Oxidation of apolipoprotein(a) inhibits kringle-associated lysine binding: The loss of intrinsic protein fluorescence suggests a role for tryptophan residues in the lysine binding site

ANITA HERMANN,¹ WILLIAM R. LAWS,² AND PETER C. HARPEL^{1,2}

¹Division of Hematology, Department of Medicine, The Mount Sinai School of Medicine, New York, New York 10029

²Department of Biochemistry, The Mount Sinai School of Medicine, New York, New York 10029

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Abstract

Lipoprotein(a) [Lp(a)] is a low-density lipoprotein complex consisting of apolipoprotein(a) [apo(a)] disulfide-linked to apolipoprotein B-100. Lp(a) has been implicated in atherogenesis and thrombosis through the lysine binding site (LBS) affinity of its kringle domains. We have examined the oxidative effect of 2,2'-azobis-(amidinopropane) HCl (AAPH), a mild hydrophilic free radical initiator, upon the ability of Lp(a) and recombinant apo(a), r-apo(a), to bind through their LBS domains. AAPH treatment caused a time-dependent decrease in the number of functional Lp(a) or r-apo(a) molecules capable of binding to fibrin or lysine-Sepharose and in the intrinsic protein fluorescence of both Lp(a) and r-apo(a). The presence of a lysine analogue during the reaction prevented the loss of lysine binding and provided a partial protection from the loss of tryptophan fluorescence. The partial protection of fluorescence by lysine analogues was observed in other kringle-containing proteins, but not in proteins lacking kringles. No significant aggregation, fragmentation, or change in conformation of Lp(a) or r-apo(a) was observed as assessed by native or SDS-PAGE, light scattering, retention of antigenicity, and protein fluorescence emission spectra. Our results suggest that AAPH destroys amino acids in the kringles of apo(a) that are essential for lysine binding, including one or more tryptophan residues. The present study, therefore, raises the possibility that the biological roles of Lp(a) may be mediated by its state of oxidation, especially in light of our previous study showing that the reductive properties of sulfhydryl-containing compounds increase the LBS affinity of Lp(a) for fibrin.

Keywords: apolipoprotein(a); essential tryptophans; kringles; lipoprotein(a); lysine binding site affinities; oxidation; protein fluorescence

Lipoprotein(a) is a low-density lipoprotein-like particle first described by Berg (1963); it is found only in humans and old world monkeys (Utermann, 1989). Although its physiological functions have not been elucidated, elevated concentrations of Lp(a) have

been shown to be a major risk factor for coronary and carotid arteriosclerosis leading to myocardial infarction and stroke (reviewed in Harpel et al., 1995; Bostom et al., 1996). Lp(a) differs from LDL by having apolipoprotein(a), a glycoprotein of variable size, disulfide linked to the apolipoprotein B-100 component of LDL (Gaubatz et al., 1987). Apo(a) is a member of the kringle-containing superfamily that comprises blood coagulation Factor XII, prothrombin, plasminogen, tissue plasminogen activator, urinary plasminogen activator, and hepatocyte growth factor (Ichinose, 1992). In this family, apo(a) is unique in that it has partial homology with plasminogen (McLean et al., 1987). Apo(a) from the individual first studied contained 37 copies of kringle IV of plasminogen with 75–85% amino acid sequence identity, one copy of kringle V of plasminogen, and the protease domain of plasminogen, but with an arginine to serine substitution to prevent its activation (McLean et al., 1987).

The mechanisms involved in the atherogenic potential of Lp(a) are not clear, but the kringle domains of apo(a) have been implicated based on clinical studies (Shandholzer et al., 1992). In vitro

Reprint requests to: Peter C. Harpel, Division of Hematology, Box 1079, The Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, New York 10029; e-mail: pharpel@smtplink.mssm.edu; or William R. Laws, Department of Biochemistry, Box 1020, The Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, New York 10029; e-mail: wlaws@smtplink.mssm.edu.

Abbreviations: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); r-apo(a), recombinant apo(a); LDL, low-density lipoprotein; HDL, high-density lipoprotein; LBS, lysine binding site; ELISA, enzyme-linked immunosorbent assay; AAPH, 2,2'-azobis-(2-amidinopropane) dihydrochloride; ϵ ACA, ϵ -amino-*n*-caproic acid; AMCHA (or tranexamic acid), trans-4-(aminomethyl)-cyclohexanecarboxylic acid; pNGB, *p*-nitrophenyl-*p'*-guanidinobenzoate; PMSF, phenylmethylsulfonylfluoride; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid; BCA, bicinchoninic acid assay; BSA, bovine serum albumin; tPA, tissue plasminogen activator; r-tPA, recombinant tPA; PAMBA, *p*-aminomethylbenzoic acid; NATA, *N*-acetyl-D-tryptophanamide.

studies have shown that the kringle domains of apo(a) participate in the interactions between Lp(a) and cells (Miles et al., 1989), and between Lp(a) and fibrin (Harpel et al., 1989; Loscalzo et al., 1990). Because plasminogen kringles I and IV bind lysine and play a role in the interaction of plasminogen with substrates, plasmin inhibitors, and cell receptors, it has been suggested that the kringles of apo(a) also have lysine binding abilities that could play a role in its atherogenicity (Miles & Plow, 1990). Three lines of evidence support this hypothesis. First, Lp(a) interacts with lysine-affinity columns and is eluted by lysine analogues (Hoover-Plow et al., 1993). Second, we have demonstrated that Lp(a) binding to immobilized plasmin-degraded fibrin can be inhibited by ϵ ACA, a lysine analogue, and that the apo(a) portion of Lp(a) was responsible for these LBS interactions (Harpel et al., 1989). Finally, recent data derived from a transgenic mouse model suggest that an apo(a) mutation that causes loss of binding to lysine-affinity columns also inhibits the accumulation of apo(a) in the mouse aorta (Boonmark et al., 1996).

