Acrylodan Can Label Amino as Well as Sulfhydryl Groups: Results with Low-Density Lipoprotein, Lipoprotein[a], and Lipid-Free Proteins†

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ABSTRACT: Human plasma lipoprotein[a] and autologous low-density lipoprotein were reacted with the fluorescent probe 6-acryloyl-2-(dimethylamino)naphthalene (acrylodan) previously reported to be specific for sulfhydryl groups. Reaction kinetics were biphasic in both cases. The reaction of bovine serum albumin with acrylodan was also biphasic. Monophasic kinetics were observed when protein free sulfhydryl groups were blocked by carboxamidomethylation prior to acrylodan reaction. A significant increase in total fluorescence was observed in the reaction of acrylodan with proteins containing no free sulfhydryl groups and with polylysine. The rates of these reactions were highly sensitive to pH. Fluorescence changes due to dissolution of probe into hydrophobic protein or lipid domains were minimal as was reaction of probe with phospholipid head groups. When isolated from acrylodan-labeled Lp[a], apo[a], which contains no free sulfhydryl groups, contained covalently bound acrylodan. These results suggest that acrylodan can modify the lysine residues of lipid-free proteins and may modify not only the free sulfhydryl groups of low-density lipoprotein and lipoprotein[a] but also reactive amino groups. We conclude that under these conditions, the use of this probe to quantify free sulfhydryl groups in these lipoproteins is infeasible.

Lipoprotein[a] (Lp[a])[1] was first discovered in human plasma by Berg et al. in 1963. Since that time, it has also been identified in monkeys and hedgehogs (Tomlinson et al., 1989; Laplaud et al., 1988). Because of the observation that elevated levels of Lp[a] have been correlated with both coronary heart disease and atherosclerosis, Lp[a] has been studied extensively (Berg et al., 1974; Dahlen et al., 1976; Kostner et al., 1981). Lp[a] contains two major apoproteins, apo[a] and apoB, which appear to be joined by one or more disulfide bonds. The lipid content of the Lp[a] particle is similar to LDL except that it has much less cholesteryl ester (Gaubatz et al., 1983). Apo[a] can be removed from the Lp[a] particle by reduction followed by ultracentrifugation, electrophoresis, or lysine–Sepharose chromatography (Fless et al., 1985; Armstrong et al., 1985). Apo[a] is highly glycosylated and has as many as 23 size polymorphs, which appear to be inherited in a Mendelian fashion (Kamboh et al., 1991). The amino acid sequence inferred from the cDNA sequence of one polymorph demonstrated that apo[a] is composed of a series of kringle structures similar to those found in plasminogen (McLean et al., 1987). The sequenced polymorph contained 37 repeats of a kringle structure similar to that of plasminogen kringle 4, 1 copy of plasminogen kringle 5, and an inactive serine protease domain homologous to the carboxy-terminal region of plasminogen.

Despite strong indications that apo[a] and apoB are linked by at least one disulfide bond, the specific cysteine residues involved in this (these) bond(s) have not yet been determined (Gaubatz et al., 1983; Utermann & Weber, 1983). Recently Sommer et al. (1991) used the fluorescent probe acrylodan to label the cysteine residues of LDL and Lp[a] to determine the number of free cysteine residues. They concluded that there were three free sulfhydryl groups in LDL, whereas only two cysteines were available in Lp[a]. The purpose of this study was to label cysteine residues specifically with acrylodan as a first step toward identifying the specific residues in apo[a] and apoB which participate in the intermolecular disulfide bond. Our results suggested that acrylodan not only may label cysteine residues but also may label lysine residues. Thus, this approach to quantitative analysis of cysteine residues may not be conclusive due to the competition of lysine residues for acrylodan binding.

EXPERIMENTAL PROCEDURES

Materials. The fluorescent probes 6-acryloyl-2-(dimethylamino)naphthalene (acrylodan) and 6-propionyl-2-(dimethylamino)naphthalene (prodan) were obtained from Molecular Probes (Eugene, OR). Stock solutions of the probes were 0.05 and 0.3 g/L in *N,N*-dimethylformamide. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and L-α-dimyristoylphosphatidylethanolamine (DMPE) were obtained from Avanti Polar Lipids (Alabaster, AL). Alcohol dehydrogenase was obtained from Millipore (Freehold, NJ). Cytochrome *c* was obtained from Sanyo (Tokyo, Japan), and ribonuclease A was obtained from Worthington (Freehold, NJ). Fatty acid free bovine serum albumin, chymotrypsinogen, trypsin (sequencing grade), triolein, and polylysine (MW 190 000) were obtained from Sigma (St. Louis, MO).

