells for 24 h.

Determination of the ApoB Concentration in the HepG2-CM. Under serum-free conditions, cultured HepG2 cells endogenously express and secrete apoB-100 in the LDL density fraction (*12*). The concentration of apoB-100 in the HepG2-CM was determined by ELISA using the 1D1 monoclonal anti-apoB-100 antibody (epitope within apoB-26) (*13*) as previously described (*14*). Purified, plasma-derived apoB-100 present in the LDL fraction was utilized as a standard for this measurement.

Covalent Lp(a) Assembly Assays. In general, in vitro covalent Lp(a) assembly assays were conducted in the following manner: ^{35}S-labeled r-apo(a) (10K or 17K) was incubated with HepG2-CM at 37 °C for variable lengths of time. At selected time points, the reactions were stopped by the addition of ϵ-ACA to a final concentration of 100 mM. The samples were immunoprecipitated with 1 μg of a sheep anti-apo(a) polyclonal antibody (Ab5−33) and re-suspended in 2X Laemmli sample buffer (*15*). Covalent Lp(a) complexes were separated from free apo(a) by SDS−PAGE on 4% polyacrylamide gels under nonreducing conditions. The gels were placed in fixing solution (25% methanol, 12.5% acetic acid in H_2O), incubated in Amplify solution (Amersham Life Sciences), dried, and exposed to a PhosphorImager screen (Bio-Rad). The screen was developed using a Bio-Rad Molecular Imager FX, and the bands were quantitated using Quantity One (Version 4.0.1) densitometry software. The extent of covalent Lp(a) particle formation was computed using the following formula: $F_{Lp(a)} = S_{Lp(a)}/(S_{Lp(a)} + S_{apo(a)}) \times 100$, where $F_{Lp(a)}$ corresponds to the fraction of Lp(a), and $S_{Lp(a)}$ and $S_{apo(a)}$ correspond to the densitometric signals for the Lp(a) and apo(a) bands, respectively. The concentration of Lp(a) in each reaction was determined according to the following formula: $[Lp(a)] = F_{Lp(a)} \times [A]_0$, where [Lp(a)] corresponds to the concentration of Lp(a), and $[A]_0$ is the total concentration of apo(a) used in the assay. The initial velocity of covalent Lp(a) particle formation (V_0) was estimated at the 15 min time point by dividing the concentration of Lp(a) formed by the time. A variety of in vitro covalent Lp(a) assembly assays were designed in order to characterize the activity that catalyzes disulfide bond formation between apo(a) and apoB-100. Although the experimental designs (see below) varied, all of the samples were treated according to the procedure described above. In all cases, samples that did not contain endogenous apoB-100 (e.g., HBS, MEM, and 5 kDa filtrate) were supplemented with purified, plasma-derived LDL to a concentration identical to that present in the HepG2-CM preparation that was utilized.

For fixed time point assays, experiments were conducted for 15 min at 37 °C. For experiments in which the concentration of HepG2-CM was varied, the concentrations of ^{35}S-labeled r-apo(a) (10K and 17K) and apoB-100 were fixed at 0.75 and 3.6 nM, respectively, and the HepG2 CM was diluted in MEM to final protein concentrations of 5−160 μg·mL^{-1}. In these experiments, dilution of the apoB-100 was compensated by supplementing the diluted HepG2-CM with purified, plasma-derived LDL. Titrations with ^{35}S-labeled r-apo(a) (10K and 17K; 0.125−4 nM of each) were performed with 0.16 mg·mL^{-1} of HepG2-CM protein and 3.6 nM apoB-100. Titrations with apoB-100 (3.6−23.6 nM) were conducted with 0.16 mg·mL^{-1} of HepG2-CM protein and 0.75 nM ^{35}S-labeled r-apo(a) (10K and 17K). In other experiments, the concentration of HepG2-CM protein was fixed at 0.14 mg·mL^{-1}, and the concentrations of ^{35}S-10K r-apo(a) (0.125−20 nM) and LDL (2.8−7.8 nM) were varied.

For variable time point assays, ^{35}S-10K r-apo(a) (0.75 nM) was incubated with apoB-100 in the presence of HBS, MEM, or HepG2-CM at 37 °C for variable lengths of time (t = 0−4 h). Reactions performed in HBS and MEM were supplemented with purified, plasma-derived LDL (2.8 nM). In one experiment, ^{35}S-10K (0.75 nM) was added to HepG2-CM in the presence and absence of HepG2 cells or in the presence of HepG2 cells to which MEM had been freshly added, and time courses (t = 0−8 h) for covalent Lp(a) assembly were performed.

Modeling the Catalysis of Covalent Lp(a) Particle Formation. A variety of possible reaction mechanisms were derived from first principles based on the assumption that a single enzyme species mediates the catalysis of covalent Lp(a) assembly (Supporting Information). It is also assumed that catalysis occurs from a ternary complex of the three components (apo(a) (*A*), apoB-100 (*B*) and enzyme (*E*)), which subsequently turns over to yield the product (covalent Lp(a) (*AB*)) with a rate constant k_{cat}. The ternary complex is assembled through binary interactions between individual components followed by interactions between the binary complexes and the third component.

Each model takes into account that covalent Lp(a) particles can be formed in a spontaneous (i.e., noncatalyzed) manner. Importantly, our previous characterization of the spontaneous reaction (*6*) can be utilized to calculate the expected rates of the spontaneous reaction ($V_{0(spon)}$) at any given concentration of apo(a) and apoB-100. As such, these values can be subtracted from the measured initial rate (V_0) to obtain values for the initial rate of catalyzed covalent Lp(a) assembly ($V_{0(cat)}$) as follows:

$$V_{0(cat)} = V_0 - V_{0(spon)} \tag{1}$$

All of the rate equations developed for $V_{0(cat)}$ are expressed in the form a typical Michaelis−Menten plot with respect to the concentration of free apo(a) ([*A*]); this general equation is shown below

$$V_{0(cat)} = \frac{V_{max(app)} \times [A]}{K_{m(app)} + [A]} \tag{2}$$

Assuming that the concentration of enzyme is less than the concentration of either apo(a) or apoB-100 used in the assay, [*A*] can be determined according to eq 3:

$$[A] \approx [A]_0 - [AB] = [A]_0 - 0.5 \times (K_D + [A]_0 + [B]_0 - \sqrt{(K_D + [A]_0 + [B]_0)^2 - 4[A]_0[B]_0}) \tag{3}$$

The validity of a given model was assessed by testing whether the predicted relationships between $V_{max(app)}$, $K_{m(app)}$, or $V_{max(app)}/K_{m(app)}$ and the concentration of apoB-100 agreed with the observed relationships.

