The Relationship between the Effect of Lysine Analogues and Salt on the Conformation of Lipoprotein(a)†

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ABSTRACT: Lipoprotein(a) [Lp(a)] exhibits many of the same properties as plasminogen, owing to a similar structural makeup from a composite of multiple kringle domains. Shared behavior includes induction of an expanded conformation by lysine analogues, inhibition of this effect, and creation of a compact conformation by NaCl. Here, we examine in detail the independent and mutual effects of NaCl and 6-aminohexanoic acid (6-AHA) on the structure of Lp(a) and the relationship between the binding of the two ligands. We find that NaCl promotes the compact conformation while binding to Lp(a) homogeneously. In the absence of salt, 6-AHA leads to the complete unfolding of Lp(a), a process that is accompanied by cooperative binding. Reversal of conformation and weakening of binding occurred when one ligand was added to Lp(a) in the presence of the other, suggesting competitive binding. High concentrations of NaCl completely reversed the expansion of Lp(a) in 100 mM 6-AHA, and high concentrations of 6-AHA unfolded Lp(a) in the presence of 100 mM NaCl, but only by 30% in the case of the 15 kringle IV Lp(a) studied. Induction of the compact form of Lp(a) appears to be an effect in common with all salts examined and cannot be attributed solely to the anion, as in the case of plasminogen. The results were summarized in terms of a model of Lp(a) depicting the conformational alterations of apo(a) caused by the binding of the two ligands. In the compact conformation in NaCl, apo(a) is apposed to the particle surface. The fully expanded form in 6-AHA results from release of both the variable and constant kringle domains. In the intermediate form in water and in a solution containing both NaCl and 6-AHA, only the variable domain is released from the particle surface.

Lipoprotein(a) [Lp(a)][1] is a cholesterol ester-rich lipoprotein composed of LDL and an additional glycoprotein, apo(a), linked to apoB with a disulfide bond (*1*−*5*). The structural makeup of apo(a) is unusual in that it consists mainly of multiple domains called kringles, which are motifs assembled from approximately 80 amino acids that are characterized by 3 highly conserved disulfide bonds (*6*). Kringle-containing proteins are found in several protein families involved in blood coagulation and fibrinolysis and include plasminogen to which apo(a) has extensive sequence homology. Like plasminogen, apo(a) contains a single kringle V and protease domain in addition to a variable number of kringle IV domains; however, it lacks kringles 1, 2, and 3 (*6*). Though nine of the subtypes of K-IV are present singly (*7, 8*), the number of K-IV_2 domains is variable and accounts for Lp(a) polymorphism. Lysine is bound in domains referred to as lysine binding sites (LBS). K-IV_{10} contains LBS I (*9*−*12*), whereas kringles of K-IV_{5-8} comprise a domain called LBS II (*13*−*17*). LBS are important in maintaining the native conformations of both Lp(a) and plasminogen and in mediating their interaction with specific target proteins.

Similar to the behavior of (Glu-)plasminogen (*18*−*25*), addition of lysine analogues to Lp(a) leads to a significant conformational change reflected by a decreased sedimentation coefficient and an increased intrinsic viscosity (*26, 27*), presumably due to disruption of the interaction of apo(a)-LBS and lysine residues of apoB, and consequent molecular expansion. It is likely that the LBS II domain on K-IV_{5-8} is involved, because rhesus Lp(a) undergoes a similar conformational change (*26*), and lacks K-V and an effective LBS on K-IV_{10} (*9, 23*). A number of ω-aminocarboxylic acids and other analogues of 6-aminohexanoic acid (6-AHA) were also found to elicit the conformational change, and those with an approximate 6 Å distance between the carboxyl and ϵ-amino function of the ligand were most effective (*27*). Maximum unfolding, characterized by an increase in the effective hydrodynamic radius of 15 K-IV Lp(a) from 135 to 177 Å, was achieved with either 4-ABA, 6-AHA, or t-AMCHA dissolved in 10 mM HEPES, 0.01% Na_2EDTA,

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[1] Abbreviations: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); apoB, apolipoprotein B; LDL, low-density lipoprotein; K, kringle; K-IV_{1-10}, apo(a) kringle domain of subtypes 1−10, all with homology to kringle 4 of plasminogen; 15 K-IV Lp(a), a Lp(a) particle containing an apo(a) molecule with 15 K-IV domains; LBS, lysine binding site; 4-ABA, 4-aminobutyric acid; 6-AHA, 6-aminohexanoic acid; t-AMCHA, *trans*-4-(aminomethyl)cyclohexanecarboxylic acid; Na_2EDTA, sodium salt of ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, sodium salt of *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HBS, HEPES-buffered saline.

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and NaN_3. However, inclusion of 100 mM NaCl in the buffer medium limited the unfolding caused by the above ligands by up to 50% and rendered the ligands glycine, 8-amino-octanoic acid, and N^{α}-acetyllysine totally ineffective (*27*).

The similarities between Lp(a) and (Glu-)plasminogen extend not only to the sequence homology and specificity to target proteins, but also to the ability of adopting an extended conformation on exposure to lysine analogues, and to the inhibition of this relaxed state and promotion of a compact conformation by NaCl. Salt inhibition of the unfolding of plasminogen was studied extensively by the laboratory of Castellino, who attributed the effect to the Cl^- anion and also demonstrated that it inhibited plasminogen activation (*29*−*33*). Inhibition by chloride was shown to be overcome at high concentrations of 6-AHA. The inhibitory effect of Cl^- was also found to extend to other anions, the magnitude of which approximated their position in the Hofmeister series. Indeed, weaker anions, such as acetate, permitted the expansion of plasminogen in the absence of 6-AHA. This finding represents one difference between plasminogen and Lp(a), since acetate promotes the compact conformation of Lp(a) (*27*).