Isolation of Lipoproteins. Experiments were performed on lipoprotein[a] and autologous low-density lipoprotein obtained from a donor homozygous for apo[a] band 3 (Gaubatz

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[1] Abbreviations: ADH, alcohol dehydrogenase; apo[a], apolipoprotein[a]; apoB, apolipoprotein B-100; BSA, bovine serum albumin; CAM, carboxamidomethylated; DMF, dimethylformamide; DMPE, L-α-dimyristoylphosphatidylethanolamine; DMSO, dimethyl sulfoxide; cDNA, complementary deoxyribonucleic acid; EDTA, ethylenediaminetetraacetate; Lp[a], lipoprotein[a]; LDL, low-density lipoprotein; PBS, phosphate-buffered saline; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; RNase, ribonuclease A.

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et al., 1990). The subject had an elevated plasma Lp[a] level (40.0 mg/dL), but was otherwise normolipidemic. Isolation of the lipoproteins was performed essentially as outlined previously (Gaubatz et al., 1987) with the following modifications. The initial ultracentrifugation step was performed at a density of 1.135 g/mL and the second step at a density of 1.050 g/mL. LDL was contained in the d = 1.020–1.050 g/mL fraction. Lp[a] was isolated from the d > 1.050 g/mL fraction by affinity chromatography. The Lp[a] fraction was dialyzed against 0.15 M NaCl and applied to a lysine–Sepharose column (20 × 1.75 cm) which had been equilibrated with 0.05 M sodium phosphate, pH 7.5; the column was washed with the same buffer, and Lp[a] was eluted with 0.05 M sodium phosphate, 0.5 M NaCl, and 100 mM ϵ-aminocaproic acid at pH 7.5. The homogeneity of LDL and Lp[a] preparations was determined by SDS gel electrophoresis. Lipoproteins were stored in a low-salt buffer containing 1 mM EDTA, 0.01% sodium azide, and 0.015 M NaCl. Protein content was determined by the Lowry method (Lowry et al., 1951).

Protein Modification. LDL and BSA were carboxamidomethylated using iodoacetamide. The reaction mixture contained 40 μg/mL protein and 50 mM iodoacetamide in phosphate-buffered saline (0.075 M phosphate/0.075 M NaCl, pH 7.2). Following incubation for 4 hours at 37 °C, excess iodoacetamide was removed by extensive dialysis against PBS.

Polarity Sensitivity Study. Five-microliter aliquots of acrylodan stock solution were dried under a stream of nitrogen and dissolved in 5 mL of hexane, isooctane, toluene, methylene chloride, acetone, acetonitrile, dimethylformamide, dimethyl sulfoxide, or water. Solutions were sonicated for 1 h, and the fluorescence emission spectra were acquired at an excitation wavelength of 377 nm.

Phospholipid Vesicle and Microemulsion Particle Preparation. Unilamellar POPC vesicles were prepared as follows: 5 mg of dry 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was suspended in 3 mL of PBS at pH 7.2 and sonicated with a Heat Systems sonifier (W-350) equipped with a microtip probe at 40% power (140 W) for 20 min at 4 °C. Multilamellar vesicles were pelleted by centrifugation at 15 000 rpm for 30 min. The supernatant containing single-bilayer vesicles was used as a stock suspension. Unilamellar POPC/DMPE vesicles were prepared by the same method using 4.75 mg of POPC and 0.25 mg of L-α-dimyristoylphosphatidylethanolamine. The triglyceride/phospholipid microemulsion was prepared as described by Mims et al. (1986). Briefly, POPC and triolein were co-suspended in 5 mL of PBS, pH 7.2, and sonicated in an ice bath at 40% power for 1 h using a microtip probe. Following sonication, the lipid mixture was centrifuged for 30 min at 15 000 rpm, 15 °C. The cloudy fraction beneath the floating pad of lipid in the tube was used as a stock suspension.