We have previously shown that the reductive capacity of sulfhydryl-containing compounds increases the relative affinity between Lp(a) and plasmin-modified fibrin approximately 20-fold, and that this increase was dependent upon LBS interactions (Harpel et al., 1992). We now report that oxidation of Lp(a) and apo(a) with a mild free radical initiator inhibits their ability to bind to lysine affinity columns and to plasmin-modified fibrin surfaces. In parallel with loss of lysine binding, we have found that oxidation caused a time-dependent loss of intrinsic tryptophan fluorescence from Lp(a) and apo(a). Lysine analogues known to bind to kringle structures prevent this inhibition of binding and protect against some of the protein fluorescence loss. Because studies show that the loss of binding is not due to gross changes in protein structure, it appears that one or more essential amino acids in the LBS are

altered. The fluorescence results suggest that one or more tryptophan residues in the LBS could be important for the initial binding of the apo(a) portion of Lp(a) to fibrin or lysine affinity columns.

Results

Effect of AAPH

The hydrophilic free radical generator AAPH has been shown to oxidize lipoproteins and cell membranes (Niki, 1990). We have tested the effect of AAPH on the lysine-dependent binding of Lp(a) by examining the capacity of AAPH-treated Lp(a) preparations to bind to lysine-Sepharose. The elution profile of Lp(a) incubated with buffer, compared to Lp(a) incubated for 2 h with 1 mM AAPH, is shown in Figure 1A. Analysis of the area under the peaks showed that 95% of the untreated Lp(a) bound to lysine-Sepharose, whereas only 6% bound after 2 h of AAPH treatment. Figure 1B demonstrates that there is a direct correlation between the decrease in the ability to bind and the AAPH incubation time. AAPH treatment also inhibited the binding of r-apo(a) to this resin. In the absence of treatment, 94% of the r-apo(a) bound to the lysine-Sepharose column, whereas only 57% r-apo(a) bound after 2 h of incubation with 1 mM AAPH (data not shown).

Our prior studies have established that the binding of Lp(a) to fibrin depends upon carboxy-terminal lysine residues produced by the proteolytic activity of plasmin (Harpel et al., 1989). To investigate the effect of AAPH on Lp(a) and its interaction with fibrin, fibrinogen was coated on the wells of microtiter plates and treated with thrombin and plasmin. Lp(a), previously reacted with AAPH at 37°C for varying periods, was then incubated with the immobilized fibrin with or without the inclusion of 0.05 M ϵ ACA. The amount of bound Lp(a) was quantified by an assay using a poly-

Fig. 1. A: Binding of Lp(a) to lysine-Sepharose; Lp(a) (500 μ g/mL) was incubated for 2 h at 37°C in the presence (●) or absence (○) of 1 mM AAPH. Arrow indicates the addition of 0.2 M ϵ ACA to elute the bound protein. B: Percent Lp(a) bound (●) as a function of AAPH incubation time, with the same experimental conditions as in A.

Fig. 2. Binding of AAPH-treated Lp(a) or r-apo(a) to plasmin-modified fibrin as detailed in Materials and methods. **A:** Control Lp(a) (■); 500 $\mu\text{g/mL}$ Lp(a) incubated with 1 mM AAPH for 0.5 h (▲) or 2 h (▼) at 37°C. **B:** Control apo(a) (■); 108 $\mu\text{g/mL}$ apo(a) treated with 2 mM AAPH for 1 h (▲) or 2 h (▼) at 37°C.

clonal rabbit anti-apo(a) IgG labeled with alkaline phosphatase (Harpel et al., 1989). As shown in Figure 2A, the specific, lysine-dependent binding of Lp(a) to plasmin-degraded fibrin was inhibited in a time-dependent process by AAPH treatment. These binding data were analyzed by the method of Eisenthal and Cornish-Bowden (1974); the results indicate that the loss in binding was due to a reduction in the number of functional Lp(a)s and not a reduction in binding affinity ($K_d \sim 3$ nM). Similar results were recovered from the EBDA ligand radioligand binding program of G.A. McPherson (Elsevier-Biosoft, Cambridge, UK).

Fibrin binding experiments were also performed with r-apo(a), which binds about 50–100 times tighter compared to Lp(a). As shown in Figure 2B, AAPH treatment of r-apo(a) also inhibited this binding. With r-apo(a), however, the rate of loss of binding capacity appeared to be slower than for Lp(a), especially because the r-apo(a) experiments were done with twice the AAPH concentration. As with Lp(a), analysis of the data suggested that the reduced binding of r-apo(a) to fibrin was due to a time-dependent decrease in the maximum number of molecules bound. There was a small increase in K_d after 2 h of AAPH treatment compared to no treatment and 1 h of AAPH, from 30 to 90 pM, but this change is within the errors of the experiment and analysis.

In these fibrin studies, the binding of Lp(a) or AAPH-treated Lp(a) was measured by the reactivity of an antibody against Lp(a). To test the possibility that AAPH might effect the antigenicity of Lp(a), increasing concentrations of Lp(a), or Lp(a) treated with 1 mM AAPH for 30 or 120 min, were added to microtiter wells coated with rabbit anti-apo(a). After incubation and washing, the polyclonal anti-Lp(a) IgG labeled with alkaline phosphatase was added. No significant difference in the binding or detection of treated Lp(a) as compared to untreated controls was observed. Thus, the impairment in binding of AAPH-treated Lp(a) to plasmin-modified fibrin was not due to a loss of structure leading to a

decrease in immunologic responsiveness. Similar findings were obtained with r-apo(a).

Oxidation of proteins can cause a decrease in intrinsic protein fluorescence, indicating destruction of tryptophan residues (Davies et al., 1987). We therefore examined the effect of AAPH on the intrinsic tryptophan fluorescence of Lp(a) and r-apo(a). The fluorescence emission spectrum (uncorrected) of Lp(a), illuminating at 295 nm to avoid exciting tyrosines, exhibited a maximum near 350 nm (Fig. 3). This spectrum for Lp(a) is at higher energies, or blue-shifted, compared to the spectrum obtained for the tryptophan model compound NATA. The emission spectrum of r-apo(a) is

Fig. 3. Uncorrected fluorescence emission spectra of Lp(a) and r-apo(a) before and after oxidation by 2 mM AAPH for 1 h at 37°C. Curve a, NATA; curve b, r-apo(a); curve c, Lp(a); curve d, oxidized Lp(a); and curve e, oxidized r-apo(a). For comparison purposes, the time zero Lp(a) and r-apo(a) spectra and the NATA spectrum have been peak normalized.

very similar to that for Lp(a) (Fig. 3), but with a small shift to lower energies (higher wavelength). Upon addition of AAPH to Lp(a) or r-apo(a), a progressive decrease in fluorescence intensity was observed as a function of time without any significant change in the shape or position of the spectrum; the fluorescence emission spectra of AAPH-treated Lp(a) and r-apo(a) are also represented in Figure 3.