Immunodepletion of Protein Disulfide Isomerase from the HepG2-CM. HepG2-CM (1 mL) was incubated in the presence (5 μL) or absence of a mouse anti-PDI antibody (Affinity Bioreagents) overnight at 4 °C. At this time, Protein A-Sepharose (Amersham Biosciences) was added, and following a 1 h incubation at 4 °C, the supernatants from a 3000*g* spin were collected. To ensure that all of the PDI was removed from the supernatant, the samples were subjected to western blot analysis using the anti-PDI antibody. Putative Lp(a) oxidase activity in the samples was assessed by performing covalent Lp(a) assembly assays for 1 h at 37 °C using methodologies described above.

RESULTS

Cultured HepG2 Cells Secrete a Putative Lp(a) Oxidase Activity that Enhances the Rate of Covalent Lp(a) Assembly. To assess the efficiency of covalent Lp(a) assembly in a human liver cell model, we transfected HepG2 cells with a plasmid corresponding to 10K r-apo(a), metabolically labeled the cells, and measured the kinetics of apo(a)/apoB-100 secretion and Lp(a) assembly. When cultured in serum-free medium, as was done in these assays, HepG2 cells endogenously express apoB-100 that is secreted in lipoprotein particles in the LDL density range (*12*). Our data show that following a 1 h lag period, which corresponds to the equilibration of the radiolabel, the total concentrations of apo(a) and apoB-100 increase linearly, whereas Lp(a) appears to accumulate exponentially over a period of 8 h (Figure 1A and B). To further explore this observation, we plotted the rates of apo(a) and apoB-100 secretion and Lp(a) assembly, calculated over 2 h time intervals, as a function of time. Using this analysis, it becomes evident that although the rates of apo(a) and apoB-100 secretion remain constant, the rate of Lp(a) assembly steadily increases throughout the experiment (Figure 1C), indicating that the increasing rate of Lp(a) assembly cannot be explained at the level of either apo(a) or apoB-100 secretion. To explain this observation, we hypothesized that HepG2 cells secrete putative Lp(a) oxidase activity into the conditioned medium (CM) that enhances disulfide bond formation between apo(a) and apoB-100 and hence accelerates the rate of Lp(a) assembly. To test this hypothesis, HepG2 cells were incubated in MEM for 24 h, during which time secreted proteins accumulated in the CM; we will refer to this preparation as HepG2-CM. At this time, purified ^{35}S-10K (0.75 nM) was added, and covalent Lp(a) particle formation with the endogenously secreted apoB-100 (3.6 nM after dilution) was monitored over 4 h at 37 °C. Two time courses, in which purified ^{35}S-10K r-apo(a) (0.75 nM) was mixed with purified plasma-derived LDL (3.6 nM) in the presence of either MEM or HBS were used as controls. The rate of covalent Lp(a) assembly conducted in the presence of the HepG2-CM was dramatically enhanced compared to the rates observed in the presence of either HBS or unconditioned MEM (Figure 2A), indicating that the putative Lp(a) oxidase activity is derived from the HepG2 cells and not present in the culture medium. To rule out the possibility that the increased rate of covalent Lp(a) assembly observed with the HepG2-CM is due to inherent differences between HepG2-derived apoB-100 and purified plasma-derived apoB-100 (in the LDL fraction), we compared the rates of covalent Lp(a) assembly using purified apoB-100 ($d < 1.063$ g·mL^{-1}) from the HepG2-CM and purified, plasma-derived LDL. The results clearly demonstrate that apoB-100 obtained from both sources forms covalent Lp(a) particles inefficiently in the absence of factors present in the HepG2-CM (Figure 2B). Because the covalent step of Lp(a) particle formation is a redox reaction, the simplest hypothesis as to the identity of the putative Lp(a) oxidase activity is that it is a small molecule, high affinity electron acceptor. Indeed, cultured cells have been previously demonstrated to actively secrete and accumulate glutathione into the culture medium (*16*). To exclude this possibility, we centrifuged HepG2-CM through a 5 kDa Centricon, collected the filtrate (<5 kDa) and the concentrate (>5 kDa), and assessed the oxidase activity in both preparations. The activity clearly resided in the concentrate (Figure 2C), suggesting that the identity of the putative Lp(a) oxidase activity is likely a protein, rather than a small molecule electron acceptor. Further support for this hypothesis is provided by the loss of activity observed upon boiling the HepG2-CM (Figure 2B). Interestingly, however, a role for a small molecule as a cofactor for the putative enzyme in the reaction is suggested by the loss of activity observed upon subjecting the HepG2-CM to dialysis (5 kDa cutoff) (Figure 2B).

It has been previously suggested that Lp(a) assembly occurs on the cell surface of baboon hepatocytes (*17*). To ascertain whether the putative Lp(a) oxidase activity is associated with the plasma membrane, covalent Lp(a) assembly assays using purified ^{35}S-10K r-apo(a) (0.75 nM) were conducted in HepG2-CM in the presence or absence of HepG2 cells. The efficiency of covalent particle formation was similar in the presence or absence of the cells (Figure 2D), indicating that the activity is secreted in the CM and is not present on the plasma membrane or is not present in sufficient amounts on the membrane to affect the assembly assay. In addition, when the efficiency of Lp(a) assembly was measured in the presence of HepG2 cells to which fresh MEM had been added, no putative Lp(a) oxidase activity could be detected (Figure 2D). This finding clearly demonstrates that enzyme activity is not the result of an incomplete removal of serum. Importantly, the viability of HepG2 cells following the 24 h CM collection was >98% as determined by trypan blue staining (data not shown), suggesting that the presence of putative Lp(a) oxidase activity in the CM is not due to cell lysis.