Fluorescence Measurements. All fluorescence studies were performed on an SLM-8000 spectrofluorometer at 37 °C. Emission and excitation spectra of a 5×10^{-5} mg/mL acrylodan standard solution were taken daily to assure monochromator calibration and for data comparison purposes. Reaction of the fluorophore with the lipoproteins was monitored at 447 nm using an excitation wavelength of 377 nm. Stock lipoprotein solutions were diluted with PBS (pH 7.2) to a final protein concentration of 40 μg/mL. The final concentration of acrylodan or prodan in the reaction mixture was 50 ng/mL. For determination of the number of labeled residues in LDL and Lp[a], the acrylodan solution was standardized using mercaptoethanol as described by Sommer et al. (1991). The quantum yield of lipoprotein-bound acrylodan was determined by reacting a known amount of acrylodan with a large excess of LDL. ADH, BSA, chymotrypsinogen, cytochrome *c*, and RNase were incubated with acrylodan or prodan for 18 h in PBS at the label and protein concentrations stated above. Protein samples were then dialyzed extensively against PBS to remove excess label.

Kinetic Analysis. Reaction rates were determined using the single- and double-exponential curve-fitting capabilities of the Enzfitter program [Leatherbarrow, R. J. (1987) Elsevier Biosoft, 68 Hills Rd., CB2 1LA, U.K.]. Statistical analysis using the Curfit program developed by Dr. Roger Knapp verified the order of the reaction kinetics.

Measurement of Apo[a] Labeling by Acrylodan. Lp[a] (200 μg/mL in PBS) was labeled for 5 h at 37 °C with acrylodan (250 ng/mL). The sample was then dialyzed overnight against PBS to remove unreacted acrylodan. Dithiothreitol was added to a final concentration of 10 mM to reduce the disulfide bond between apo[a] and apoB, the Lp[a] solution was incubated for an additional hour at 37 °C, and the density of the solution was adjusted to 1.07 g/mL with solid potassium bromide. The sample was subjected to centrifugation for 18 h at 40 000 rpm (Beckman SW 50.1 rotor). Under these conditions, the Lp[a$^-$] particle migrates to the top of the centrifuge tube while free apo[a] pellets to the bottom of the tube. Five fractions were isolated from the tube; the topmost fraction contained greater than 75% of the reisolated apoB, and the bottom fraction contained all of the recovered apo[a]. The fluorescence spectra of the top and bottom fractions were measured following dialysis against PBS to remove potassium bromide and dithiothreitol. Protein concentrations were measured using the BCA protein assay (Smith et al., 1985).

Tryptic Mapping of RNase and Acrylodan-Labeled RNase. RNase (200 μg/mL in PBS) was labeled overnight at 37 °C with a 3.5-fold molar excess of acrylodan. An equal amount of RNase was treated with DMF and incubated overnight at 37 °C. Excess acrylodan and/or DMF were removed by dialysis against 0.25 M Tris/1 mM EDTA, pH 8.6. Acrylodan-labeled and native RNase samples were reduced and carboxymethylated as described by Crestfield et al. (1963) and then dialyzed against three changes of PBS. Trypsin was dissolved at 100 μg/100 μL of 0.001 N HCl. One hundred and fifty-microgram aliquots of acrylodan-labeled and native RNase were incubated with 6 μg of trypsin at 37 °C for 18 h. After digestion, the reaction mixtures were chromatographed on a Vydac C-4 analytical column (4.6 × 250 mm) equilibrated in 0.1% trifluoroacetic acid with a flow rate of 1.5 mL/min, using a linear gradient of 2-propanol (0–70%) in 0.1% trifluoroacetic acid over a 45-min period. The eluate was monitored at 220, 275, and 377 nm in order to detect both protein and acrylodan absorbance.

RESULTS

The fluorescent probe acrylodan was used to label human Lp[a] and autologous LDL. Modification of these lipoproteins by acrylodan was observed as a time-dependent increase in fluorescence intensity. The reaction of acrylodan with LDL and Lp[a] displayed biphasic kinetics (Figure 1A,B; Table I). The number of acrylodan-modified residues in LDL and Lp[a] was estimated from the fluorescence intensity at 450 nm to be 3.2 and 4.2 per mole of protein, respectively. Possible sources of the two phases included covalent bonding of acrylodan to more than one class of cysteine residues, bonding of the probe to phospholipid head groups or to lysine residues, and noncovalent association of acrylodan with hydrophobic lipid and/or protein domains.