The purpose of the current investigation is to more completely characterize the relationship between the binding of salts and the lysine analogue 6-AHA on the global configuration of Lp(a) and to gain insight into the disposition of apo(a) on the lipoprotein surface. The present report represents the key findings of this investigation.

EXPERIMENTAL PROCEDURES

Preparation of Lipoprotein(a). Lp(a) was purified from the plasma of four human subjects, all of whom gave informed consent prior to blood donation or plasmapheresis. One subject, whose plasma contained two apo(a) polymorphs having either 15 or 27 K-IV domains, at a respective relative abundance of 10 to 1, served as main donor for the preparation of Lp(a) and LDL. Lp(a) (15 K-IV) was isolated from plasma by a combination of lysine Sepharose chromatography and density gradient centrifugation using previously described methods (5, *34*, *35*). Lp(a) from each of the other donors was isolated similarly. The lipoprotein was dialyzed against 33 mM sodium phosphate containing 0.01% Na_2EDTA and NaN_3, pH 7.4, filter-sterilized, and stored in sterile 2 mL Sarsted vials filled to the top to allow no air space.

Lipoprotein purity was established by SDS−PAGE using 3.5% T, 2.66% C, 1.5 mm polyacrylamide gels in a Novex X Cell II gel electrophoresis apparatus (Novex, San Diego, CA). The Lp(a) particles were phenotyped by SDS−PAGE using the Phast system (Pharmacia) and conditions described by Molinari et al. (*36*). The Phast gels were calibrated using four different homogeneous Lp(a) particles of which the apo(a) molecular weight, ranging from 2.89×10^5 to 4.88×10^5, had been determined previously by sedimentation equilibrium (*37*). The number of K-IV domains in each apo(a) polymorph was calculated from the measured protein molecular weight after subtracting the portion (23%) due to carbohydrate (*37*).

Analytical Centrifugation. Sedimentation velocity experiments were performed with a Beckman Optima XLA ultracentrifuge interfaced to a Dell Optiplex XMT 590 personal computer, an AN-60 Ti four-place rotor, and analytical double-sector cells equipped with aluminum-filled Epon centerpieces and quartz windows (*26*). The rotor speed was 40 000 rpm, and the temperature was controlled at 20 °C. Both rotor and cells were preequilibrated to 20 °C before starting the sedimentation velocity runs. The cells were scanned at 8 min intervals; usually 16 data sets were collected after either an 8 or a 16 min delay. Data from sedimentation velocity experiments were analyzed using the second moment/boundary spreading program of the Optima XL-A data acquisition and analysis software, version 3.01 h (Beckman, Palo Alto, CA), in conjunction with Origin software, version 3.78 (Microcal Software, Northhampton, MA). Solvent density and viscosity were determined as described previously (*26*, *27*).

Before each set of sedimentation velocity experiments, Lp(a) was dialyzed against 100 mM 6-AHA, pH 7.0, or other appropriate media. To eliminate potential inhibitors of the unfolding of Lp(a), Na_2EDTA and NaN_3 were not added to the dialysate. Therefore, to prevent oxidation and bacterial degradation of Lp(a), the dialysate was saturated with oxygen-free nitrogen and the dialyzed Lp(a) was filter-sterilized and stored in 0.7 mL aliquots in Sarsted tubes with no air space. Once opened, a vial was kept for no longer than 24 h. The Lp(a) solutions were checked periodically by SDS−PAGE for proteolytic degradation and for increases in the absorbance at 234 nm, which is a measure of an increased diene content and of oxidation (*51*); none were found. Salts were added from 1−2 M stock solutions. The pH of individual working solutions was checked before and after centrifugation to ensure that values did not deviate from 7.0. For plots of the viscosity-corrected sedimentation coefficient (ηs) vs density or ligand concentration, depending on the experiment, the ligand or salt concentration ranged from 200 to 1000 mM.

Unlike the other salts tested, the effect of NaI on the sedimentation behavior of Lp(a) was difficult to evaluate because of great variability in $s'_{20,w}$ values. This appeared to be related to the period of time between making up NaI solutions and the sedimentation velocity runs, because those measured with older NaI solutions that started to attain a slightly yellow coloration gave usually lower s values. Only by minimizing this interval by making NaI solutions immediately before centrifugation runs could reliable sedimentation coefficients be obtained.

Data Analysis. Plots of ηs vs density, for each salt examined, were extrapolated to unity. These values were corrected to water at 20 °C using the relation:

$$s_{20,w} = s_{obs}\frac{\eta_s(1 - \bar{v}\rho_w)}{\eta_w(1 - \bar{v}\rho_s)}$$

where η_s and η_w are, respectively, the viscosities of the solvent and water at 20 °C; ρ_s and ρ_w are, respectively, the densities of solution and pure water at 20 °C; and $\bar{v}$ is the anhydrous lipoprotein partial specific volume determined in D_2O. The effective hydrodynamic radius of Lp(a), R_h, was calculated from the relation:

$$R_h = \frac{f}{6\pi\eta}$$

where f is the translational friction coefficient and η is the

solution viscosity. The frictional coefficient, *f*, was determined from the molecular weight and partial specific volume obtained by sedimentation equilibrium in D_2O, and the Svedberg equation:

$$f = \frac{M(1 - \bar{v}\rho)}{Ns}$$

where M is the molecular weight, $\bar{v}$ the partial specific volume, and s the sedimentation coefficient of the lipoprotein; ρ is the solvent density, and N is Avogadro's number. A value of 3.28×10^6 was used for the molecular weight of Lp(a) and a $\bar{v}$ of 0.9425 mL/g for the partial specific volume (*27*). Although these values are not constant for different Lp(a) preparations obtained from the same donor over time, the observed variability affects R_h by no more than 5% (*26*, *27*, *38*). Preferential hydration was obtained from the horizontal intercept of ηs vs density plots as described by Cox and Schumaker (*39*).