Dependence of Putative Lp(a) Oxidase Activity on the Concentrations of Apo(a) and ApoB-100. Having demonstrated that the HepG2-CM contains putative Lp(a) oxidase activity that dramatically enhances the rate of covalent Lp(a) assembly, we characterized this activity by individually varying each of the components in the reaction (i.e., HepG2-CM, apo(a), and apoB-100) and measuring the initial rate of covalent Lp(a) particle formation (V_0). Because we have previously shown that the conformational status of apo(a) (i.e., open or closed) influences the efficiency of covalent Lp(a) particle formation in the noncatalyzed reaction (*7*), we used two r-apo(a) variants (10K and 17K) in our analysis. Importantly, the 10K r-apo(a) adopts an open conformation, whereas 17K r-apo(a) adopts a closed conformation (*6*, *7*). Titration of the HepG2-CM protein from 0 to 160 μg·mL^{-1} yielded linear increases in V_0 for both the 10K and 17K r-apo(a) variants (Figure 3A). Linear regression analyses yielded slopes of 5.48 ± 0.35 and 2.53 ± 0.16 nM

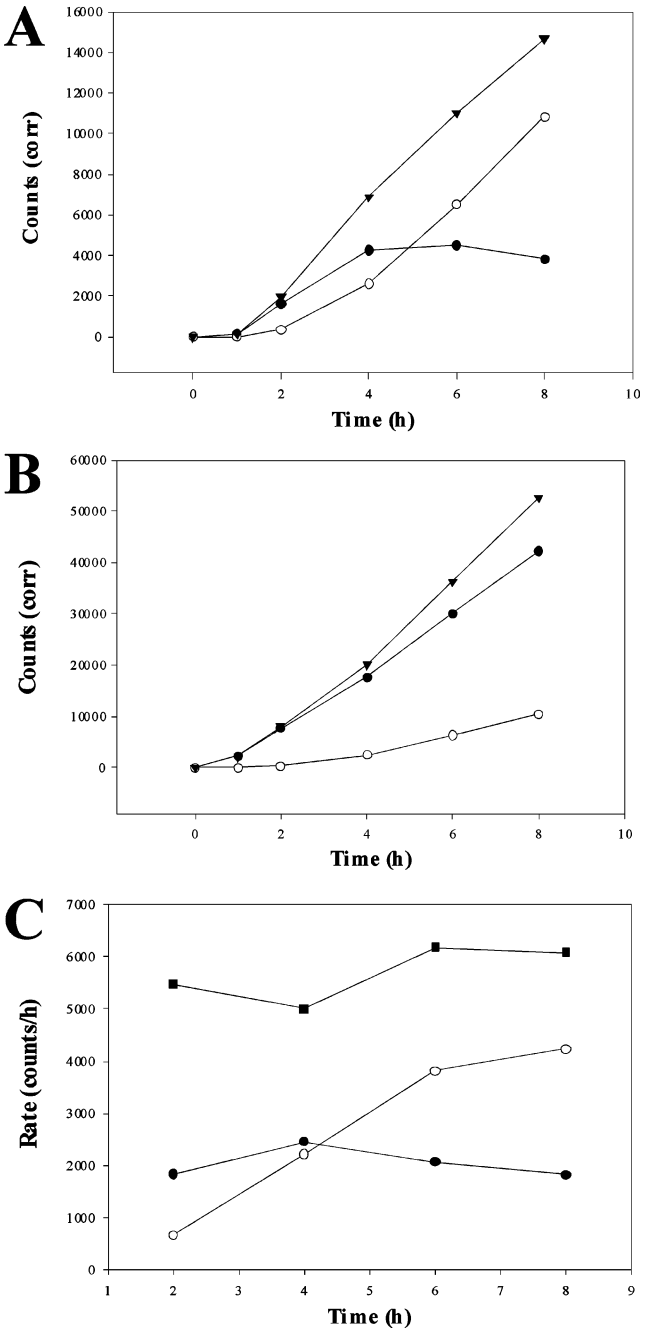


FIGURE 1: Assessment of Lp(a) assembly in a HepG2 cell model. HepG2 cells, which endogenously express apoB-100, were transiently transfected with a plasmid encoding 10K r-apo(a), metabolically labeled with ^{35}S-Cys/Met, and the kinetics of apo(a)/apoB-100 secretion as well as covalent Lp(a) assembly were assessed. The contribution of radioactive apo(a) (or apoB-100) to the total radioactivity associated with the Lp(a) band was calculated by correcting the radioactivity in the Lp(a) band for the number of Cys/Met residues in apo(a) (or apoB-100) versus Lp(a). Panel A: kinetics of uncomplexed apo(a) (●), apo(a) in the Lp(a) fraction (○), and total apo(a) (▼). Panel B: kinetics for uncomplexed apoB-100 (●), apoB-100 in the Lp(a) fraction (○), and total apoB-100 (▼) in the medium. Panel C: Rates of apo(a) secretion (●), apoB secretion (■), and Lp(a) assembly (○) calculated over each 2 h time interval by dividing the amount of Lp(a) formed or total apo(a) secreted during the time period. It is important to note that because the concentration of the putative Lp(a) oxidase in the HepG2-CM is not known, the activity present within two HepG2-CM preparations cannot be compared directly. The results obtained in this Figure and all subsequent Figures are representative of at least three independent experiments using different HepG2-CM preparations.