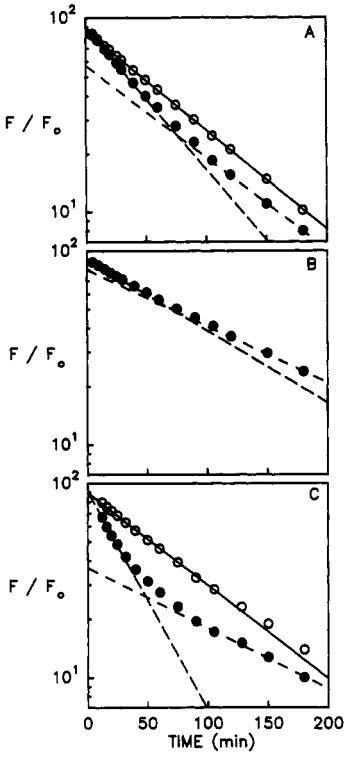

FIGURE 1: Kinetic analysis of acrylodan reactions with (A) native (●) and blocked (○) LDL, (B) Lp[a], and (C) native (●) and blocked (○) BSA. All reactions were performed with 40 μg/mL protein and 50 ng/mL acrylodan in PBS (pH 7.2) at 37 °C. An excitation wavelength of 377 nm was used, and fluorescence emission was monitored at 447 nm for LDL and Lp[a], and at 490 nm for BSA. The semilog plot of the fraction of fluorescence change versus time was used to determine the number of phases involved in the reaction as well as their rates.

Table I: Rates of Fluorescence Increase Due to Acrylodan Reaction with Proteins and Lipid Complexes

sample	n^a	rate × 10^3 (min^{-1})^b fast	slow
LDL (Sommer et al.)^c		14.5	1.4
LDL	10	18.4 ± 3.6	3.2 ± 1.5
CAM-LDL	3	11.6 ± 0.7	
Lp[a] (Sommer et al.)		14.2	0.7
Lp[a]	7	14.2 ± 2.9	3.2 ± 1.8
BSA	2	59.0 ± 7.5	6.3 ± 0.3
CAM-BSA	2	12.1 ± 1.0	
POPC		0.9	
POPC/DMPE		2.7	
POPC/triolein		13.9	

^{a} Number of experimental repetitions. ^{b} All reactions were performed with 40 μg/mL protein, or 160 μg/mL phospholipid, and 50 ng/mL acrylodan in PBS (pH 7.2) at 37 °C. The excitation wavelength was 377 nm, and fluorescence emission was monitored at 450 nm in the Sommer et al. experiments, at 447 nm for LDL and Lp[a], at 490 nm for BSA, and at 485 nm for the phospholipids. ^{c} These rates were estimated by kinetic analysis of the figures reported by Sommer et al. (1990).

In order to determine whether one or both of the two phases corresponded to the covalent binding of acrylodan to sulfhydryl groups, LDL and BSA were treated with iodoacetamide to block any free cysteines. The modified proteins were then treated with acrylodan, and the kinetic results were compared to those of the native proteins. The reaction of acrylodan with native proteins was biphasic; however, after these proteins were treated with iodoacetamide, the reaction became monophasic. The rate of the single reaction was intermediate between those of the two phases observed in the native proteins (Figure 1A,C; Table I). The number of acrylodan-modified residues in the carboxamidomethylated LDL was estimated to be 2.3 per mole of protein.

Table II: Maximum Emission Wavelengths of Acrylodan Dissolved in Solvents of Various Polarities

solvent	polarity	max emission wavelength (nm)^a
isooctane	0.1	427
toluene	2.4	428
methylene chloride	3.4	430
acetone	5.4	453
acetonitrile	6.2	465
DMF	6.4	471
DMSO	7.2	476
water	10.0	525

^{a} All measurements were taken at 37 °C with an excitation wavelength of 377 nm.

Unreacted acrylodan was dissolved in a variety of solvents to determine if its emission spectrum was sensitive to polarity. As solvent polarity was increased, the fluorescence emission maximum red-shifted (Table II). The fluorescence emission maximum of the lipoprotein-bound acrylodan was at 447 nm, which falls into the range of solvents of moderate polarity. Since fluorescence changes were observed at a single wavelength, it is possible that changes in emission maxima resulting from changes in the environment of the unreacted, but slightly fluorescent probe molecule might appear to be increases in fluorescence. Thus, synthetic phospholipid particles were prepared to determine whether dissolution of acrylodan in the hydrophobic lipid phase or covalent bonding of acrylodan to reactive lipids would alter acrylodan fluorescence. Unilamellar vesicles of POPC and POPC/DMPE and a POPC/triolein microemulsion at lipid concentrations comparable to those found in the lipoprotein particles were treated with acrylodan. All three reactions were monophasic (Table I), and although the apparent reaction rates were similar to those of native Lp[a] and LDL, the total fluorescence change observed was 10-fold lower than that observed with the lipoproteins.