For the purpose of examining the effect of ligand or salt on the sedimentation coefficient of Lp(a), the hydrodynamic partial specific volume, $\bar{v}_h$, was used instead of $\bar{v}$ in correcting s_{obs}; $\bar{v}_h$ is the reciprocal of the hydrated density of Lp(a), i.e., $\bar{v}_h = 1/\rho_h$. This yields a slightly different corrected sedimentation coefficient, $s'_{20,w}$, which is defined by the following:

$$s'_{20,w} = s_{obs}\frac{\eta_s(1 - \bar{v}_h\rho_w)}{\eta_w(1 - \bar{v}_h\rho_s)}$$

Ligand titration data were analyzed using the Mac software Kaleidagraph and the appropriate equation for the relationship between ligand concentration and $s'_{20,w}$. A standard error for the binding parameters and limiting s values was calculated from a set of 10−15 data points by the curve-fitting routines.

Chemical Analysis. Protein content was determined by the method of Lowry et al. (*40*), as modified by Markwell et al. (*41*), using bovine serum albumin as standard, as previously described (*5*).

RESULTS

The binding of a number of ω-aminocarboxylic acids or other analogues of 6-AHA induces a conformational change in Lp(a), from a compact to an extended form, which can be detected by measuring the sedimentation coefficient of Lp(a) as a function of ligand concentration. In studying the specificity of this ligand-induced conformational change, we found that binding of salt present at relatively modest concentrations appeared to inhibit the change promoted by ω-aminocarboxylic acids (*27*). This finding indicated that two classes of small molecules, acting in opposition, were able to regulate the conformation of Lp(a). Consequently, the effect of binding 6-AHA and NaCl by Lp(a) was evaluated independently of each other, and under conditions where both molecules were bound to Lp(a).

Independent Effects of 6-AHA and NaCl on Lp(a) Conformation. The effect of 6-AHA on the conformation of 15 K-IV Lp(a) in the absence of salt is shown in Figure 1A. It is characterized by a remarkable expansion of Lp(a), represented by a decrease of nearly 5 S, from an $s'_{20,w}$ value of 11.5 S in water to an $s'_{20,w}$ of 6.8 S in saturating

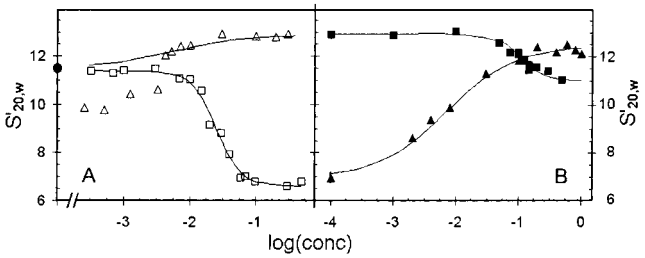

FIGURE 1: (A) Independent effects of 6-AHA and NaCl on the $s'_{20,w}$ of Lp(a). In the complete absence of salt, 6-AHA (open squares) induces expansion of Lp(a) as evidenced by a decrease in the sedimentation coefficient of 4.7 S from the value of 11.5 S in water (solid circle). In contrast, the addition of NaCl induces the formation of a compact structure as reflected by an increase of 1.4 S (open triangles). The effect on Lp(a) produced by both 6-AHA and NaCl is considered to be a consequence of reversible binding, except at NaCl concentrations lower than 5 mM where reduced sedimentation is most likely due to the primary charge effect (*44*). The solid lines represent a fit of the data to eqs 1 and 2, respectively. The derived binding parameters are shown in Table 1. (B) Effect of cobinding of NaCl and 6-AHA on the $s'_{20,w}$ of Lp(a). The data set representing the unfolding of Lp(a) in 100 mM NaCl upon the addition of 6-AHA (solid squares) was fit to eq 3. The other data set (solid triangles), representing the transformation of Lp(a) in 100 mM 6-AHA to a compact form by the addition of NaCl, was fit by eq 4. The derived binding parameters are shown in Table 1.

concentrations of 6-AHA. In the absence of 6-AHA, the addition of NaCl increased sedimentation more modestly to a value of 12.9 S which is characteristic of a more compact configuration. The concentration dependence of the 6-AHA mediated effect was analyzed in terms of the following equilibrium:

$$\text{Lp(a)} + \text{6-AHA} \overset{K_e}{\rightleftharpoons} \text{Lp(a)}_e\text{−(6-AHA)}_x$$

from which it follows that f_e, the fraction of Lp(a) in the expanded form, is determined by

$$f_e = (s_{max} - s)/(s_{max} - s_{min}) = 1/\{1 + (K_e/[\text{6-AHA}])^{n_e}\} \quad (1)$$

where K_e is the (midpoint) dissociation constant, n_e is the cooperativity index, s_{max} is the sedimentation coefficient at infinite 6-AHA dilution, and s_{min} is the sedimentation coefficient at infinite 6-AHA concentration.