Lp(a)·μg^{-1}·h^{-1} and *y*-intercepts of 0.218 ± 0.023 and 0.198 ± 0.022 nM Lp(a)·h^{-1} for the 10K and 17K r-apo(a) variants, respectively. These data indicate that in the absence of HepG2-CM, covalent Lp(a) particle formation proceeds in a spontaneous fashion with a rate dictated by the *y*-intercept; the addition of HepG2-CM to the reaction elicits a dose-dependent increase in V_0, where the slope reflects the degree of enhancement observed. In this set of experiments, both the putative Lp(a) oxidase and apoB-100 are diluted. Dilution of apoB-100 is overcome by supplementing the HepG2-CM protein with purified, plasma-derived LDL. As such, the linear dependence of V_0 on the concentration of the HepG2-CM also indicates that purified plasma-derived LDL and apoB-100 secreted by HepG2 cells are equally capable of supporting catalyzed covalent Lp(a) assembly. Interestingly, catalyzed covalent Lp(a) assembly proceeded more efficiently with 10K versus 17K r-apo(a), indicating that the conformational status of apo(a) plays an important role in determining the efficiency of catalyzed covalent Lp(a) assembly. Next, we kept the concentrations of HepG2-CM protein (160 μg·mL^{-1}) and apoB-100 (3.6 nM) constant and varied the concentrations of ^{35}S-10K and ^{35}S-17K r-apo(a) from 0 to 4 nM. The relationships between V_0 and the concentration of r-apo(a) obeyed typical Michaelis−Menten kinetics (Figure 3B). Nonlinear regression analysis of the data according to a hyperbolic relationship yielded $K_{m(app)}$ values of 1.60 ± 0.14 and 5.11 ± 0.51 nM and $V_{max(app)}$ values of 5.43 ± 0.22 and 3.93 ± 0.26 nM Lp(a)·h^{-1} for the 10K and 17K r-apo(a), respectively. As such, it is apparent that the decreased efficiency with which the 17K r-apo(a) (versus 10K) forms covalent Lp(a) particles in the catalyzed reaction can be explained primarily by an elevated $K_{m(app)}$ value. Finally, we kept the concentrations of HepG2-CM (160 μg·mL^{-1}) and apo(a) (0.75 nM) constant and varied the concentration of apoB-100 from 3.6 to 23.6 nM. Surprisingly, increasing the concentration of apoB-100 yielded a dose-dependent, saturable decrease in V_0 to a nonzero value for both r-apo(a) species tested (Figure 3C). Once again, the 10K r-apo(a) formed covalent Lp(a) particles more efficiently than 17K at all concentrations of apoB-100 tested. It is important to note that apoB-100 concentrations lower than 3.6 nM could not be tested because that is the concentration of apoB-100 present within this particular preparation of HepG2-CM.

Inhibition of Catalyzed Covalent Lp(a) Assembly with High Concentrations of ApoB-100 Can Be Alleviated by Increasing the Concentration of Apo(a). To more thoroughly investigate the dependence of activity on the concentration of apoB-100, we performed apoB-100 titrations (2.8−22.8 nM) at varying concentrations of 10K r-apo(a) (0.125−20 nM) and measured V_0. Importantly, because the concentrations of apo(a) and apoB-100 were varied, we would expect that the amount of covalent Lp(a) formed from the spontaneous reaction would change throughout the titrations. To account for this, the observed initial rate (V_0) was converted to a catalytic initial rate ($V_{0(cat)}$) by correcting for the initial rate of the spontaneous reaction ($V_{0(spon)}$) (see eq 1), which was calculated on the basis of our previous characterization of covalent Lp(a) assembly using purified 10K r-apo(a) and LDL (*6*). The assays were conducted with 10K r-apo(a) because this species yields rates of covalent Lp(a) assembly that are more easily measured. At low concentrations of apo(a) (less than 1 nM), the addition of increasing amounts of

