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Sulfhydryl-Selective Fluorescence Labeling of Lipoprotein(a) Reveals Evidence for One Single Disulfide Linkage between Apoproteins(a) and B-100†

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ABSTRACT: Human lipoprotein(a) and low-density lipoprotein were labeled with two different sulfhydryl-selective fluorescence markers. The hydrophilic fluorophore lucifer yellow iodoacetamide and the apolar compound 6-acryloyl-2-(dimethylamino)naphthalene were used to derivatize free -SH groups in the lipoproteins. Three sulfhydryls could be detected in low-density lipoprotein, whereas only two cysteines were available in lipoprotein(a). One of the three -SH groups in low-density lipoprotein was shown to be located in close proximity to the particle surface. We suggest that this surface-exposed cysteine of apoprotein B-100 serves as a component for the disulfide linkage to apoprotein(a) in lipoprotein(a).

Lipoprotein(a) [Lp(a)][1] represents a class of human plasma lipoprotein particles associated with premature coronary heart disease and stroke. When the plasma level of Lp(a) is above 30 mg·dL^{-1}, the relative risk of coronary atherosclerosis rises about 2-fold (Kostner et al., 1981; Armstrong et al., 1986; Dahlen et al., 1986). The molecular basis of these findings is still under investigation.

Lipoprotein(a) consists of two polypeptide chains. One of these is identical with apolipoprotein B-100 (apo B), the

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[1] Abbreviations: Lp(a), lipoprotein(a); LDL, low-density lipoprotein; apo(a), apolipoprotein(a); apo B, apolipoprotein B-100; acrylodan, 6-acryloyl-2-(dimethylamino)naphthalene; LY, lucifer yellow iodoacetamide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

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apoprotein of low-density lipoprotein (LDL). The other protein component of Lp(a), apolipoprotein(a) [apo(a)], is a hydrophilic glycoprotein exhibiting remarkable size heterogeneity with a molecular mass ranging from 400 to 700 kDa, depending on the donor (Utermann et al., 1987).

Indirect experimental evidence has been obtained that these two apoproteins might be linked via a disulfide bond. Apolipoprotein(a) can be separated from lipoprotein(a) after reductive treatment (Armstrong et al., 1985). Other authors have suggested such a type of linkage from the electrophoretic mobility of apo(a) in SDS–polyacrylamide gels in the reduced and nonreduced states (Gaubatz et al., 1983). On the other hand, upon gel filtration in either low or high salt concentrations, apo(a) failed to separate from reduced Lp(a), indicating that apo(a) might be bound to the lipoprotein surface also by other forces, e.g., noncovalent interactions (Fless et al., 1985).

It was the aim of our work to assess the type of linkage between the apoproteins in Lp(a). In a comparative study, we applied two SH-sensitive fluorescence markers of different polarity for the determination of free sulfhydryl groups in LDL and Lp(a). From the results obtained, it can be inferred that a surface-exposed SH group in apo B is involved in disulfide bond formation to apo(a) in Lp(a).

Experimental Procedures

Materials. The fluorescence labels 6-acryloyl-2-(dimethylamino)naphthalene (acrylodan) and lucifer yellow iodoacetamide dipotassium salt (LY) were obtained from Molecular Probes, Eugene, OR. Stock solutions (1 mM) in *N*,*N*-dimethylformamide (acrylodan) and water (LY) were prepared. Standard laboratory chemicals were purchased from Merck, Darmstadt, Germany.