Likewise, the effect of NaCl was analyzed according to

$$\text{Lp(a)} + \text{salt} \overset{K_c}{\rightleftharpoons} \text{Lp(a)}_c\text{−(salt)}_m$$

from which it follows that f_c, the fraction of Lp(a) in the compact form, is determined by

$$f_c = (s - s_{min})(s_{max} - s_{min}) = 1/\{1 + (K_c/[\text{salt}])^{n_c}\} \quad (2)$$

where K_c is the (midpoint) dissociation constant, n_c is the cooperativity index, s_{min} is the sedimentation coefficient at infinite salt dilution, and s_{max} is obtained at saturating salt concentration. A value of n greater than 1 is interpreted as positive cooperativity, a value equal to 1 as homogeneous binding, and a value less than 1 as negative cooperativity or heterogeneous binding (*42*).

Alteration of the sedimentation coefficient of Lp(a) is due to binding of either 6-AHA or NaCl, except for the effect of

Table 1: Characteristics of NaCl and 6-AHA Binding to Lp(a)[a]

	NaCl[b]					6-AHA[c]				
	no 6-AHA		in 100 mM 6-AHA			no NaCl		in 100 mM NaCl		
	K_c (mM)	n_c	K'_c (mM)	n'_c	K'_c (mM), calcd	K_n (mM)	n_e	K'_e (mM)	n'_e	K'_e (mM), cald
	4.7 ± 0.6	1.1 ± 0.2	7.6 ± 1.1	0.67 ± 0.06	9.6	24.2 ± 2.1	2.4 ± 0.4	108 ± 12	1.7 ± 0.3	100
s_{min} (S)	11.5 ± 0.1		7.1 ± 0.2			6.8 ± 0.2		10.8 ± 0.2		
s_{max} (S)	12.9 ± 0.0		12.6 ± 0.1			11.5 ± 0.2		13.0 ± 0.1		
Δs (S)	1.4 ± 0.1		5.5 ± 0.3			4.7 ± 0.4		2.2 ± 0.3		

[a] Values represent the mean ± standard error of the mean. [b] Binding parameters were determined from eq 2, except for the calculated K'_c, determined from eq 4. [c] Binding parameters were determined from eq 1, except for the calculated K'_e, determined from eq 3.

NaCl at concentrations lower than 5−10 mM where anomalously reduced sedimentation is most likely due to the primary salt effect (*43*). The minimum value at lower NaCl concentrations is taken to be the same as that found for H_2O and low concentrations of 6-AHA, a zwitterion which does not produce a primary charge effect. The data points for NaCl at concentrations lower than 5 mM were ignored, and only those at higher concentrations, as well as the minimum value, were used to evaluate the binding. This is shown graphically in Figure 1A where the data points below 5 mM NaCl deviate from the computer-fitted line generated at higher concentrations of NaCl. The quantitative characteristics of the independent interactions with Lp(a) of 6-AHA and NaCl appear in Table 1. 6-AHA binding exhibited positive cooperativity as indicated by a n of 2.4. NaCl binding, on the other hand, appeared homogeneous; however, the paucity of data below 5 mM NaCl does not permit a rigorous analysis.

We evaluated the effect of salt on the hydrodynamic properties of LDL by measuring its sedimentation coefficient at concentrations of NaCl ranging from 3.25 to 1000 mM. The resulting ηs vs ρ plot was linear, with no sign of curvature, indicating that LDL conformation was not affected by salt, in contrast to Lp(a).

Cobinding of 6-AHA and NaCl to Lp(a). The expanded form of Lp(a) was prepared by dialysis against 100 mM 6-AHA in order to study the reversal of this expansion from increasing concentrations of NaCl by measuring the sedimentation coefficient of Lp(a). The results are shown in Figure 1B along with those obtained from the addition of 6-AHA to Lp(a) in 100 mM NaCl. These data were fit to the same equations as above, and the binding parameters, designated with primes, are shown in Table 1. In each case, the affinity was diminished by the presence of the other ligand. NaCl binding became heterogeneous, as indicated by an n'_c of 0.67. Furthermore, it is clear that NaCl completely reverses the expansion of Lp(a) induced by 6-AHA. In contrast, 6-AHA is considerably less effective in reversing the effect of NaCl, unfolding Lp(a) by only about 30%.

A binding model consistent with the above results is shown in Figure 2. Ligand-free Lp(a) is designated $Lp(a)_o$, with $s'_{20,w}$ = 11.50 S. Interaction with NaCl yields a compact species, designated $Lp(a)_c$, with $s'_{20,w}$ = 12.90 S. Interaction with 6-AHA forms the expanded species, designated $Lp(a)_e$, with $s'_{20,w}$ = 6.80 S. Addition of salt, at sufficiently high concentration, to $Lp(a)_e$−$(6\text{-AHA})_n$ completely eliminates expansion, by competing with 6-AHA for binding. On the other hand, addition of high concentrations of 6-AHA to $(salt)_m$−$Lp(a)_c$ reverses the compact form by only 30%, to $s'_{20,w}$ = 11.10 S, presumably because salt binding renders the remaining 6-AHA sites inaccessible.