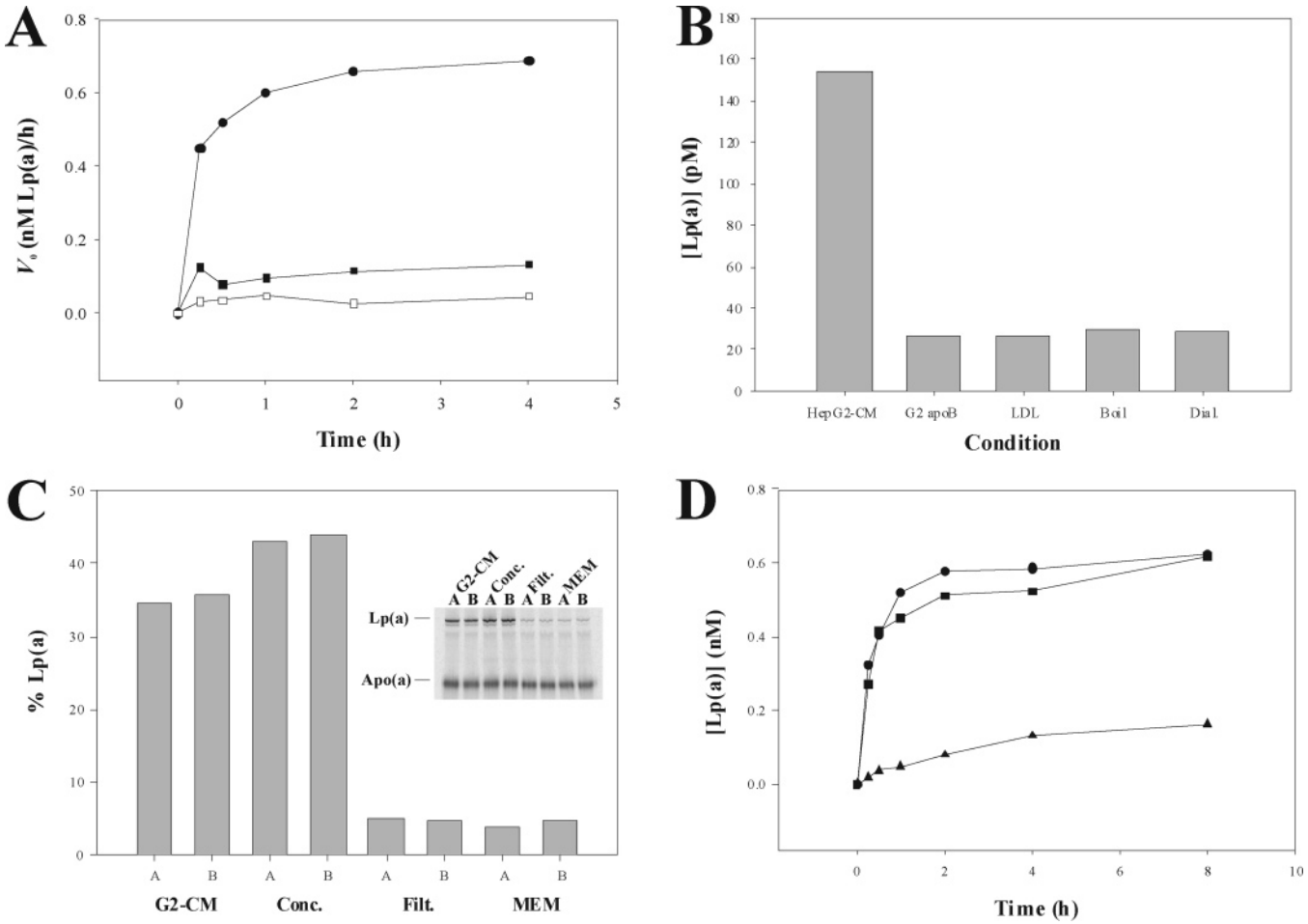


FIGURE 2: HepG2 cells secrete a putative Lp(a) oxidase activity that dramatically enhances covalent Lp(a) particle formation. In vitro covalent Lp(a) assembly assays were used to study the kinetics of covalent Lp(a) assembly. Panel A: Purified, ^{35}S-labeled 10K r-apo(a) (0.75 nM) was incubated with HepG2-CM (●) at 37 °C for variable lengths of time and covalent Lp(a) assembly was allowed to proceed with the endogenously secreted apoB-100 (3.6 nM after dilution). Two additional experiments in which ^{35}S-10K r-apo(a) was incubated with either MEM (■) or HBS (□), supplemented with purified plasma-derived LDL (3.6 nM), were included as controls. Panel B: Covalent Lp(a) assembly assays were conducted using HepG2-CM, apoB-100 (3.6nM) purified from HepG2-CM (d < 1.063 g·mL^{-1}; G2 apoB) or plasma (1.02 g·mL^{-1} < d < 1.063 g·mL^{-1}; LDL), HepG2-CM that had been boiled (Boil), or HepG2-CM that had been dialyzed (5 kDa cutoff membrane; Dial.). Panel C: HepG2-CM was centrifuged through a 5 kDa cutoff centricon (Millipore) and the filtrate (<5 kDa) and concentrate (>5 kDa) collected. Covalent Lp(a) assembly assays using ^{35}S-10K r-apo(a) (0.75 nM) were conducted in the presence of HepG2-CM (G2-CM), concentrate (Conc.), filtrate (Filt.), and MEM in duplicate (A,B) for 0.25 h at 37 °C. The inset shows the fluorogram from which these data were obtained. Panel D: ^{35}S-labeled 10K r-apo(a) (0.75 nM) was incubated with HepG2-CM either in the presence (●) or absence (■) of HepG2 cells for the indicated lengths of time. In addition, covalent Lp(a) assembly assays were conducted in the presence of HepG2 cells that had not been preincubated in MEM (▲); for this experiment, the MEM was supplemented with purified, plasma-derived LDL (3.6 nM).

apoB-100 elicits a decrease in the initial rate of covalent Lp(a) assembly, whereas at higher apo(a) concentrations (2.5 to 20 nM), the opposite effect is observed (Figure 4A). When the relationship between $V_{0(cat)}$ and the concentration of free apo(a) ([A]) (see eq 2) is examined, characteristic Michaelis−Menten plots are observed at each concentration of apoB-100 tested (data not shown). Modeling of the data according to hyperbolic relationships yielded $K_{m(app)}$ and $V_{max(app)}$ values that vary with the concentration of apoB used in the reaction (Table 1). The ratio $V_{max(app)}/K_{m(app)}$, however, remained constant over a 10-fold apoB-100 concentration range (Table 1).

Protein Disulfide Isomerase Does Not Enhance Covalent Lp(a) Assembly. One attractive hypothesis for the identity of the putative Lp(a) oxidase activity in the HepG2-CM is protein disulfide isomerase (PDI). PDI has been well-characterized as an important enzyme in mediating disulfide bond formation during protein folding in the endoplasmic reticulum (reviewed in *1*), and we have demonstrated its presence in the HepG2-CM (Figure 5A). To rule out PDI as the identity of the putative Lp(a) oxidase activity that we report in this study, we measured the ability of HepG2-CM that had been immunodepleted of PDI to enhance covalent Lp(a) assembly. The results clearly demonstrate that the removal of PDI from the HepG2-CM, as evidenced by western blotting (Figure 5A), had no effect on the ability of the HepG2-CM to up-regulate covalent Lp(a) assembly (Figure 5B).