Isolation of Lipoproteins. Lipoprotein(a) and low-density lipoprotein were isolated from plasma of fasting normolipemic volunteers selected according to their plasma Lp(a) concentrations. Experiments were carried out with a donor homozygous for the apo(a) isoform B (Utermann et al., 1987). Lp(a) was purified essentially as described earlier (Steyrer & Kostner, 1990): Immediately after being drawn, the plasma was stabilized with EDTA and sodium azide (1 mg/mL) and subjected to density gradient ultracentrifugation in an SW-40 rotor (Beckman) for 24 h at 40000 rpm (Knipping et al., 1986). The fraction at density 1.070–1.125 g/mL was passed over an immunoadsorber specific for apo(a). Adsorbed Lp(a) was eluted with glycine hydrochloride buffer, pH 2.5, yielding preparations of more than 95% purity. LDL was harvested from a fraction corresponding to densities 1.025–1.055 g/mL of the density gradient and recentrifuged under identical conditions.

All buffers and solutions used for lipoprotein preparation contained EDTA and sodium azide (1 mg/mL) and were deoxygenated in vacuum after saturation with nitrogen. All purification steps were performed at 4 °C, and preparations were used within 1 week.

The purity of the Lp(a) and LDL fractions was assayed by double-decker rocket immunoelectrophoresis and SDS–polyacrylamide gel electrophoresis as described (Laurell, 1966; Gaubatz et al., 1983; Armstrong et al., 1985). Protein content was determined by the method according to Lowry (Lowry et al., 1951).

Fluorescence Measurements. All fluorescence experiments were carried out on a Shimadzu RF-540 spectrofluorometer. Immediately before use, the lipoprotein fractions were dissolved in 10 mM Tris-HCl, pH 7.4, and desalted by gel chromatography using a Sephadex G-25 column. A 5 molar excess of acrylodan or LY was added to 2 mL of a lipoprotein suspension with a final protein concentration of 40 μg/mL. Incubations were carried out at 37 °C. After a maximum of fluorescence intensity had been reached (for acrylodan), excess label was removed by gel chromatography on Sephadex G-25.

The time-dependent increase of the fluorescence intensity upon incubation of acrylodan with lipoproteins was measured at 450 nm using an excitation wavelength of 372 nm. Excitation and emission slits were set at 10 and 5 nm, respectively.

The purity of commercially available acrylodan was confirmed using 2-mercaptoethanol as a substrate. The label–mercaptoethanol conjugate was prepared from acrylodan and a defined amount of mercaptoethanol in aqueous buffer. The concentration of free –SH groups in this sample was independently determined by a photometric method with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), using a molar absorption coefficient of 14150 M^{-1} cm^{-1} at 412 nm (Riddles et al., 1979). Then, the quantum yield of lipoprotein-bound acrylodan was determined by reacting known amounts of acrylodan with a large excess of lipoprotein. Finally, the maximum number of reactive cysteines in the lipoproteins tested could be determined by labeling lipoproteins with an excess of acrylodan.

After incubation of lucifer yellow iodoacetamide with the lipoproteins, excess fluorophore was removed by gel chromatography as described for acrylodan. Lucifer yellow iodoacetamide is water-soluble and, therefore, this label should still be exposed to water if bound to the LDL surface. Thus, the quantum yield of LY in solution and bound to LDL should be the same. On the basis of the fluorescence intensity of a given concentration of LY in aqueous buffer, the amount of LY bound to LDL could be directly calculated. The monochromators were set at 520 nm for emission and at 426 nm for excitation, respectively. Experimental conditions for labeling of LDL with both probes were identical to the incubation procedures described. Fluorescence intensities of both labels were determined at the wavelengths indicated above.

For the measurement of the fluorescence anisotropies of LY-labeled LDL, the excitation and emission slits were set at 5 and 20 nm, respectively. Fluorescence anisotropies (r) were determined according to

$$r = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} \quad (1)$$

$$G = I_{hv}/I_{hh} \quad (2)$$

I_{vv} and I_{vh} are the fluorescence intensities parallel and normal, respectively, relative to the vertically polarized excitation light. I_{hv} and I_{hh} are the fluorescence intensities determined with the emission polarizer oriented vertically and horizontally, respectively, when the excitation polarizer was set in the horizontal position.

Results

Human lipoprotein(a), phenotype B according to Utermann (Utermann et al., 1987), and low-density lipoprotein were labeled with two different sulfhydryl-selective fluorescent probes.