Since acrylodan fluorescence emission is sensitive to polarity, noncovalent interaction of acrylodan with the hydrophobic protein domain(s) might also alter the apparent rate of the labeling reaction. To determine the relative amounts of fluorescence associated with covalent bonding of acrylodan to the protein, as opposed to its noncovalent interaction with hydrophobic domains, ADH, BSA, chymotrypsinogen, cytochrome *c*, and RNase were treated extensively with both acrylodan and prodan. Any probe not strongly bound to the protein was removed by dialysis. Table III shows the peak emission wavelength and fluorescence intensity for each of the proteins before and after dialysis, and the percent of the total fluorescence which was retained following dialysis. In all cases, greater than 80% of the prodan fluorescence could be removed from the proteins by dialysis. For acrylodan, however, 40–91% of the fluorescence remained bound to the proteins, some of which contained no free cysteine residues (cytochrome *c*, chymotrypsinogen, and RNase). These results suggested that other nucleophilic moieties such as ϵ-NH_2 groups of lysine might react with acrylodan.

To determine if acrylodan could bind to lysine, polylysine (MW 190 000) was reacted with the fluorophore. A significant increase in the total fluorescence and a relatively rapid reaction rate were observed (Table IV). The kinetics of acrylodan reaction with LDL, polylysine, and RNase at pH 7.2 and 6.0 were studied to determine the extent of low-level lysine bonding. The reaction rates were decreased at least 2-fold in all cases when the pH was lowered to 6.0.

Table III: Spectral Properties of Various Protein/Fluorophore Adducts Pre- and Postdialysis

sample	max emission wavelength (nm)[a]		max fluorescence (rel units)		% fluorescence retained following dialysis
	predialysis	postdialysis	predialysis	postdialysis	
acrylodan labeling					
ADH	475	470	3010	2750	91
BSA	490	485	2090	1840	88
chymotrypsinogen	515	490	320	130	40
cytochrome *c*	520	475	210	90	43
RNase	505	485	630	490	78
prodan labeling					
ADH	515	470	3150	280	9
BSA	510	455	2960	125	4
chymotrypsinogen	515	455	2580	80	3
cytochrome *c*	515	445	910	150	16
RNase	515	440	1900	100	5

[a] All reactions were performed with 40 μg/mL protein and 50 ng/mL acrylodan in PBS (pH 7.2) at 37 °C. An excitation wavelength of 377 nm was used. Pre- and postdialysis measurements were made on the same day.