To obtain more information about the reaction, 100 nM Lp(a) was incubated at 37 °C with varying concentrations of AAPH and the intrinsic tryptophan fluorescence was monitored at 345 nm as a function of time. Following addition of AAPH, there was a progressive decrease in fluorescence intensity (Fig. 4). The rate of loss was also dependent upon the AAPH concentration. At 1 and 2 mM AAPH, the decrease in fluorescence appeared to be a first-order process; at 4 and 8 mM AAPH, however, the decrease in $\ln$ (% fluorescence) was nonlinear with time as indicated using a linear regression. All subsequent loss of fluorescence experiments were performed at the lower AAPH concentrations, where a linear regression is sufficient for the relative effects reported.

Effect of Trolox

Our studies suggested that the loss of binding and intrinsic protein fluorescence was due to the free radical-generating effects of AAPH. To rule out other inhibitory mechanisms, Lp(a) was incubated with AAPH in the presence of the antioxidant Trolox, a water-soluble vitamin E analogue (Cao et al., 1993). Trolox (100 μ M) was found to preserve >95% of the Lp(a) binding affinity to lysine-Sepharose when treated with 1 mM AAPH for 2 h at 37 °C (data not shown). To insure that AAPH was not exerting its effect on Lp(a) intrinsic fluorescence by another mechanism, such as being a collisional quenching agent, Lp(a) and r-apo(a) were monitored in the fluorometer in the presence of both AAPH and Trolox. As shown in Figure 5, 100 μ M Trolox prevented the decrease in intrinsic protein fluorescence caused by the interaction of Lp(a) or r-apo(a) and 2 mM AAPH. Further evidence indicating that Trolox overcomes the effect of AAPH is found from its ability to inhibit lipid hydro-

Fig. 4. Change in intrinsic fluorescence upon addition of AAPH to Lp(a): 100 μ g/mL Lp(a) was incubated in buffer (0.05 M phosphate, pH 7.4) containing 0 mM (■), 1 mM (▲), 2 mM (▼), 4 mM (◆), or 8 mM (*) AAPH at 37 °C. Lines represent linear least-squares analyses assuming that the intercepts are $\ln(100)$ and first-order kinetics apply (see text).

Fig. 5. Effect of Trolox on the change in intrinsic tryptophan fluorescence of Lp(a) and r-apo(a) induced by AAPH; 100 μ g/mL Lp(a) (▲, △) or r-apo(a) (▼, ▽) was incubated with 2 mM AAPH at 37 °C in the presence (solid symbols) or absence (open symbols) of 100 μ M Trolox. Lines represent linear least-squares analyses assuming that the intercepts are $\ln(100)$.

peroxide formation in Lp(a). In the absence of Trolox, 1 mM AAPH incubated with Lp(a) at 37 °C for 2 h produced an increase of 42 nmol lipid hydroperoxides/mg Lp(a) measured as described (El-Saadani et al., 1989). In the presence of Trolox, however, there was no increase in lipid hydroperoxide formation under similar conditions. Background lipid hydroperoxide concentrations in Lp(a) ranged from 0 to 10 nmol/mg protein.

Protection by lysine analogues

The ability of AMCHA, a lysine analogue known to interact with kringles and inhibit lysine binding, to preserve Lp(a) binding to lysine-Sepharose during treatment with AAPH was examined next. As shown in Figure 6, Lp(a) treated with 1 mM AAPH for 1 h at 37 °C reduced the binding from 95% (Fig. 1A) to 24%. The presence of 100 mM AMCHA during the incubation allowed 70% of the Lp(a) to retain its affinity for the matrix. In contrast, AMCHA did not alter the amount of lipid hydroperoxides formed.

To test whether lysine analogues could also protect against the loss of intrinsic protein fluorescence, the fluorescence of 100 μ g/mL (100 nM) Lp(a) incubated at 37 °C in the presence of 2 mM AAPH was measured as a function of time. As shown in Figure 7, the ability of AAPH to decrease Trp fluorescence was reduced when lysine analogues were also present in the incubation mixture. In the absence of a lysine analogue, there was a 55% loss of protein fluorescence based on the slope of its fit (Fig. 7). In contrast, only 25% and 39% of the fluorescence was lost after 2 h incubation in the presence of either 100 mM AMCHA or ϵ ACA, respectively.

Other proteins were examined to establish whether the protection by lysine analogues is a general phenomenon or is specific to Lp(a). AMCHA was able to protect not only r-apo(a) (Fig. 8A), but also Lys-plasminogen (Fig. 8B), which binds lysines through its kringle domains (Sottrup-Jensen et al., 1978), from the AAPH-induced loss of fluorescence. However, two proteins that lack kringle structures, BSA (Fig. 8C) and LDL (Fig. 8D), are not protected from loss of intrinsic protein fluorescence by the presence of

Fig. 6. Lysine-Sepharose binding of Lp(a) incubated for 2 h with 2 mM AAPH at 37 °C in the absence (●) or presence (○) of 100 mM AMCHA. Arrow indicates the addition of 0.2 M ϵ ACA to elute the bound protein.

AMCHA. Table 1 summarizes our data concerning the capacity of this lysine analogue to protect against oxidation-induced loss of protein fluorescence. The values presented are a ratio of the rate of loss of fluorescence in the presence of AMCHA compared to the rate of loss in its absence. Proteins known to have kringles with

Fig. 7. Effect of two lysine analogues on the change in intrinsic fluorescence upon the addition of AAPH; 100 μ g/mL of Lp(a) was incubated with 2 mM AAPH at 37 °C in the absence of a lysine analogue (■) or in the presence of 0.1 M ϵ ACA (▲) or 0.1 M AMCHA (●). Lines represent linear least-squares analyses assuming that the intercepts are $\ln(100)$.

lysine binding activity [Lp(a), r-apo(a), plasminogen, and tPA] demonstrated protection by AMCHA from loss of fluorescence with ratios from 0.4 to 0.57. In two proteins containing kringles without known lysine binding activity (prothrombin and Factor XII), the protection by AMCHA was less (0.67 and 0.81, respectively). The AAPH-induced loss of fluorescence of proteins not containing kringles (BSA, LDL, HDL, and α_2 -macroglobulin) and of the tryptophan model compound NATA was unaffected by the presence of AMCHA (ratios of 0.88 to 1.0).