The hydrophobic compound acrylodan is not fluorescent. Fluorescence can be observed only after reaction with sulfhydryl groups (Prendergast et al., 1983). Thus, reaction of acrylodan with free sulfhydryls in the lipoproteins can be followed by observation of the time-dependent increase in fluorescence intensity. Figure 1 shows the time course of the fluorescence intensity at 450 nm measured during the reaction of acrylodan with LDL and Lp(a) at 37 °C.

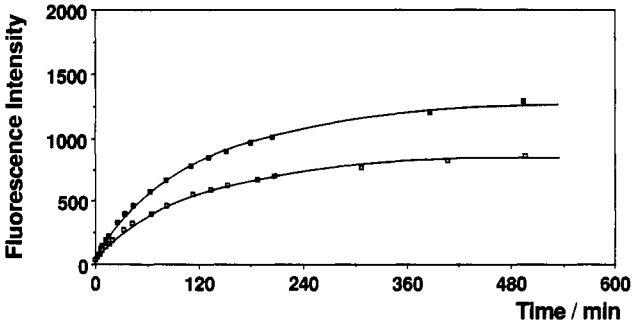

FIGURE 1: Time-dependent increase in fluorescence intensity at 450 nm during incubation of acrylodan with lipoproteins, normalized for protein content: (□) Lp(a); (■) LDL.

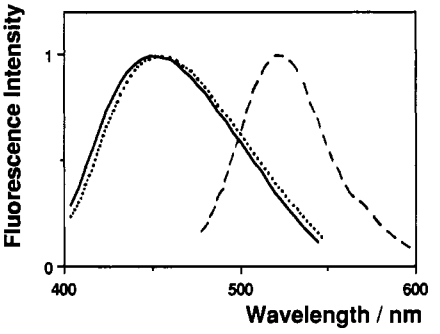

FIGURE 2: Normalized fluorescence emission spectra of acrylodan bound to Lp(a) (—) and LDL (···) and of lucifer yellow iodoacetamide bound to low-density lipoprotein (- - -).

The intensities in Figure 1 are normalized with respect to the protein content of the sample. This figure shows that the acrylodan fluorescence intensity reaches a plateau with both lipoprotein classes after an incubation time of approximately 8 h. The final intensity of the label in the presence of LDL is approximately 1.5 times higher than with Lp(a).

The normalized fluorescence emission spectra for LDL and Lp(a) labeled with acrylodan at 37 °C are illustrated in Figure 2. Both emission maxima are located at rather short wavelengths, indicating a nonpolar environment of the lipoprotein-bound label in both systems. The emission maximum of acrylodan in Lp(a) (450 nm) is somewhat blue-shifted compared with LDL (456 nm). According to spectral data obtained for the mercaptoethanol adduct of the fluorophore in organic solvents, the environmental polarity experienced by the label in lipoproteins is comparable to that of the mercaptoethanol–acrylodan adduct in acetonitrile (Prendergast et al., 1983).

The number of free -SH groups available for reaction with acrylodan was obtained from the fluorescence intensities at 450 nm and related to moles of apolipoprotein using the following values. Molecular masses of 513 and 460 kDa were assumed for apo B (Knott et al., 1986) and apo(*a*), phenotype B (Utermann, 1989), respectively. The calculations for Lp(a) are based on a stoichiometry of 1:1 (mol/mol) for apo(a) and apo B in Lp(a) (Utermann, 1989; MBewu & Durrington, 1990). The stoichiometry of lipoprotein -SH groups labeled with acrylodan is summarized in Table I. Acrylodan reacts with two cysteines in the Lp(a) particle (1.84 ± 0.19 mol/mol of protein, $n = 4$) and three cysteines in LDL (2.84 ± 0.23 mol/mol of protein, $n = 4$).