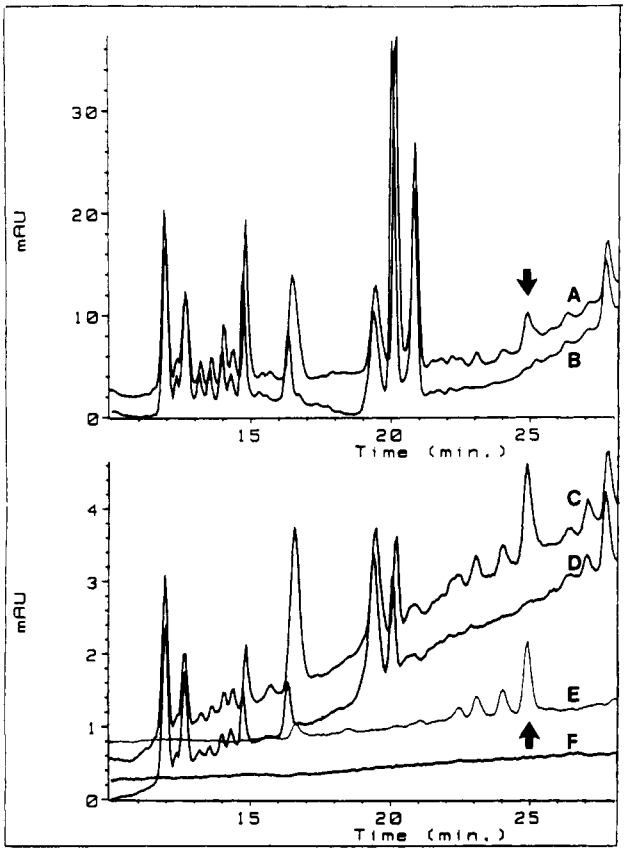

FIGURE 2: Tryptic maps of acrylodan-labeled and native RNase. Acrylodan-labeled and native RNases were reduced, carboxymethylated, and digested with trypsin. The resulting peptides were resolved by reverse-phase HPLC as described under Experimental Procedures. Eluant absorption was monitored at 220 (A, B) and 275 nm (C, D) to detect peptide absorbance and at 377 nm (E, F) to detect absorbance by acrylodan. Traces B, D, and F represent native RNase; traces A, C, and E represent acrylodan-labeled RNase. The arrow indicates the position of the major peptide which is modified by acrylodan.

To confirm that acrylodan–lysine interactions were in fact responsible for the acrylodan labeling observed in non-cysteine-containing proteins, acrylodan-labeled RNase was digested with trypsin and subjected to HPLC to separate the resulting peptides. Since trypsin cleaves at arginine and lysine residues, modification of a lysine residue with acrylodan should prevent trypsin cleavage at that site and generate a peptide not present in the tryptic digest of native RNase. Figure 2 shows the peptide maps of both native and acrylodan-modified RNase. The chromatographic profiles reveal a major peak at 25 min,

Table IV: Rates of Fluorescence Increase Due to Acrylodan Reaction with Selected Proteins at pH 7.2 and 6.0

sample	pH	rate × 10^3 (min^{-1})[a]	
		fast	slow
LDL	7.2	18.4	3.3
	6.0	3.0	1.3
polylysine	7.2	8.3	
	6.0	1.9	
RNase	7.2	4.4	
	6.0	2.1	

[a] All reactions were performed with 40 μg/mL protein and 50 ng/mL acrylodan in PBS at 37 °C. An excitation wavelength of 377 nm was used, and fluorescence emission was monitored at 447, 520, and 500 nm for LDL, polylysine, and RNase, respectively.

and several minor peaks at 16.5, 22.5, 23, and 24 min which absorb at 220, 275, and 377 nm and are present in the acrylodan-modified sample, but not in the native RNase digest. Further analysis of the acrylodan-labeled RNase sample by electrospray mass spectrometry revealed an ion of molecular mass corresponding to the addition of one acrylodan group to RNase. No such ion was seen in the mass spectrum of the native RNase (data not shown).

As a final demonstration that acrylodan might be capable of labeling lysine residues in apo[a], acrylodan-labeled Lp[a] was reduced with dithiothreitol followed by ultracentrifugal separation of reduced apo[a] and Lp[a^-]. This experiment demonstrated that both the apo[a] recovered from the bottom of the tube and the Lp[a^-] particle which floated to the top of the tube contained covalently bound acrylodan. After correction for protein concentration, apoB fluorescence was 9.6-fold greater than that of apo[a]. Fluorescence spectra of both Lp[a^-] and apo[a] demonstrated a single broad emission maxima at 445–450 nm (data not shown).

DISCUSSION

Prendergast et al. (1983) have reported that acrylodan preferentially labels cysteine residues in proteins. More recently, Sommer et al. (1991) used this fluorescent compound to label the cysteine residues in lipoprotein[a] and low-density lipoprotein. Our interest in acrylodan was for use in identifying the cysteine residues involved in the disulfide bond(s) between the two major apoproteins of Lp[a], apo[a] and apoB. As a first step, LDL and autologous Lp[a] were treated with acrylodan using conditions identical to those used by Sommer et al. Kinetic analysis demonstrated that the labeling time courses for both lipoproteins were biphasic. In an attempt to

determine the source of the two phases, several possible fluorophore–lipoprotein interactions were explored: covalent bonding of acrylodan to multiple classes of cysteine residues; noncovalent association of acrylodan with hydrophobic lipid and/or protein domains; covalent bonding of acrylodan to phospholipid head groups; and covalent bonding of acrylodan to lysine residues.

In order to evaluate the role of cysteine residues in this reaction, LDL was treated with iodoacetamide to block this amino acid. The reaction of acrylodan with blocked LDL was monophasic, suggesting that the cysteine residues were not solely responsible for the fluorescence change observed. Treatment of LDL with iodoacetamide not only resulted in the disappearance of one phase of the acrylodan labeling reaction but also significantly altered the rate of the remaining phase.