Protection as a function of AMCHA concentration was evaluated to demonstrate that the protection against loss of Trp fluorescence was due to the binding of AMCHA to Lp(a). Three different AMCHA concentrations are shown in Figure 9A, and indicate that there is a measurable AMCHA concentration effect. It is apparent that the slope for the control oxidation curve is greater than those observed in Figures 4, 5, and 7. This may be due to inherent variation in the sample because the Lp(a), although from the same donor, was from a different preparation. The binding analysis presented below is valid, however, because all the data in Figure 9 were obtained with this particular Lp(a) preparation.

To determine the K_d for AMCHA binding, the differences in the slopes of the $\ln(\% \text{ fluorescence})$ versus time plots (as in Fig. 9A) were examined as a function of AMCHA concentration. As shown in Figure 9B, the protection against AAPH-induced loss of Trp fluorescence by AMCHA generated a typical binding profile. Analysis of the binding isotherm, assuming that there is a single class of binding sites, yielded an apparent K_d of 22 mM. Previously, it was reported that the IC_{50} for the inhibition by AMCHA of Lp(a) binding to lysine-Sepharose is ~ 1 mM (Hoover-Plow et al., 1993). It is difficult to compare our K_d , which is based upon protection of AAPH-induced loss of fluorescence by AMCHA, with an IC_{50} that is based upon inhibition of binding.

Effect of AAPH oxidation on Lp(a) structure

Oxidation of Lp(a) has been reported to cause both protein aggregation and fragmentation (Naruszewicz et al., 1992). To examine whether the mild oxidizing conditions created by the free radical generator AAPH that were used in this study affected Lp(a) similarly, various Lp(a) samples were analyzed by native gradient (2.5–7.5%) PAGE in 0.09 M Tris-HCl, 0.08 M boric acid, and 0.0025 M NaEDTA, pH 8.4, to assess Lp(a) aggregation and fragmentation. In this study, Lp(a) was incubated at 37 °C with 2 mM AAPH for either 2 or 20 h. In addition, Lp(a) was incubated at 37 °C with 0.05% glutaraldehyde for 10 min to promote Lp(a) aggregation. Higher molecular weight aggregates, as assessed by densitometric scan, were not observed in the Lp(a) sample incubated for 2 h with AAPH compared to the control incubated with buffer (Fig. 10). In contrast, Lp(a) treated for 20 h with AAPH or with glutaraldehyde demonstrated higher molecular weight aggregates. Analysis of similar samples by SDS-PAGE (4–8% gradient) (Borth et al., 1991), with or without disulfide bond reduction by dithiothreitol, showed neither covalent aggregates nor protein fragmentation in the 2-h sample, whereas covalent aggregation was observed in the 20-h sample (not shown).

Light scattering was also used to test for aggregation or fragmentation: an increase/decrease in the size of the scattering particle should cause a corresponding increase/decrease in light scattering. The amount of light scattering for Lp(a) did not change ($100 \pm 1\%$ of an untreated control) over 4 h at 37 °C following the addition of AAPH to a final concentration of 2 mM. In contrast, a

Fig. 8. Effect of the absence (■) and presence (●) of 100 mM AMCHA on the change in intrinsic fluorescence of (A) apo(a), (B) Lys-plasminogen, (C) BSA, and (D) LDL induced by 2 mM AAPH at 37°C. Lines represent linear least-squares analyses assuming that the intercepts are $\ln(100)$.

Table 1. Effect of AMCHA on the AAPH-induced loss of intrinsic protein fluorescence

Sample ^a	Ratio ^b
Lp(a)	0.57
r-Apo(a)	0.45
lys-Plasminogen	0.4
r-tPA	0.55
Prothrombin	0.67
Factor XII	0.81
NATA	1.0
LDL	0.93
HDL	0.96
BSA	0.88
Thyroglobulin	1.0
α_2 -Macroglobulin	0.92

^aSamples have been grouped based upon the presence of kringle with known lysine binding sites (first four samples), the presence of kringle without demonstrated lysine binding activity (next two samples), and without kringle structures (remaining samples). All samples had an OD₂₈₀ of 0.15–0.2.

^bSlope (best fit by linear regression analysis) of the time-dependent loss of intrinsic tryptophan fluorescence in the presence of 100 mM AMCHA divided by the slope in its absence. Ratios are averages of two to five experiments. A ratio of 1.0 indicates lack of protection by AMCHA.

sample treated with 2 mM AAPH for 20 h demonstrated a slight increase in light scattering (110% of control), and a glutaraldehyde-treated sample had a significant increase (180% of control). These results are in qualitative agreement with the native PAGE results (see above).

Discussion

Lp(a) is a member of a superfamily of kringle-containing proteins (Ichinose, 1992). Plasminogen is the prototype for this family because kringles were first identified in this protein (Sottrup-Jensen et al., 1978) and the interaction of these structures with lysine and lysine analogues has been studied extensively (for a review, see McCance et al., 1994). Plasminogen contains five different kringles that comprise the nonprotease region. Human tPA and prothrombin each contain two kringles (Pennica et al., 1983), one kringle is found in human urinary plasminogen activator (Steffens et al., 1982) and coagulation Factor XII (McMullen & Fujikawa, 1985), and four kringles are in hepatic growth factor (Nakamura et al., 1989). Apo(a), in contrast, has multiple repeats of kringles that are 75–85% homologous to kringle IV of plasminogen (McLean et al., 1987). The ability of Lp(a) and r-apo(a) to bind to plasmin-modified fibrin/fibrinogen or to lysine affinity columns depends upon LBS interactions (Harpel et al., 1989; Koschinsky et al.,

Fig. 9. A: Effect of AMCHA concentration on the loss of intrinsic fluorescence from Lp(a); 100 μ g/mL of Lp(a) was incubated with 2 mM AAPH at 37°C in the presence of 0 mM (■), 5 mM (▲), 80 mM (●), and 150 mM (▼) AMCHA. Lines represent linear least-squares analyses assuming that the intercepts are $\ln(100)$. B: Change in slope, representing relative protection from loss of Trp fluorescence, as a function of AMCHA concentration. Each data point is an average of three different experiments; individual values were determined by subtracting the slope obtained in the absence of AMCHA, as in A, from the slope in the presence of a known concentration of AMCHA. The line is a fit to the data assuming a single class of AMCHA binding sites on Lp(a).