DISCUSSION

Lp(a) assembly is known to occur via a two-step process in which an initial noncovalent interaction between apo(a) and apoB-100 is followed by the formation of a single disulfide bond (*18*). Although numerous studies suggest that the efficiency of the covalent step of Lp(a) assembly is dictated by the affinity of the noncovalent step (*2*, *19*, *20*),

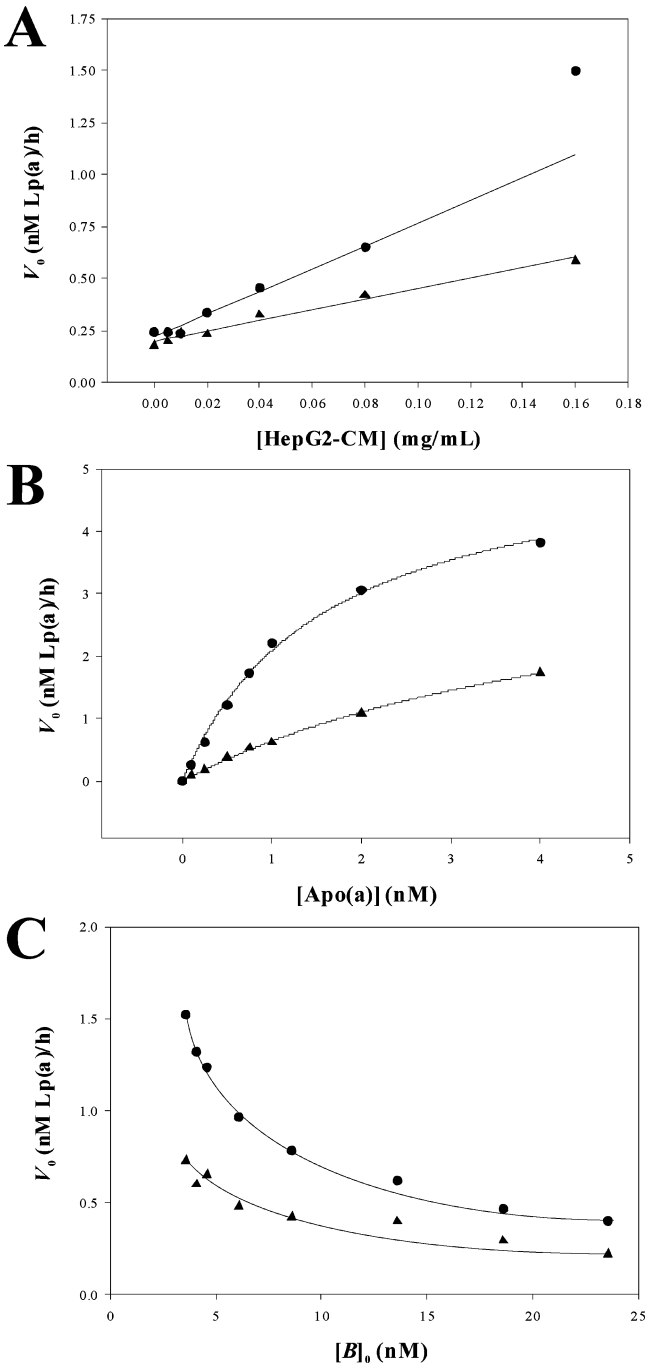


FIGURE 3: Characterization of the putative Lp(a) oxidase activity secreted by HepG2 cells. In vitro covalent Lp(a) assembly assays were performed with ^{35}S-10K (●) and ^{35}S-17K (▲) r-apo(a) to characterize the oxidase activity secreted by HepG2 cells. The initial rate of covalent Lp(a) particle formation (V_0) was calculated by dividing the concentration of Lp(a) formed (nM) by the time (0.25 h). Panel A: Amount of HepG2-CM titrated from 0 to 0.16 mg·mL^{-1} using fixed concentrations of apo(a) (0.75 nM) and apoB-100 (3.6 nM). The lines represent the fits obtained by subjecting the data to linear regression. Panel B: Concentrations of apoB-100 (3.6 nM) and HepG2-CM (0.16 mg·mL^{-1}) were kept constant, and apo(a) was titrated from 0 to 4 nM. The lines correspond to the fits obtained by modeling the data according to Michaelis−Menten equations to obtain values for $K_{m(app)}$ and $V_{max(app)}$. Panel C: Concentrations of apo(a) (0.75 nM) and HepG2-CM (0.16 mg·mL^{-1}) were kept constant and apoB-100 titrated from 3.6 to 23.6 nM.

we have recently shown that the rate of disulfide bond formation between apo(a) and apoB-100 can be enhanced

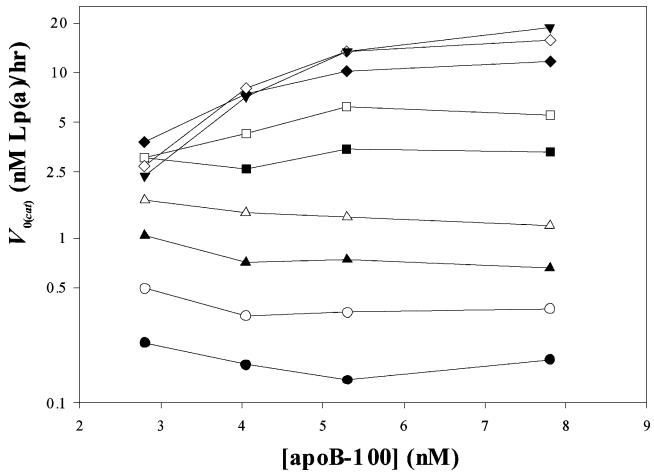


FIGURE 4: Kinetics of catalyzed covalent Lp(a) assembly.The effect of varying the concentrations of apo(a) and apoB-100 on the initial rate of catalyzed covalent Lp(a) assembly ($V_{0(cat)}$) was assessed by performing in vitro covalent Lp(a) assembly assays at 37 °C for 0.25 h. Because covalent Lp(a) particles can form spontaneously (i.e., in the absence of enzyme), V_0 values were converted to $V_{0(cat)}$ values by correcting for the theoretical rate of spontaneous covalent Lp(a) assembly determined in our previous study (*6*). The concentration of HepG2-CM was fixed at 0.14 mg·mL^{-1}, whereas apoB-100 was titrated from 2.8 to 7.8 nM at varying concentrations of ^{35}S-10K r-apo(a) (0.125 nM, ●; 0.25 nM, ○; 0.5 nM, ▲; 1 nM, △; 2.5 nM, ■; 5 nM, □; 10 nM, ◆; 15 nM, ◇; 20 nM, ▼).