Free cysteines on lipoproteins might react with acrylodan at different rates depending on their accessibility to the fluorophore. The presence of only one kinetic phase in the reaction of blocked LDL [run under conditions used by Sommer et al. (1991)] could be explained by incomplete reaction of iodoacetamide with all of the cysteine residues. Therefore, the carboxamidomethylation was performed under conditions stringent enough to assure that all free cysteines were blocked. Again, acrylodan labeling of the blocked lipoproteins resulted in a monophasic increase in fluorescence.

Acrylodan labeling was also performed on BSA, a protein containing only one free cysteine, to determine whether the biphasic kinetics were a phenomenon unique to lipoprotein particles. Experiments were performed at the same protein and acrylodan concentrations used with the lipoproteins. As with LDL, biphasic kinetics were observed with the native protein, whereas reaction of acrylodan with carboxamidomethylated BSA was monophasic.

Prodan, from which acrylodan is synthesized (Prendergast et al., 1983), is an unreactive fluorescent compound used to probe hydrophobic lipid and protein domains (Weber et al., 1979). The fluorescence emission spectrum of prodan is extremely sensitive to solvent polarity. In more polar environments, the spectrum is blue-shifted. Similar effects have been seen with acrylodan–mercaptoethanol adducts (Prendergast et al., 1983). Although unreacted acrylodan is much less fluorescent than the protein or mercaptoethanol adducts (Prendergast et al., 1983), its fluorescence emission was also sensitive to polarity. The peak emission wavelength of the acrylodan-labeled lipoproteins was 447 nm. This emission is blue-shifted approximately 50 nm from the observed spectral emissions of other acrylodan–protein complexes (Prendergast et al., 1983). This shift could be due either to the sulfhydryl-bound acrylodan residing in hydrophobic domains or to the fluorescence of a large quantity of noncovalently bound acrylodan dissolved in the lipid matrix or in hydrophobic protein domains.

Acrylodan treatment of unilamellar vesicles of POPC was intended to mimic fluorophore incorporation into the lipid domain of the lipoprotein particle. The degree of dissolution of acrylodan into the neutral lipids present in the core of the lipoprotein particles was estimated from the labeling of a POPC/triolein microemulsion. Both the vesicle and microemulsion reactions were monophasic. Although the rates determined were similar to those of the lipoprotein reactions, the increase in total fluorescence measured was 10-fold lower than expected if dissolution into lipid domains was solely responsible for the second phase of the labeling reaction. The possibility that acrylodan was covalently modifying the polar head groups of lipoprotein phospholipids was evaluated by labeling unilamellar POPC/DMPE vesicles with acrylodan. Again, the observed monophasic rate was comparable to the lipoprotein labeling rates, but the total increase in fluorescence was extremely low.

Some of the fluorescence seen in the labeled proteins may have been due to the presence of a large number of unreacted acrylodan molecules dissolved in hydrophobic protein domains. Thus, several proteins were treated with acrylodan (the excess fluorophore removed through dialysis) to determine the relative amount of fluorescence attributable to molecules covalently attached to or in strong hydrophobic interaction with the protein. For this purpose, proteins were chosen that contained many, one, and no sulfhydryls. After acrylodan treatment, but before dialysis, ADH and BSA, which contain free cysteines, had up to a 15-fold greater total fluorescence than the non-sulfhydryl-containing proteins. These two proteins retained 90% of their fluorescence following dialysis. All but 40% of the fluorescence could be dialyzed away from cytochrome *c* and chymotrypsinogen, but RNase retained 78% total fluorescence after dialysis. When the same experiment was performed using prodan instead of acrylodan, only 3–16% of the total fluorescence remained associated with any of the proteins following dialysis. Since the structure of acrylodan is quite similar to that of prodan, the amount of each fluorophore bound in the hydrophobic sites of the protein should be similar.