1991; Rouy et al., 1992). A number of studies have documented that the apo(a) portion of Lp(a) is responsible for this binding to lysyl residues (Harpel et al., 1989; Rouy et al., 1992). The kringle domains of apo(a), in analogy to the known properties of kringle IV in plasminogen (Scanu et al., 1993), are therefore implicated in lysine binding interactions.

The time-dependent loss of lysine binding functions of Lp(a) and r-apo(a) to both plasmin-degraded fibrin and lysine-Sepharose suggests that the kringles in the apo(a) portion of Lp(a) are a target of AAPH. Although we have verified that the free radicals generated by AAPH are able to modify the lipid portion of Lp(a), as measured by lipid hydroperoxide formation, it is reasonable to assume that the modification of protein is the major cause in the loss of the ability of Lp(a) to bind to lysines because apo(a) does not contain lipids. That AAPH acts only as an oxidizer was demonstrated by the retention of binding in the experiments using the water soluble antioxidant Trolox, which has been shown to protect proteins from oxidative damage by AAPH (Dean et al., 1991).

Fig. 10. Native PAGE of Lp(a) samples. Lp(a) (lane 1) was incubated with 2 mM AAPH at 37°C for 2 h (lane 2) and 20 h (lane 3), or treated with 0.05% glutaraldehyde at 37°C for 10 min (lane 4).

Additional evidence that the loss of binding is due to the attack of AAPH on the LBS domains in the kringles comes from the ω -aminocarboxylic acid experiments. These lysine analogues bind to the kringles of plasminogen and inhibit its interaction with plasminogen activators, fibrinogen, and fibrin (McCance et al., 1994). Lysine analogues also interact differentially with Lp(a) (Hoover-Plow et al., 1993), and are able to inhibit Lp(a) from binding to lysine-Sepharose. It has been postulated that these lysine analogues inhibit by binding to the LBS of Lp(a) kringle IV type 10 (Scanu et al., 1993). [For a review of the types and organization of kringles in apo(a) and their suggested nomenclature, see Scanu and Edelstein (1995).] Our results support this concept, because AMCHA was able to protect Lp(a) from AAPH-induced loss of binding to lysine-Sepharose. This suggests that amino acids essential for binding interactions in a kringle are protected from AAPH-generated free radicals by the presence of a lysine analogue in the binding site. That AMCHA was not a nonspecific antioxidant was demonstrated by its failure to inhibit lipid hydroperoxide formation.

Mild oxidation of LDL *in vivo* has been proposed to be pathogenic in human atherogenesis based upon laboratory studies indicating multiple inflammatory effects associated with oxidative changes in the LDL particle (Witztum & Steinberg, 1991). The effects of oxidation on Lp(a) are not studied as extensively. Oxygen radicals have been reported to damage proteins causing aggregation, fragmentation, and amino acid modification, depending upon the agent used and the conditions (Davies, 1987). For example, 10 mM AAPH has been reported to damage both BSA and monoamine oxidase, causing protein fragmentation and a decrease in tryptophan fluorescence (Dean et al., 1991). We did not observe either protein fragmentation or aggregation of Lp(a) treated with 2 mM AAPH for up to 2 h of incubation at 37°C, as assessed by native PAGE, by SDS-PAGE, or by light scattering studies. Aggregation of Lp(a) was observed, however, at longer incubation periods or when crosslinking was induced with glutaraldehyde. Because the binding of Lp(a) to lysine beads was essentially abolished at 2 h with 1 mM AAPH, our data show that neither lipoprotein aggregation nor fragmentation were responsible. The retention of antigenicity and the constant fluorescence emission wavelength maximum (see below) also indicate that the overall structure of Lp(a) was maintained. Another possible target for oxidation by AAPH would be the sialic acid residues on apo(a);

however, their location in the interkringle regions (McLean et al., 1987) would not be likely to affect LBSs.

In the absence of major structural perturbations, the loss of binding to fibrin or lysine-Sepharose could be the result of the modification of one or more amino acids in Lp(a) and r-apo(a) in the LBS. For example, in studies utilizing BSA, tryptophan, tyrosine, histidine, and cysteine were especially sensitive to oxidation (Davies et al., 1987), although all amino acids may be susceptible to oxidative modification (Davies et al., 1987; Stadtman, 1992). Based on the results of the intrinsic protein fluorescence studies, one or more Trp residues in the LBS could participate in the binding interaction. The decrease in tryptophan fluorescence is most probably due to the destruction of Trp residues and not due to other effects such as a loss of protein structure. If protein structure was perturbed sufficiently to alter the quantum yields of Trp residues, then changes in other properties of the intrinsic protein fluorescence should also have been observed. For example, the maximum wavelength of the fluorescence emission spectrum of Lp(a) or apo(a) should have moved from near 350 nm toward 360 nm, the maximum observed for NATA, if the addition of AAPH induced changes in protein structure that exposed Trp residues to solvent. Because the emission spectrum did not change either its shape or position, it is reasonable to assume that protein structure is maintained under the mild conditions used in these studies. Another possibility to explain a decrease in fluorescence intensity would be a collisional quenching interaction between AAPH and a Trp residue. This explanation, however, would require that the fluorescence remains constant with time. Because the loss of fluorescence was time dependent, it is apparent that a reaction must be responsible. Finally, the ability of the antioxidant Trolox to eliminate the loss of protein fluorescence argues for an AAPH-induced oxidation of Lp(a) and r-apo(a) Trp residues.