Lucifer yellow is a brightly fluorescent water-soluble compound. In its functional form, containing the iodoacetamide residue, it can be used for fluorescence labeling of water-accessible sulfhydryl groups. In contrast to acrylodan, it should not penetrate into the deeper apolar domains of the protein in the lipoprotein particle.

Table I: Number of Free Sulfhydryl Groups in Lipoprotein(a) and Low-Density Lipoprotein As Determined by Lucifer Yellow Iodoacetamide (LY) and Acrylodan

lipoprotein	mol of -SH/mol of protein	
	LY	acrylodan
Lp(a)	0	1.84 ± 0.19
LDL	0.91 ± 0.05	2.84 ± 0.23
LY-labeled LDL		1.78 ± 0.21
acrylodan-labeled LDL	0	

Reaction of LDL with LY for 8 h at 37 °C, followed by separation of excess label, gave a fluorescent lipoprotein showing a characteristic lucifer yellow emission spectrum (Figure 2). The fluorescence anisotropy of lipoprotein-bound LY (0.204 ± 0.017 at 37 °C) was elevated relative to the anisotropy for the freely rotating label molecule dissolved in water, which is practically zero. On the basis of the emission intensity of LY in buffer, a label to protein stoichiometry of 1:1 can be assumed for the fluorescent lipoprotein conjugate (0.91 ± 0.05 mol/mol of protein, $n = 2$; see Table I). Under the same conditions as employed for LDL, Lp(a) could not be derivatized with LY.

Thus, LDL possesses a water-accessible free sulfhydryl group that is not available in Lp(a). Moreover, iodoacetamide lucifer yellow does not reach the apoprotein cysteines in the more apolar region of the lipoprotein that are accessible for acrylodan.

In order to prove this finding, LDL was prelabeled with acrylodan. Subsequently, it did not react with LY. Therefore, the buried and the water-accessible -SH groups reacted with acrylodan, leading to the same inert behavior toward the lucifer yellow reagent as already observed with lipoprotein(a). On the other hand, when LDL was prelabeled with LY, only two cysteines (1.78 ± 0.21 mol/mol of protein, $n = 2$) could be detected with acrylodan.

Discussion

In order to assess whether a disulfide linkage exists between apoproteins(a) and B-100 in Lp(a), we investigated the sulfhydryl chemistry of Lp(a) and LDL in a comparative study. Our first attempt to determine free -SH groups by the photometric assay with DTNB (Riddles et al., 1979; Cardin et al., 1982) was not feasible because the method was ill-reproducible and not very sensitive since the amount of free sulfhydryls in lipoproteins is very small (A. Sommer et al., unpublished results). Thus, we decided to apply a more sensitive fluorescence technique, using two different sulfhydryl-selective markers with different hydrophilicity which should detect -SH groups of different localization in the lipoproteins.

One of these fluorescent compounds, lucifer yellow iodoacetamide, is a hydrophilic molecule and should not be able to penetrate into the hydrophobic interior of the particles. In fact, one accessible -SH group could be derivatized in LDL by LY, indicating the existence of a cysteine residing in close proximity to the lipoprotein surface. The same label did not react with Lp(a). As a consequence, the exposed sulfhydryl either is not accessible to the reagent in this particle, e.g., due to the physical presence of apolipoprotein(a), or is involved in chemical bonding. The first possibility could be excluded from our results obtained with the second fluorescence marker, acrylodan.

Acrylodan is a rather apolar compound which should also reach the cysteines of the more hydrophobic peptide segments in the lipoprotein.

Three free -SH groups could be derivatized in LDL whereas only two free sulfhydryls were labeled in Lp(a). Furthermore,

when the surface-exposed cysteine in LDL was blocked by reaction with lucifer yellow iodoacetamide, the number of cysteines for subsequent labeling with acrylodan was reduced to the extent that was observed for Lp(a). In other words, LY-labeled LDL behaved like Lp(a) with respect to its capacity to react with acrylodan. From these findings, it is plausible to assume that the failure of labeling Lp(a) with LY was not due to inaccessibility of a free -SH to the hydrophilic probe. On the other hand, acrylodan-labeled LDL in which all available sulfhydryls were blocked no longer bound lucifer yellow iodoacetamide.