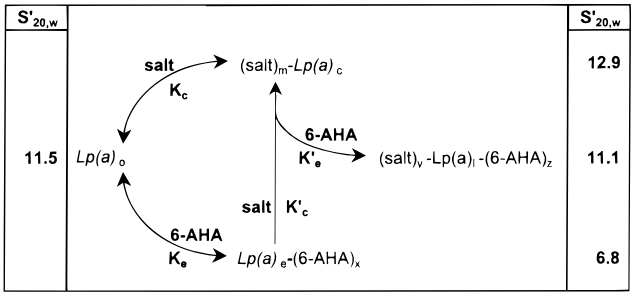

FIGURE 2: Relationship between binding-induced conformations of Lp(a). $Lp(a)_o$ is the "nascent" state in water with a sedimentation coefficient of 11.5 S. $Lp(a)_e$ denotes the expanded form with a reduced sedimentation coefficient of 6.8 S, induced by the binding of x moles of 6-AHA with a dissociation constant K_e. $Lp(a)_c$ represents the compact form with an increased sedimentation coefficient of 12.9 S, produced on binding m molecules of salt with a dissociation constant K_c. $Lp(a)_e$ can be transformed to $Lp(a)_c$ by the addition of excess salt. However, excess 6-AHA cannot completely convert $Lp(a)_c$ to $Lp(a)_e$; instead, an intermediate $Lp(a)_i$ is formed with a maximum z molecules of 6-AHA displacing salt so that y molecules of salt remain bound.

The above partially competitive binding model was used as the basis for evaluating the effect of each ligand on the binding characteristics of the other ligand. The competitive binding of 6-AHA in the presence of salt, in terms of fraction of accessible sites bound (f'_e), is therefore described by

$$f'_e = ([6\text{-AHA}]/K_e)^{n_e}/\{1 + ([salt]/K_c)^{n_c} + ([6\text{-AHA}]/K_e)^{n_e}\}$$

from which it follows that the apparent K'_e (the 6-AHA concentration at which f'_e equals 1/2) is determined by

$$K'_e = K_e\{1 + ([NaCl]/K_c)^{n_c}\}^{1/n_e} \quad (3)$$

Likewise, if f'_c is the fraction of $(salt)_m$−$Lp(a)_c$, in the presence of 6-AHA, and taking into account that 6-AHA is only partially (30%) competitive with NaCl binding, then it follows that

$$f_c = 0.7([salt]/K_c)^{n_c}/\{1 + ([salt]/K_c)^{n_c}\} + 0.3([salt]/K_c)^{n_c}/\{1 + ([salt]/K_c)^{n_c} + ([6\text{-AHA}]/K_e)^{n_e}\}$$

and K'_c ([NaCl] at $f'_c = 1/2$) is determined from the resulting quadratic equation:

$$0 = (K'_c/K_c)^{2n_c} + 0.4(K'_c/K_c)^{n_c}([6\text{-AHA}]/K_e)^{n_e} - \{1 + ([6\text{-AHA}]/K_e)^{n_e}\} \quad (4)$$

K' values, calculated from the above equation, for the case

Table 2: Hydrodynamic and Salt Binding Properties of Lp(a)

compounds	$s_{20,w}$ (S)	R_h (Å)	hydrated density[a] (g/mL)	K'_c[c]	n[c]
6-AHA	7.04 ± 0.23	242	1.048 ± 0.001		
NaCl	12.80 ± 0.08	133	1.058 ± 0.001		
NaF/6-AHA[b]	12.95 ± 0.08	132	1.059 ± 0.001	18 ± 2.9	0.95 ± 0.13
NaCl/6-AHA	12.93 ± 0.11	132	1.058 ± 0.001	7.6 ± 1.1	0.67 ± 0.06
NaI/6-AHA	12.42 ± 0.22	132	1.061 ± 0.001	57.1 ± 18.6	0.57 ± 0.09
LiCl/6-AHA	12.61 ± 0.20	135	1.055 ± 0.001	9.4 ± 1.6	0.87 ± 0.13
CsCl/6-AHA	13.29 ± 0.05	128	1.060 ± 0.001	7.1 ± 1.5	0.72 ± 0.10
Na phospate/6-AHA	12.37 ± 0.15	138	1.053 ± 0.001	7.1 ± 1.6	0.75 ± 0.13
Na glutamate/6-AHA	12.90 ± 0.02	132	1.054 ± 0.000	10.2 ± 2.2	0.81 ± 0.14
Na citrate/6-AHA	13.29 ± 0.24	128	1.053 ± 0.001	7.1 ± 1.9	0.87 ± 0.17

[a] Values represent the mean ± SEM. [b] The concentration of 6-AHA present in the different salt solutions was 100 mM. Binding parameters were determined from eq 2.

of 6-AHA in the presence of 100 mM NaCl and for the case of NaCl in the presence of 100 mM 6-AHA are shown in Table 1. Agreement with the experimental values supports the binding model.

Effect of Other Salts. A systematic study of the effect of different salts on the conformation of Lp(a) in 6-AHA was undertaken for comparison with the corresponding findings for plasminogen (*29*−*33*). Salts were selected to cover a range of effects, based on their relative strengths in inhibiting plasminogen unfolding, e.g., from sodium citrate, which was very weak, to NaI, which was a strong inhibitor (*31*). They were also chosen in order to determine whether the effect is, likewise, primarily due to anions. The results of this investigation are presented in Table 2. The $s'_{20,w}$ values of Lp(a) in 6-AHA and NaCl, representing the respective fully extended and compact states, were determined by extrapolation from ηs vs ρ plots. Only the linear portion of these plots was used, usually corresponding to ligand concentrations ranging from 200 to 1000 mM. The table also lists the hydrated densities necessary for determining the effective hydrodynamic radius and $s'_{20,w}$ values. The effects of different salts on the $s'_{20,w}$ values of Lp(a) in 100 mM 6-AHA, representing the most compact state, were obtained similarly and are given in Table 2. All the salts tested were equally effective inhibitors of Lp(a) expansion as indicated by $s'_{20,w}$ and R_h values that were within 4% of the minimum value obtained with NaCl in the absence of 6-AHA. This small variation in R_h appears insignificant compared to the 82% increase achieved when Lp(a) is allowed to expand in 6-AHA in the absence of salt.