Table 1: Dependence of $V_{max(app)}$, $K_{max(app)}$, and $V_{max(app)}/K_{max(app)}$ on the Concentration of ApoB-100

[apoB-100] (nM)	$K_{m(app)}$ (nM)	$V_{max(app)}$ (nM Lp(a)·h^{-1})	$V_{max(app)}/K_{m(app)}$
2.8	1.48 ± 0.26[a]	4.29 ± 0.32	2.90 ± 0.19
4.05	4.8 ± 1.4	10.6 ± 1.1	2.21 ± 0.31
5.3	7.4 ± 1.8	18.8 ± 2.0	2.54 ± 0.27
7.8	12.6 ± 3.5	26.6 ± 4.0	2.11 ± 0.32
12.8	13.2 ± 2.8	31.6 ± 3.8	2.40 ± 0.24
22.8	10.2 ± 6.6	31.9 ± 11.7	3.13 ± 0.74

[a] The values are presented ± the standard errors of the estimates as provided by the regression algorithm.

by altering the conformation of apo(a) (*6*, *7*). This observation suggests the potential for the regulation of Lp(a) particle formation by factors that influence the covalent step of the process. In the present study, we elaborate on this notion by providing the first evidence that Lp(a) assembly is a catalyzed reaction that is dramatically enhanced by putative Lp(a) oxidase activity secreted by human hepatoma (HepG2) cells. This oxidase activity is apparently possessed by a protein that also requires a small molecular cofactor. Our initial characterization of this complex, multi-substrate reaction lays the foundation for the subsequent purification and identification of this enzyme activity.

We have previously demonstrated that although apo(a) and apoB-100 noncovalently interact with high affinity ($K_D \sim$ 20 nM; *2*, *7*), the rate of spontaneous disulfide bond formation is extremely slow ($k \sim 0.05$ h^{-1}; *7*). Given the inefficiency of in vitro disulfide bond formation, one would expect to observe a significant accumulation of uncomplexed apo(a) in vivo. Previous data, however, indicate that the opposite is the case; concentrations of uncomplexed apo(a) in human plasma fall barely above the detection limit by western blot analysis (*8*). Importantly, because the rate of apoB-100 secretion exceeds the rate of apo(a) secretion both

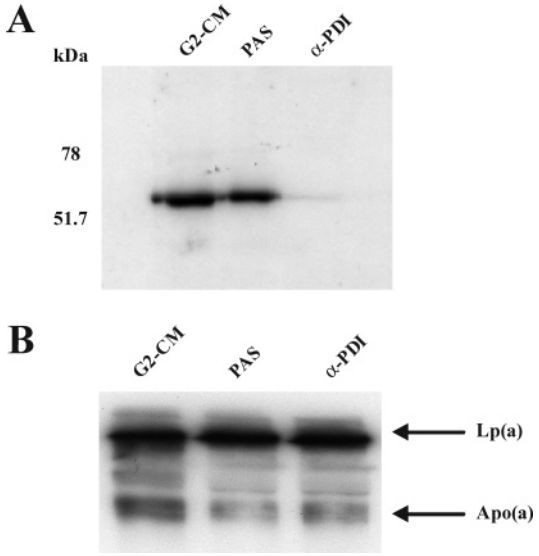
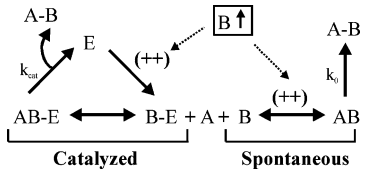


FIGURE 5: PDI does not catalyze covalent Lp(a) assembly. Panel A: Western blot analysis of HepG2-CM (G2-CM), HepG2-CM incubated with Protein A-Sepharose beads (PAS) and HepG2-CM immunodepleted of PDI (α-PDI) using a mouse anti-PDI antibody followed by Protein A-Sepharose beads. Panel B: Covalent Lp(a) assembly assays were conducted for 1 h at 37 °C, and covalent Lp(a) complexes were separated from uncomplexed 10K r-apo(a) on 5% polyacrylamide gels.

in vivo and in our HepG2 cell model (Figure 1), the relative rates of apo(a) secretion and Lp(a) assembly determine whether uncomplexed apo(a) accumulates. A real-time analysis of Lp(a) assembly in a HepG2 cell model suggests an explanation for this apparent inconsistency (Figure 1). At early time points after the addition of fresh medium, (t < 4 h), the concentration of the putative Lp(a) oxidase in the medium is low, and covalent Lp(a) formation proceeds via a slower spontaneous pathway. As predicted, the relative inefficiency of this reaction results in the accumulation of uncomplexed apo(a). As the concentration of the putative Lp(a) oxidase in the medium increases, however, the rate of covalent Lp(a) assembly is enhanced, resulting in the disappearance of free apo(a); a complete disappearance of uncomplexed apo(a) is observed by 24 h (data not shown). It is important to note that in vivo, concentrations of the enzyme would be at steady-state levels, and thus, uncomplexed apo(a) would not accumulate.

Our identification of putative Lp(a) oxidase activity in the conditioned medium of HepG2 cells is consistent with the assembly of Lp(a) particles in the extracellular compartment. Although covalent Lp(a) particles were present within the intracellular compartment of human liver cells (*21*) in one study using a small, nonphysiological apo(a) variant, the preponderance of the evidence in the literature points to an extracellular assembly process (*3*−*5*). Interestingly, studies by White and colleagues proposed that Lp(a) assembly occurs on the cell surface of baboon hepatocytes (*17*). In this model, apo(a) bound to the plasma membrane could be released into the lipoprotein fraction by the addition of apoB-100, thereby resulting in the formation of covalent Lp(a) particles. Our data, however, failed to substantiate this notion because we did not detect appreciable putative Lp(a) oxidase activity on the plasma membrane of HepG2 cells (Figure 2D). This may reflect the inherent difference between the human and baboon systems. However, in a flowing system represented by the liver versus the static system employed here, a cell-surface pool of the enzyme might be expected to play a larger role. Identification of the enzyme responsible for the putative Lp(a) oxidase activity will greatly facilitate addressing these questions.