Since the olefinic group of acrylodan is electrophilic toward amino groups as well as sulfhydryl moieties, there was a possibility that acrylodan was reacting not only with cysteine residues but also with lysine residues (Prendergast, personal communication). In this vein, it is significant to note that reduced apo[a] isolated from acrylodan-labeled Lp[a] contained covalently bound acrylodan. When bound to apoB in Lp[a], apo[a] is reported to contain no free cysteine residues, suggesting that some other amino acid residue of apo[a] is labeled by acrylodan. To support the suggestion that lysine residues are susceptible to labeling by acrylodan, polylysine was treated with acrylodan. There was significant labeling of polylysine, indicating that in fact lysine amino groups were being covalently labeled. This covalent labeling could explain the higher level of fluorescence retained by non-free-sulfhydryl-containing proteins such as RNase. This protein retained a greater amount of acrylodan fluorescence following dialysis than the other non-free-sulfhydryl-containing proteins. A significant feature of RNase is a highly reactive lysine residue at position 41 (Blackburn & Moore, 1982). If the fluorescence of the dialyzed proteins were caused by low-level reactivity with lysine, RNase would have an increased fluorescence due to this reactive residue. The tryptic map of acrylodan-labeled RNase revealed a major peptide at 25 min with absorbance at 377 nm which was not present in the digest of native RNase. This result suggests that at least one lysine residue in RNase has been modified by acrylodan. Lesser amounts of several other fluorescent peptides are also present in the chromatogram, suggesting that other lysine residues in RNase may be susceptible to modification by acrylodan.

Finally, if lysine bonding were responsible for the additional fluorescence associated with the proteins, the reaction rate should be decreased when the pH of the reaction is lowered. At lower pH, the lysine residues will be more protonated, less nucleophilic, and thus not as reactive with acrylodan. The same is true for cysteine residues, but to a lesser extent.

Reaction rates for LDL, polylysine, and RNase were reduced up to 6-fold upon lowering the pH from 7.2 to 6.0.

ApoB in LDL contains 25 cysteine residues and 357 lysine residues (Chen et al., 1986). It has been reported that at least 16 of the cysteines are involved in intramolecular disulfide bonds (Yang et al., 1990), leaving only 9 sulfhydryls available for covalent bonding to acrylodan. Lund-Katz et al. (1988) have found that approximately 225 (63%) of the lysine residues on LDL were accessible to reductive methylation. Thus, there are 24-fold more lysines available for modification than cysteines. Although cysteine SH groups are more chemically reactive than lysine NH_2 groups, the abundance of lysine residues present in LDL could account for much of the increased fluorescence remaining after iodoacetamide treatment.

The same argument is true for the acrylodan treatment of Lp[a] and BSA. Lp[a] is thought to contain one molecule each of apo[a] and apoB. Apo[a] has a minimum of 79 cysteine residues, with the actual number dependent on the number of repeats of kringle type 2 in any given polymorph (McLean et al., 1987). It appears that only one of those residues, Cys-4057, is not involved in intramolecular disulfide bonds (McLean et al., 1987). Since there are 22 lysine residues present in apo[a], the ratio of cysteines to lysines in Lp[a] is not significantly different from that found in LDL. If, as a number of publications suggest, Cys-4057 is involved in an intermolecular disulfide bond with apoB, the apo[a] labeling observed must be due to an amino acid residue other than cysteine. The much lower level of apo[a] labeling as compared to apoB labeling observed in the reduction and separation of labeled Lp[a] may be explained by the large difference in the number of lysine residues in apo[a] and apoB. BSA contains 35 cysteines and 58 lysines (Brown, 1975; Hiyarama et al., 1990); however, all of the cysteine residues, with the exception of Cys-34, are involved in intramolecular disulfide bonds. Thus, the ratio of lysine residues to available sulfhydryl residues in BSA (58:1) is greater than that of the lipoproteins. In the cases of LDL and BSA, labeling of lysine residues could play a significant part in the overall increase in fluorescence following acrylodan treatment.

In the reaction of acrylodan with the lipoproteins, the slow phase is responsible for 65% of the total fluorescence observed. The contribution of the slow phase in the reaction of acrylodan with BSA is only 43%. It is not evident whether the two phases observed in the reaction of lipoproteins with acrylodan are caused by the same chemical processes as the two phases observed in the reaction of BSA with acrylodan. If the origins of the two reaction phases are the same for LDL and BSA, then the difference in total fluorescence due to each phase could be caused by differences in the accessibility of the lysine residues.

Thus, it appears that acrylodan cannot be used for quantitative analysis of cysteine residues under the conditions suggested by Sommer et al. (1991). Reaction of acrylodan with lysine residues occurs at sufficient rates and to a sufficient extent to alter significantly the total fluorescence and observed reaction rate. We conclude that unambiguous measurement of free cysteine residues using acrylodan is not possible under these conditions in the presence of lysine.

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