The dissociation constants determined for the different salts are also shown in Table 2. As with the effectiveness in decreasing the hydrodynamic radius, the binding affinity and cooperativity index of all of the salts tested were approximately equivalent, except for NaF and NaI. The effects produced do not seem to be determined by specific properties of anions or cations. However, among the halides, the affinity for chloride was greatest.

Effect of K-IV Number on the $s'_{20,w}$ of Lp(a) in the Absence of Salt. As shown previously (*26*), in 100 mM NaCl, the expansion induced by high concentrations of 6-AHA involves the release of only the variable portion of apo(a) represented by the K-IV_2 domains. This was demonstrated by plotting either the sedimentation coefficient or the frictional ratio of both the compact (100 mM NaCl) and extended (100 mM NaCl/saturating concentrations of 6-AHA) forms of Lp(a) as a function of K-IV number. The lines defining the two states intersected at a K-IV number between 7 and 8, the point at which Lp(a) in either medium has the same shape because of equal sedimentation and frictional coefficients. Hence, it was concluded that the constant portion of apo(a), represented by the K-IV_{3-9} domain, did not participate in the expansion of Lp(a) but remained apposed to the lipoprotein surface (*26*).

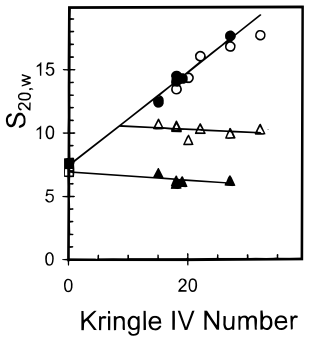

FIGURE 3: Effect of salt and 6-AHA on the sedimentation of Lp(a) as a function of K-IV number. The sedimentation coefficients of different Lp(a) phenotypes dissolved in saturating concentrations of either NaCl (solid circles) or 6-AHA (solid triangles) were plotted as a function of number of K-IV domains. Data points obtained in NaCl (open circles) and in 100 mM NaCl and saturating concentrations of 6-AHA (open triangles) were taken from a previous study (*26*). Results for LDL in NaCl (closed square) or in 6-AHA (open square) are plotted at a K-IV number of 0. The lines represent linear regression of the data. Note that in the presence of NaCl, the line depicting the 6-AHA data intersects the line for the NaCl data at 8 rather than 0 kringles. The significance of this is discussed in the text.

In a manner similar to that described above, we evaluated the extent of expansion of Lp(a) induced by 6-AHA in the total absence of salt, by determining the sedimentation coefficient of the compact and expanded forms of Lp(a) as a function of K-IV number in saturating concentrations of either NaCl or 6-AHA. If Lp(a) were fully unfolded, then the lines should intersect at zero, and as the results of Figure 3 show, and taking into account experimental error, this appears to be the case, thus demonstrating that 6-AHA completely unfolds Lp(a) in the absence of salt. Figure 3 includes data from the previous study and clearly shows that the data points obtained for the compact form of Lp(a) in NaCl from both studies overlapped. In contrast, the data set of 6-AHA-expanded Lp(a) (in the absence of NaCl) produced a line which was displaced by 4 S from the line obtained when the 6-AHA-induced expansion of Lp(a) was partially inhibited by NaCl. The figure also clearly shows the movement of the intersection point from 8 K-IV's for the partially inhibited Lp(a) to 0 K-IV's for the fully extended

form of Lp(a) that is obtained in the complete absence of salt. The results given for a kringle number of 0 are for LDL obtained either in NaCl or in 6-AHA. The nearly identical values demonstrate that LDL conformation is not affected by either agent and that changes observed with Lp(a) must be due to changes in apo(a) conformation.

DISCUSSION

We previously showed that salt and ω-aminocarboxylic acids have opposite effects on the conformation of Lp(a) (*27*). These studies were carried out in a background of buffer and preservative salts. However, because of potentially confounding effects, we eliminated them in order to determine the sole effect of NaCl and 6-AHA on the structure of Lp(a). In the absence of salts, the sedimentation coefficient of Lp(a) was 11.5 S, a value consistent with a conformation intermediate between the compact and expanded states of the lipoprotein (see Figure 1A), and a R_h of 157 Å. The addition of NaCl effected a modest increase in the sedimentation velocity of Lp(a), consistent with a more compact conformation and a R_h of 133 Å, as a consequence of homogeneous binding. In contrast, 6-AHA alone reduced the sedimentation velocity of Lp(a) by nearly 5 S, consistent with enormous expansion and a R_h of 242 Å, as a consequence of cooperative binding. The ligand-induced conformational changes of Lp(a) are considerably greater than those of plasminogen (*21*) and are, in fact, some of the largest ligand-induced conformational changes of any protein known to date. The striking difference in the magnitude of the change between plasminogen and Lp(a) is most probably due to the larger size of apo(a) used here (16 kringles), compared to plasminogen with only 5 kringles. Lp(a)s with longer apo(a) would produce an even greater effect.