As shown by the higher AAPH concentrations in Figure 4, the loss of Trp fluorescence from Lp(a) is not a simple first-order kinetic process; a higher-order reaction has also been reported for the AAPH-induced loss of intrinsic fluorescence in BSA (Dean et al., 1991). One explanation for this observation would be the existence of several "classes" of tryptophan environments in Lp(a), such as exposed, partly exposed, and not exposed to solvent. Each class, therefore, would have a different rate for the reaction based on the ability of AAPH-generated free radicals to reach the various Trp residues. The blue-shifted fluorescence emission spectra of Lp(a) and r-apo(a), compared to the spectrum of NATA, indicate that a majority of the emitting Trp residues are in a less polar environment than solvent. It is not surprising, therefore, that the loss of fluorescence was kinetically complex and not complete even after 2 h of incubation under the conditions used. Additional possibilities that could explain the nonlinear kinetic data include a higher-order reaction mechanism and a time-dependent loss of AAPH.

This slow, incomplete loss of protein fluorescence should be contrasted with the more rapid, essentially complete loss of lysine binding capability. A similar comparison should also be made concerning the extent of the protection provided by lysine analogues. Whereas essentially all of the binding capability was retained when Lp(a) or r-apo(a) were incubated with AMCHA during AAPH treatment, AMCHA and ϵ ACA were able to provide only partial protection against the loss of protein fluorescence. The different protective effects of AMCHA can be explained by the fact that not all Trp residues are in LBS domains. Therefore, saturating concentrations of a lysine analogue would provide only partial pro-

tection against the loss of fluorescence, but could provide full protection against the loss of function. The differences between the proteins listed in Table 1 as to the amount of fluorescence lost to oxidation and protected by a lysine analogue would depend upon the number of kringles, the number of tryptophans in "protected" kringles, the total number of tryptophan residues, and the fluorescence quantum yield of each tryptophan.

It was also found that, although both apo(a) and Lp(a) lost protein fluorescence at a similar rate due to the action of AAPH, there was a distinct difference in the rate of loss of binding affinities for the two samples. The protein fluorescence signal is made up of all of the Trp residues that are fluorescent, whether they are in a kringle or not. The rate of loss of the intrinsic fluorescence will depend on the relative solvent exposure of those fluorescent Trp residues. Because both apo(a) and Lp(a) have so many Trp residues, most of which are not in kringles, the relative quantum yields and solvent exposures average out and similar rates of loss are observed. To compare the rate of loss of binding with the rate of loss of Trp residues in the LBS would require a way to observe only those Trp residues in the LBS.

Another consideration is that there are other LBSs in apo(a) that appear to be exposed in apo(a) but are masked when it is complexed to apo B-100 in the intact Lp(a) (reviewed in Klezovitch et al., 1996). This fact may explain the tighter affinity of apo(a) for fibrin. These extra LBS kringles in apo(a) are different than kringle IV-type 10 in that they do not possess tryptophan at residue 72. If one modification by AAPH treatment is the destruction of Trp residues in an LBS, then apo(a) should also be more resistant to loss of lysine binding than the corresponding Lp(a) particle, as we observed.

AMCHA provided a small amount of protection against tryptophan oxidation for the kringle-containing proteins prothrombin and Factor XII, two proteins not previously shown to bind to lysine residues (Table 1). Crystallographic studies, however, have raised the possibility that both human and bovine prothrombin fragment 2 (the second kringle) have properties similar to an LBS (Arni et al., 1993). This could imply that lysine binding is weak, and the concentrations of AMCHA used in our studies were able to provide partial protection for Trp residues in prothrombin fragment 2. At this time, we can only speculate that a kringle on Factor XII has a weak affinity for lysine, leading to partial protection of tryptophans from oxidation. The small protection noted for Factor XII is similar to that observed with BSA (Table 1), a protein without kringles but known to bind a wide variety of ligands with weak affinity.

Kringles are highly homologous structures consisting of ~90 amino acids with three disulfide bonds (Sottrup-Jensen et al., 1978). An examination of the structure of human apo(a) kringle IV type 10 (Mikol et al., 1996), outlined in view C of Figure 11, shows the three main regions that make up the LBS. The hydrophobic pocket consists of the aromatic rings of Trp 62, Trp 72, and Phe 64. At either end of this hydrophobic region are charged amino acids: one end has the positively charged side chains from Arg 71 and Arg 35, and the other end has the negatively charged side chains from Asp 55 and Asp 57. These ionized side chains can interact with the carboxyl and amino ends of the ligand, respectively, whereas the aromatic rings of the LBS are able to interact favorably with the hydrophobic middle of the ligand.

Scanu et al. (1993) have observed that Rhesus monkey Lp(a) binds with low affinity to lysine-Sepharose compared to human Lp(a). They suggested that this decreased affinity is a consequence

Fig. 11. Five views of apo(a) kringle IV type 10 generated using RasMol and the coordinates determined by Mikol et al. (1996). In all views, blue represents Arg 35 and Arg 71; red, Asp 55 and Asp 57; orange, Phe 64; yellow, Trp 62; and green, Trp 72. View C shows the lysine binding site consisting of the hydrophobic core flanked by charged side chains. Views A and E are rotations of view C to demonstrate the exposure of the Trp 72 indole ring to solvent. Views B and D are the complexes with the lysine analogues ϵ ACA and PAMBA, respectively (in black).

of the Trp to Arg substitution at position 72 found in kringle IV type 10 of the monkey Lp(a). Scanu et al. (1994) have identified Lp(a) from two humans also possessing low affinity to lysine-Sepharose and that have the Trp to Arg change at residue 72 of kringle IV type 10. Klezovitch and Scanu (1996) also recently have expressed the wild type and the Trp to Arg mutant kringle IV type 10 and demonstrated that only the wild type had lysine binding capacity. These findings demonstrate that Arg cannot substitute for Trp 72 in kringle IV type 10.