Scheme 1



Multi-substrate enzyme reactions are inherently complicated because many situations arise where increases in the enzyme or substrate concentration results in a decreased initial rate. Our preliminary analysis of the dependence of the rate of catalyzed Lp(a) assembly ($V_{0(cat)}$) on the concentrations of apo(a) and apoB-100 that were utilized (Figure 4) offers important insights into the mechanism of catalysis. Because the concentration of the putative Lp(a) oxidase in the HepG2-CM is not known, it is difficult to fit the data to discrete rate equations. Therefore, we considered whether the data were consistent with different types of reaction mechanisms based on the relationships between $K_{m(app)}$, $V_{max(app)}$, and $V_{max(app)}/K_{m(app)}$ (obtained from the apo(a) titrations) and the concentration of apoB-100 ([*B*]). The mechanisms considered were of the sequential, nonsequential, and ping-pong types, and rate equations were developed that describe each of them (Supporting Information). A ping-pong reaction mechanism would be consistent with all of the three required relationships (Table 1 and data not shown) and thus serves as the best candidate model at this time. In fact, a constant $V_{max(app)}/K_{m(app)}$ value is diagnostic of ping-pong kinetics.

If the ping-pong model involving a covalent apoB−enzyme (B−E) complex applies, then a B−E complex should be present in the HepG2-CM because during this preparation, apoB-100 and the enzyme are co-incubated at 37 °C for 24 h. Intriguingly, the presence of a preformed B−E complex in the HepG2-CM offers a simple explanation (Scheme 1) for the unusual dependence of $V_{0(cat)}$ on the concentrations of apoB-100 and apo(a) utilized (Figure 4A).

In this scheme, the initial rate of covalent Lp(a) assembly is determined by the distribution of apo(a) between the spontaneous and catalyzed reactions. Addition of apoB-100 stimulates (++) the catalyzed arm of the reaction by increasing the rate of formation of the B−E complex, but it also up-regulates the spontaneous arm by driving the formation of the noncovalent Lp(a) complex (AB). When the B−E complex is preformed, the concentration of apo(a) determines which arm is predominant. At low concentrations of apo(a), $V_{0(cat)}$ would be less dependent on enzyme recycling, and the addition of apoB-100 would simply favor the spontaneous reaction. At higher concentrations of apo(a), however, $V_{0(cat)}$ is more dependent on enzyme recycling, and thus, supplementing the HepG2-CM with additional apoB-100 would increase the rate by driving the formation of the B−E complex following enzyme turnover.

Interestingly, a ping-pong mode of catalysis for the enhancement of disulfide bond formation is not unprecedented. In fact, it is very reminiscent of the PDI-mediated

catalysis of disulfide bond formation that takes place during protein (re)folding (*22*). Given the similarities between our proposed reaction mechanism and that previously suggested for PDI, it is tempting to speculate that PDI enhances the rate of covalent Lp(a) assembly. Indeed, the presence of PDI in the CM of cultured cells has been reported by numerous groups (*23*, *24*), and we have confirmed its presence in the HepG2-CM by western blot analysis (Figure 5A). We can, however, definitely exclude a role for PDI in the catalysis of covalent Lp(a) formation because immunodepletion of PDI from the HepG2-CM does not abrogate activity (Figures 5A, 5B).

Plasma levels of Lp(a) vary considerably, from almost undetectable to greater than 100 mg/dL within the human population. The concentration of Lp(a) in plasma is in general inversely correlated with apo(a) isoform size (*25*), which in turn is determined by the number of KIV_2 repeats present within the amino-terminal half of the molecule (*26*). Although the molecular mechanisms underlying this inverse relationship have not been examined thoroughly to date, one study reported an inverse correlation between apo(a) isoform size and the rate of secretion using a HepG2 cell model (*27*). Recent findings from our group suggest that the rate of covalent Lp(a) assembly is inversely related to apo(a) isoform size (*6*), and thus, the efficiency of Lp(a) assembly may also contribute to the determination of plasma concentrations of Lp(a). To explain our findings, we have proposed a model in which the closed conformation of apo(a) negatively regulates covalent Lp(a) assembly by restricting the access of apoB-100 to the free cysteine residue in apo(a) KIV_9; importantly, the extent of this inhibition is governed by apo(a) isoform size (*6*). Interestingly, the rate of catalyzed covalent Lp(a) assembly is also affected by the conformational status of apo(a) (Figure 3). Furthermore, preliminary data indicate that apo(a) isoform size is also inversely correlated with the efficiency of catalyzed Lp(a) assembly (Becker, L., and Koschinsky, M. L. unpublished results). Given these new findings, it is likely that the plasma levels of Lp(a) are determined by both the efficiency of apo(a) secretion as well as Lp(a) assembly. Indeed, synergy would be expected between these two processes because smaller apo(a) isoforms would be secreted more efficiently, yielding faster rates of Lp(a) particle formation due to elevated substrate (apo(a)) concentrations. The increased rate of Lp(a) assembly would be further enhanced on the basis of our observation that smaller apo(a) isoforms form covalent Lp(a) particles more efficiently at a given substrate concentration (Becker and Koschinsky, unpublished results).

An increasing number of studies have reported the extracellular enzymatic manipulation of protein disulfide bonds. For example, one study demonstrated that cultured HT1080 (Chinese hamster ovary) cells secrete plasmin reductase activity that enhances the generation of angiostatin (*28*). In another report, it was shown that extracellular molecular chaperones (PDI and BiP) present within the follicular lumen regulate covalent multimerization of thyroglobulin (*29*). The identification of the putative Lp(a) oxidase activity that we report as well as the elucidation of the mechanism of catalysis of covalent Lp(a) assembly provides important information regarding the general manipulation of disulfide bond formation in the extracellular milieu.

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SUPPORTING INFORMATION AVAILABLE

Illustration of the different kinetic models for the catalysis of Lp(a) assembly as well as information on their derivation. This material is available free of charge via the Internet at http://pubs.acs.org.

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