Lee and co-workers (Huang et al., 1988; Lee & Singh, 1988; Lee, 1991) have identified thiol ester linked lipids in apo B. It has been suggested that thiol ester bound fatty acids may play an important role in contributing to the hydrophobic nature of this protein. In the context of our work, it is important to say that reaction of acylated sulfur atoms with the probes acrylodan and lucifer yellow iodoacetoamide under the experimental conditions employed in our work is not very likely. If they did react, we should have detected a much larger number of reactive sulfurs compared with the three -SH groups reported here. An estimate of roughly 10 fatty acyl thioesters was found by Huang et al. (1988), and the respective reactions required much larger pH values in order to remove the acyl groups.

The emission maxima of acrylodan in Lp(a) and LDL are located at very low wavelengths (450 and 456 nm, respectively). This indicates that the labeled cysteines are located in a rather hydrophobic environment. The emission maxima found in the lipoproteins are similar to those found by other authors for acrylodan-labeled cysteines in hydrophobic pockets of proteins, e.g., 480 nm in oligomycin sensitivity conferring protein (Duszynski et al., 1988) or 465 nm in rabbit skeletal muscle F-actin (Marriott et al., 1988). Nevertheless, one of the three cysteines detected in LDL by acrylodan must be in closer proximity to the particle surface since one SH group is available for LY labeling. Thus, it is tempting to suggest that the same surface-exposed -SH group of apo B in Lp(a) is involved in disulfide linkage to apo(a).

The localization of a possible disulfide-linkage site on apo(a) has been suggested. Sequencing of the apo(a) cDNA (McLean et al., 1987) showed a cysteine at position 4057, which is to be found on the penultimate N-terminal kringle 4 of the protein.

No information is available about the localization of the (exposed) cysteine residue in apo B which in Lp(a) may be linked to apo(a) via a disulfide bond. It has been proposed that a possible site involved in covalent protein linkage resides in the C-terminal part of apolipoprotein B (MBewu & Durrington, 1990), probably near the region responsible for lipoprotein binding to the B/E receptor. This could explain the fact that Lp(a) is a poor competitor of LDL binding to the B/E receptor (Steyrer & Kostner, 1990).

In order to find a possible position in the apo B sequence that is involved in disulfide linkage to apo(a), we analyzed the hydrophobicity profile of the protein (Kyte & Doolittle, 1982). There are 25 cysteine residues on apo B with an unusual clustering in the N-terminal region of the protein (Yang et al., 1986). For the reasons mentioned above, we drew attention only to the cysteines on the C-terminal half of apo B including the putative receptor binding region which is supposed to be near residue 3249 (Knott et al., 1986; Innerarity et al., 1987). The criterion for the cysteine search was a high polar index, since, according to our fluorescence data, the respective amino acid should be located near the lipoprotein surface.

However, the results did not reveal any outstanding candidate for such a cysteine residue in the apo B sequence. Nevertheless, our derivatization experiments show that apo B contains a sulfhydryl group which is particularly accessible to hydrophilic agents such as lucifer yellow iodoacetamide. On the other hand, it seems to be buried to some extent. Labeling with acrylodan results in a rather "hydrophobic fluorescence" (see above), and reaction with a pyridyl disulfide derivative of B-phycoerythrin (Oi et al., 1982), a very large hydrophilic sulfhydryl-selective marker, failed (R. Gorges et al., unpublished results).