We also studied the binding characteristics and the effect on Lp(a) conformation of each ligand in the presence of the other. As shown in Figure 1B and Table 1, the addition of either ligand reduced the affinity of, and reversed the effect on conformation induced by, the other ligand. Though NaCl totally reversed the effect of 6-AHA on Lp(a) conformation, 6-AHA only incompletely reversed the effect of NaCl. These results suggested a partially competitive relationship between the two ligands in binding to Lp(a), and a corresponding binding model (Figure 2) successfully accounted for the results. An additional finding was that NaCl binding became heterogeneous in the presence of 6-AHA. Apparently, all of the 6-AHA sites are shared by, or are identical to, or are influenced by, NaCl sites. On the other hand, only some of the NaCl sites are shared by the 6-AHA sites.

The effect of salt on the conformation of Lp(a) is similar in part to the role Cl^- and other anions have in determining the structure of plasminogen (*33*). As with Lp(a), NaCl, or more specifically the Cl^- anion, induces Glu-plasminogen to take on a compact conformation that is resistant to proteolytic activation. However, on binding ω-aminocarboxylic acids, plasminogen expands and becomes more flexible, a conformation which allows a much more rapid activation. A mechanism proposed for the inhibitory effect of Cl^- was that it competes with the carboxyl moiety of 6-AHA for interacting with Arg_{70} in the LBS of both K1 and K4.

Like with plasminogen, the Cl^- binding sites on Lp(a) also bind a number of other anions. In contrast to the findings

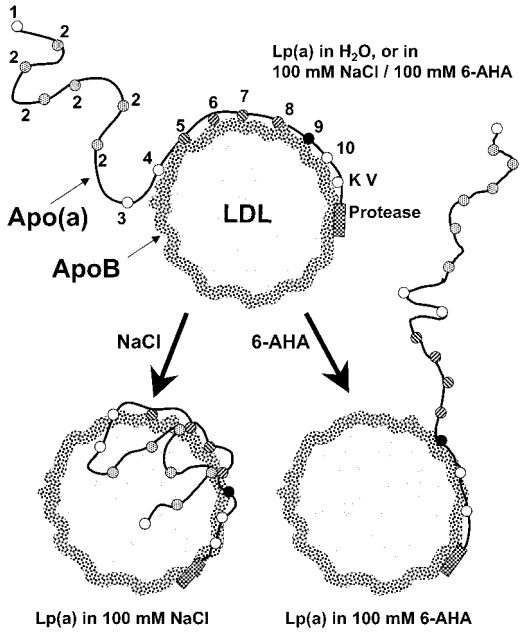

FIGURE 4: Model depicting the different conformational states of Lp(a) in water, salt, and/or 6-AHA. The numbers in the upper molecule represent the types of K-IV domains of apo(a). ApoB is represented by a stippled belt around the LDL moiety of Lp(a).

with plasminogen, all salts inhibited the 6-AHA-induced expansion of Lp(a) to a similar extent and had similar affinities (see Table 2). Hence, Lp(a) is in a compact state in the presence of almost any salt, in contrast to plasminogen, which is in the expanded state in some salts, such as citrate and acetate. These observations lead us to conclude that the effect of salts in inhibiting the 6-AHA-induced expansion of Lp(a) and in inducing the compact state is not related to position in the Hofmeister series, and that the effects cannot be ascribed specifically to either anions or cations. However, if the effect of salt in promoting the compact conformation is indeed due to anions, as observed with plasminogen, then anion binding must be nonspecific.

Earlier, we found that the 8 K-IVs of the constant region of apo(a) remained apposed on the Lp(a) surface and only the variable region kringles were released to contribute to frictional resistance in the presence of 6-AHA and 100 mM NaCl. The R_h of the 15 K-IV Lp(a) was 162 Å under this condition. We determined the extent of apo(a) unfolding in the absence of salt, in a similar manner, by plotting the sedimentation coefficient of Lp(a) in both 6-AHA and NaCl as a function of K-IV number. In contrast to the previous result where the lines generated by the two different conditions intersected at a K-IV number of 8, the intersection at a K-IV number of 0 (Figure 3) demonstrated that apo(a) was completely released from the Lp(a) surface up to the disulfide linkage with apoB in $K\text{-}IV_9$.

A structural model of Lp(a) summarizing our findings is shown in Figure 4, with the putative conformation of apo(a) determined by the ligand present in the medium. In 6-AHA, and the absence of salt, apo(a) unfolds maximally. Though, it is possible that some inter-kringle interactions remain in 6-AHA, because we have found that saturating concentrations of either 4-ABA or t-AMCHA unfold Lp(a) somewhat more (Fless, G. M., unpublished observations).

In the partially expanded conformation, formed upon the addition of 6-AHA to Lp(a) in NaCl, the variable domain

of apo(a) is released from the surface of the Lp(a) particle with the constant domain remaining apposed. A similar sedimentation rate suggests a similar conformation in water.

In 100 mM NaCl, both the variable and constant domains of apo(a) are apposed to the lipoprotein surface. This structure is supported by a frictional ratio for Lp(a) of 1.2 (*26*), a value consistent with a compact shape of 133 Å, only about 30% greater that of LDL, despite a larger lipid core and the presence of the highly glycosylated and hydrated apo(a), and also by small-angle X-ray scattering studies (*44*). Furthermore, because the frictional ratio is constant regardless of apo(a) phenotype, the variable moiety of apo(a), consisting of K-IV_2 domains, cannot project into the medium but instead must interact with the surface.