Our studies raise the additional possibility that a reduction in binding affinity to lysine could occur as a result of the oxidation of Trp residues in an LBS, including Trp 72 in kringle IV type 10. To evaluate the potential of the three Trp residues in kringle IV type 10 undergoing an interaction with solutes, we have utilized the three-dimensional coordinates of human apo(a) kringle IV type 10 detailed by Mikol et al. (1996). As shown in Figure 11, Trp 62 is partially buried as it forms the bottom of the hydrophobic region of the LBS (Mikol et al., 1996). Because Trp 25 is buried in the hydrophobic core of the kringle (not shown), it is very unlikely that it could be a target for solutes. The third Trp residue, Trp 72, forms

one side of the hydrophobic region of the LBS (Mikol et al., 1996). Views A and E in Figure 11 also document that Trp 72 is well exposed to solvent with the plane of the indole ring essentially orthogonal to the surface of the protein. Based on solvent accessibility, therefore, it appears that Trp 72 would be an excellent target for AAPH-generated free radicals and that Trp 62 could also be attacked, but at a slower rate.

As discussed above, AMCHA in the LBS of Lp(a) or r-apo(a) was able to protect partially against the loss of intrinsic fluorescence but yet provide complete protection against the loss of binding. Upon binding the lysine analogues ϵ ACA or PAMBA, the hydrophobic binding pocket is masked (Fig. 11, views B and D). It should be noted that the carboxyl end of ϵ ACA is in solution and not interacting with either Arg 35 or Arg 71; this is different than the ϵ ACA complex with kringle IV of plasminogen (Wu et al., 1991). In contrast, the carboxyl end of PAMBA does interact with these arginines (Fig. 11, view D). In either the ϵ ACA or the PAMBA complex, however, both Trp 62 and Trp 72 appear to be protected equally (Fig. 11, views B and D). The observed difference in protection between equimolar amounts of ϵ ACA and AMCHA

(Fig. 4) might be explained by a difference in K_d for these ligands binding to the LBS. The difference in binding affinity between the two lysine analogues could be largely a result of the carboxyl end of AMCHA, a saturated ring analogue of PAMBA, interacting with the arginines.

Three possible explanations for loss of lysine binding upon chemical modification of a Trp residue are apparent. First, destruction of a major component of the hydrophobic region of the binding site would obviously affect the ability of the hydrophobic portion of lysine or a lysine analogue from forming a favorable interaction. Second, modification of a Trp residue could generate another charged group. Although we have not determined the product or products [note that in r-apo(a) alone there are 190 Trp residues (McLean et al., 1987)], the oxidation of the tryptophan side chain could result in the production, for example, of an amino group from one of the indole nitrogens. A new, incorrect distribution of charges would result, similar to the Trp to Arg change in the Lp(a)s found to have reduced lysine binding. The third possible explanation is that replacement or modification of a Trp residue could lead to a conformational change that effectively destroys the stereochemistry of the binding site. Realignments around the binding site, however, are likely to be minimal because the three disulfides will maintain overall backbone structure of the kringle. In fact, as shown by Mikol et al. (1996), due to the disulfides, the structure of apo(a) kringle IV type 10 is not altered even upon the binding of a ligand to the LBS.

To assess these three explanations, it will be necessary to construct kringle IV type 10 site-directed mutants replacing Trp 72 with a series of different hydrophobic amino acids as well as with either Asp or Glu; a similar set of experiments has been proposed previously (Scanu & Edelstein, 1994). Analogous experiments would be required to establish if Trp 62 is also involved. In this way, it should be possible to begin to resolve whether the loss of lysine binding is a result of a loss of the hydrophobic region, an altered distribution of charged groups, a conformational change, or some combination of these events, occurring in or around the LBS. To test this approach, we have performed preliminary experiments on purified kringle IV of plasminogen produced by elastase cleavage. AAPH-induced oxidation destroyed, in a time-dependent manner, both the intrinsic tryptophan fluorescence of kringle IV and the binding to lysine-Sepharose. The presence of AMCHA completely protected against the loss of both fluorescence and binding. These results suggest that studies involving a single kringle will help clarify the molecular mechanisms underlying the state of oxidation of the LBS.

In the present study, we have documented that mild oxidative conditions are associated with loss of lysine binding activity of the Lp(a) particle. The effect of oxidation on LBS activity of Lp(a) may explain in part the large variations in the lysine-Sepharose binding activity of Lp(a) purified from different individuals (Armstrong et al., 1990; Leerink et al., 1992). In our studies, these variations in binding appeared not to be related to apo(a) size polymorphism. Other studies, however, have indicated that heterogeneity in binding to either lysine-Sepharose or fibrin is related to apo(a) size (Fless & Snyder, 1994; Hervio et al., 1995). Our results raise the additional possibility that oxidation of Lp(a), either in vivo or in vitro during purification, may provide another mechanism for altered LBS reactivity. Whether oxidation produces a form of Lp(a) that is less atherogenic, as opposed to the increased atherogenicity postulated for oxidized LDL, is not known. Preliminary data suggest that mild oxidation of Lp(a) with AAPH

increases its binding to human umbilical vein endothelial cells in tissue culture monolayers. Thus, oxidation may cause a targeting of Lp(a) from fibrin to cell surfaces. Our previous findings, that mild reduction enhances the LBS reactivity of Lp(a), combined with the present report, indicate that alterations in the oxidative state of Lp(a) affect its function significantly. These studies raise the possibility that the catabolism as well as the atherogenicity of Lp(a) may be related, in part, to its oxidative state.

Materials and methods

Reagents

The following chemicals were purchased and used without further purification: AAPH from Polyscience (Warrington, Pennsylvania), plasmin from American Diagnostica (Greenwich, Connecticut), bicinchonic acid from Pierce (Rockford, Illinois), cholesterol color reagent from Merck (Darmstadt, Germany), Trolox and AMCHA from Aldrich Chemistry Co. (Milwaukee, Wisconsin), ϵ ACA, *p*-nitrophenylphosphate, pNGB, PMSF, and BSA from Sigma (St. Louis, Missouri), NATA from Vega Biochemicals (Tucson, Arizona), and prothrombin and coagulation Factor XII from Enzyme Research Laboratories, Inc. (South Bend, Indiana). Human fibrinogen (Kabi Diagnostica AB, Mölndal, Sweden) was depleted of fibronectin and plasminogen as described previously (Harpel et al., 1989). Lys-plasminogen was a generous gift from Immuno AG (Vienna, Austria), and recombinant tPA, r-tPA, and recombinant apo(a), r-apo(a), $M_r = 500$ kDa (Koschinsky et al., 1991), were generously provided by Genentech, Inc. (San Francisco, California). All other chemicals were of reagent grade.