The site of disulfide bond formation between apo(a) and apo B in vivo is unknown. Lp(a) is synthesized in the liver and is not a metabolic product of other apo B containing lipoprotein classes (Krempler et al., 1979). Recently, a clinical study with *N*-acetylcysteine, a mild disulfide bond reducing agent, has been reported (Gavish & Breslow, 1991). In two patients, Lp(a) levels could be significantly lowered after treatment with *N*-acetylcysteine. It was suggested that this reagent might provide a sulfhydryl acceptor and therefore prevent Lp(a) formation. Since *N*-acetylcysteine is thought to act in the liver, this indicates that the liver could be the organ where apoproteins(a) and B-100 are linked to each other.

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Functional Analysis of the Domains of Dihydrolipoamide Acetyltransferase from *Saccharomyces cerevisiae*†

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ABSTRACT: The *LAT1* gene encoding the dihydrolipoamide acetyltransferase component (E_2) of the pyruvate dehydrogenase (PDH) complex from *Saccharomyces cerevisiae* was disrupted, and the *lat1* null mutant was used to analyze the structure and function of the domains of E_2. Disruption of *LAT1* did not affect the viability of the cells. Apparently, flux through the PDH complex is not required for growth of *S. cerevisiae* under the conditions tested. The wild-type and mutant PDH complexes were purified to near-homogeneity and were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, immunoblotting, and enzyme assays. Mutant cells transformed with *LAT1* on a unit-copy plasmid produced a PDH complex very similar to that of the wild-type PDH complex. Deletion of most of the putative lipoyl domain (residues 8–84) resulted in loss of about 85% of the overall activity, but did not affect the acetyltransferase activity of E_2 or the binding of pyruvate dehydrogenase (E_1), dihydrolipoamide dehydrogenase (E_3), and protein X to the truncated E_2. Similar results were obtained by deleting the lipoyl domain plus the first hinge region (residues 8–145) and by replacing lysine-47, the putative site of covalent attachment of the lipoyl moiety, by arginine. Although the lipoyl domain of E_2 and/or its covalently bound lipoyl moiety were removed, the mutant complexes retained 12–15% of the overall activity of the wild-type PDH complex. Replacement of both lysine-47 in E_2 and the equivalent lysine-43 in protein X by arginine resulted in complete loss of overall activity of the mutant PDH complex. These observations indicate that the lipoyl domain of protein X can substitute, at least in part, for the lipoyl domain of E_2 and that the lipoyl domain of protein X can couple with the catalytic domain of the truncated E_2. Deletion of residues 8–181, encompassing the lipoyl domain, first hinge region, and the subunit binding domain, resulted in loss of ability of the truncated E_2 to bind E_1. Similar results were obtained by deleting the putative subunit binding domain itself (residues 145–181). This domain apparently plays an important role in binding E_1.

Mammalian and *Saccharomyces cerevisiae* pyruvate dehydrogenase (PDH)[1] complexes are organized about a 60-subunit E_2 core, to which multiple copies of E_1, E_3, and protein X are bound by noncovalent bonds (Reed & Hackert, 1990). Protein X binds and positions E_3 to the E_2 core, and this specific binding is essential for a functional PDH complex (Powers-Greenwood et al., 1989; Gopalakrishnan et al., 1989; Lawson et al., 1991).

Studies involving limited proteolysis, molecular genetics, and ^{1}H NMR spectroscopy have provided evidence that dihydrolipoamide acyltransferases possess a unique multidomain structure (Reed & Hackert, 1990; Guest et al., 1989; Perham & Packman, 1989). The amino-terminal lipoyl domain (or domains) is followed by a putative E_3 and/or E_1 binding domain and then by the carboxyl-terminal catalytic domain. The domains are connected by conformationally flexible segments (hinge regions).

An intriguing aspect of structure–function relationships in dihydrolipoamide acyltransferases is the nature and function of the putative E_3 and/or E_1 binding domain. This conserved domain is apparently involved in binding E_3 in the *Escherichia*

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[1] Abbreviations: PDH complex, pyruvate dehydrogenase complex; E_1, pyruvate dehydrogenase; E_2 dihydrolipoamide acetyltransferase; E_3, dihydrolipoamide dehydrogenase; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

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