In apparent contradiction to these conclusions are results from reassembly studies in salt, seeming to suggest a lack of interaction between the variable domain and LDL (*45*−*47*), and that 6-AHA inhibits the interaction of the constant domain with apoB (*13*−*16*, *26*). However, these results revealed that in the first step of Lp(a) formation, i.e., the noncovalent association of apo(a) and LDL, the contribution by the variable domain is not absent, even though it is small (*46*). This finding is not in contradiction with, but indeed supports, the physicochemical studies, which clearly show that the variable domain is apposed to the particle surface, though perhaps binding relatively weakly. These results also demonstrated that 6-AHA prevented the noncovalent association of the constant domain of apo(a) with LDL, seeming to suggest that 6-AHA would totally unfold Lp(a). However, since we found that 6-AHA in the presence of salt did not release the constant domain, its LBS apparently are not accessible to 6-AHA in native Lp(a) as they are in free apo(a). Indeed, it has been reported that some apo(a)-LBS are masked in intact Lp(a) (*17*). In essence, salt strengthens the interaction between the two apoproteins, thereby preventing access of 6-AHA to these LBS. An involvement of Cl^- in strengthening inter-kringle interactions by binding at the edges of the interface near LBS has been proposed (*48*−*50*). It is not immediately obvious how chloride could prevent access of 6-AHA to LBS in an apparently irreversible manner, for chloride binding itself is clearly a reversible process and during periods of dissociation/reassociation 6-AHA may gain access to the LBS and thereby disrupt the interaction between apo(a) LBS and the apoB complementary ligand. However, because the dissociation rate of this complex is undoubtedly much slower than the reassociation rate of chloride, the LBS are masked, i.e., never exposed for interaction with 6-AHA. Another set of unmasked LBS is available for interaction with 6-AHA in the presence of salt.

Apposition of apo(a) with the particle surface in 100 mM NaCl requires interaction of both the constant and variable domains with LDL. The interaction of the constant domain of apo(a) with apoB involves relatively specific and strong electrostatic and hydrophobic forces between K-IV LBS and certain apoB amino acid residues (*13*−*17*), which we found to be disrupted by 6-AHA only in the absence of salt.

Though the variable domain of apo(a) must interact with LDL based on previous (*26*) and present hydrodynamic studies and by small-angle X-ray scattering (*44*), the nature of the interaction and the exact arrangement of the variable moiety of apo(a) on the particle surface in 100 mM NaCl are unknown. However, there are several possibilities: the variable portion of apo(a), consisting of K-IV_2 domains, may interact with apoB and/or the lipid surface; alternatively, it may interact with the constant moiety by folding over or by encircling the Lp(a) particle.

Unfolding of the variable region by the removal of salt (i.e., in H_2O) suggests a role of salt in promoting hydrophobic interactions, and perhaps in shielding electrostatic repulsion. The net interaction force is likely weak and nonspecific and may be with the lipid surface even though we have shown that complete hydrolysis of phosphatidylcholine by phospholipase A_2 has no effect on the conformation of Lp(a) in either NaCl or 6-AHA (*51*). Interactions with remaining ionic/polar moieties may still occur. Nevertheless, the release of the variable domain of apo(a) from the lipoprotein surface by 6-AHA in 100 mM NaCl is curious since K-IV_2 apparently contains neither a functional LBS (*52*) nor lysine residues (*6*). This paradox can be resolved by suggesting, as in the case of plasminogen (*32*, *33*), that arginine (together with a proximate aspartate residue) may form a "ligand" capable of interacting with a LBS, so that the variable region of apo(a) folds back over the constant portion where some K-IVs of the K-IV_{5-8} domain interact with the variable domain and the others interact with apoB (as depicted in Figure 4). It is also possible that apo(a) extends completely around the Lp(a) particle so that the tail end of apo(a) interacts with LBS in the same manner as suggested above. It has been shown that the structure of a 17 K-IV free apo(a) is highly asymmetric and extended with a length of 800 Å (*53*). Therefore, most apo(a)s, which range in size from 12 to 51 K-IV domains, are sufficiently long to encircle an LDL particle with a circumference of 600−700 Å. In fact, many apo(a)s are considerably longer than the 17 K-IV variant, and the largest apo(a) could in theory encircle LDL up to 3 times. It is not clear whether such a structure is possible because extension of apo(a) on the LDL surface is most likely limited since interactions of the variable domain with the lipid surface and/or apoB are nonspecific so that the "head to tail" distance would be determined by "random walk" considerations. Although the findings of Phillips et al. (*53*) indicate that there are few if any interactions between different domains within free apo(a) in solution, it is conceivable that when apo(a) is surface-bound that kringle−kringle interactions of the type proposed by Mulichak et al. (*48*) and Padmanabhan et al. (*54*) occur which would shorten the total length of apo(a).

The finding of positive cooperativity and a Hill coefficient with a value of approximately 2 for the binding of 6-AHA to two different sets of LBS implies that there are at least two sites for each set. Since the LBS II domain of apo(a) most likely contains a total of four LBS, one each per K-IV_{5-8} kringle, then each set of LBS must contain two sites. The masked LBS interact with apoB and the unmasked interact with K-IV_2. The proposed model is in keeping with the putative manner in which Lp(a) interacts with fibrin under physiological conditions. Binding of fibrin C-terminal lysines to Lp(a) is thought to involve LBS of the K-IV_{5-8} domain (*17*). Some of these are masked, because Lp(a) has fewer binding sites for fibrin than does apo(a). In our model, it is the masked LBS which interact with apoB and only the remainder which interact with K-IV_2, 6-AHA, or fibrin.

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