Isolation of proteins

Lp(a) was purified in the presence of 0.001 M NaEDTA as detailed previously (Harpel et al., 1989) from a donor who demonstrated two Lp(a) isoforms: one representing 90% of the total apo(a) with an apparent M_r of 600 kDa, and the other isoform representing 10% of the apo(a) with an apparent M_r of 800 kDa. The isoforms were identified by western immunoblot analysis (Gaubatz et al., 1990). Lp(a) was stored in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl and 1 mM Na₂EDTA. The concentration of Lp(a) was measured using the bicinchonic acid assay (BCA) with BSA as the standard (Smith et al., 1985) and reported as mg protein/mL. α_2 -Macroglobulin (Harpel, 1976) and LDL (Harpel et al., 1989) were purified as described previously. HDL was purified from a donor's plasma by ultracentrifugation in sodium bromide followed by Sephacryl-400 chromatography.

Preparation of samples

Prior to incubation with AAPH, all protein samples were dialyzed in oxygen-depleted 0.05 M potassium phosphate buffer, pH 7.4, for 24 h in the dark. NATA was dissolved in the same buffer immediately before use. A reaction at 37°C was initiated by the addition of an aliquot from an AAPH stock solution. A 100-mM AAPH stock solution was prepared by dissolving AAPH in water and freezing 1-mL portions. A fresh tube was used for each experiment. For binding experiments, the reaction was terminated by gel filtration chromatography using Sephadex PD10 columns (Pharmacia, Piscataway, New Jersey). Unless otherwise stated, all kinetic studies were performed at least twice, and a representative experiment has been presented.

Binding of Lp(a) and r-apo(a) to lysine-Sepharose

Binding of untreated or AAPH-treated Lp(a) to lysine-Sepharose 4B (Pharmacia) was performed on a 1-mL column equilibrated with 10 mM phosphate, 0.15 M NaCl, 1 mM Na₂EDTA, 0.01% NaN₃, pH 7.4, as described (Scanu et al., 1993). Approximately 50 µg of Lp(a) were applied after removal of the AAPH by gel filtration (see above). The column was washed with equilibration buffer until no further protein was detected. Bound Lp(a) was then eluted with 0.2 M εACA in equilibration buffer. The concentration of protein in the collected fractions was determined by BCA. The resulting optical densities were plotted against the elution volume; to calculate the percent bound, the area under the peaks were determined by weight. The same procedures were done with r-apo(a), but with approximately 30 µg applied to the lysine-Sepharose column.

Binding of Lp(a) and r-apo(a) to immobilized fibrin

Binding was studied as described (Harpel et al., 1989), using ELISA plate wells (NUNC, Naperville, Illinois) that were coated with fibrinogen and then incubated with human thrombin (1 IU/mL, a gift of Dr. John Fenton) for 30 min. After washing with buffer, the fibrin was incubated with plasmin (5 ng) for an additional 30 min. The reaction was terminated by adding protease inhibitors (pNGB and PMSF, both at 10⁻⁴ M) and 0.05 M εACA. Serial dilutions of Lp(a) and AAPH-treated Lp(a) from 0.3 µg/mL to 20 µg/mL were prepared and 50-µL aliquots were added to the fibrin-coated wells. After a 90-min incubation at room temperature, polyclonal rabbit anti-Lp(a) IgG labeled with alkaline phosphatase prepared as detailed (Harpel et al., 1989) was added to detect the bound Lp(a) or r-apo(a). After an 18-h incubation and subsequent washing, the substrate *p*-nitrophenyl phosphate was added and color development followed at 405 nm in a kinetic microplate reader (Molecular Devices, Palo Alto, California). The absorbance values indicating the binding of Lp(a) in the presence of 0.05 M εACA were subtracted from the values obtained without this inhibitor as described previously (Eisenthal & Cornish-Bowden, 1974). Studies of the binding of r-apo(a) or AAPH-treated r-apo(a) to fibrin were performed as detailed for Lp(a) using serial dilutions of 3 ng/mL to 200 ng/mL. For comparison of the binding of Lp(a) or r-apo(a) to fibrin, the absorbance units were converted to moles of Lp(a) or r-apo(a) bound using a curve constructed by comparing color development with the binding of radiolabeled r-apo(a) as described previously (Harpel et al., 1992). All experiments were performed in triplicate.

To assess the effect of AAPH treatment on the antigenicity of Lp(a), a sandwich ELISA was performed. The coating antibody was rabbit polyclonal anti-Lp(a) absorbed with immobilized LDL to remove reactivity against apolipoprotein B-100. After the addition of the Lp(a) sample, the rabbit antibody against Lp(a) labeled with alkaline phosphatase was added.

Light scattering

The scattering of 320-nm light from samples (200 µL) in quartz fluorescence cuvettes (4 × 10 mm pathlengths with illumination along the 10 mm axis) was detected at 90° (through a monochromator) by single photon counting. The light was generated by a frequency-doubled, pulsed dye laser (Spectra-Physics 375B) pumped by a frequency-doubled, mode-locked Nd-YAG laser (Spectra-

Physics series 3000). The laser system has been described in more detail previously (Hasselbacher et al., 1991).

Fluorescence measurements

To measure the change in intrinsic tryptophan fluorescence induced by AAPH treatment, 0.9 mL of a sample was incubated in a quartz cuvette (4 × 10 mm pathlengths) maintained at 37°C with an external water bath. Either buffer or AAPH was then added. Fluorescence was detected at right angles through a monochromator set at 345 nm in a spectrofluorometer (SLM/Aminco SPF-500C) using 295-nm excitation (along the 10-mm axis) and 7.5-nm bandpasses for each monochromator. To prevent photobleaching during the experiments, the excitation beam was blocked except when taking measurements. Each data point was an average of five sequential readings. The relative fluorescence intensities were then converted to ln% of the initial (time zero) fluorescence in order to compare the results from different experiments.

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