Electron Spin Resonance Studies of Lipid-Protein Interaction in Human Serum Lipoproteins

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Abstract. Spin-labeled derivatives of high density (HDL) and low density (LDL) lipoproteins from human serum were prepared with N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide. At the low ratios of spin label to protein employed, no changes in circular dichroism or immunochemical reactions were detected. The lipoprotein derivatives, labeled in their protein moieties, exhibited electron spin resonance spectra containing signals of strongly immobilized and weakly immobilized components. The strongly immobilized component was relatively more prominent in high density than in low density lipoproteins. Delipidation or addition of detergent greatly reduced the relative magnitude of the highly-immobilized signal. Reaction with specific antibodies did not alter the signals of spin-labeled lipoproteins or apoproteins. The signals of the reconstituted complex of spin-labeled apoHDL-phospholipid were indistinguishable from those of a spin-labeled apoHDL in the absence of lipid.

With the use of nuclear magnetic resonance (nmr), it has recently been shown that lipid moiety of the plasma low density (LDL) and high density (HDL) lipoproteins behaves as thought it were dissolved in an organic solvent relatively free of constraint; i.e., in a magnetically isotropic environment. Lipid constituents account for about 95 per cent of the nmr absorption in the hydrocarbon region. Since the spectrum of sonically dispersed lipids from lipoproteins and that of native lipoproteins is quite similar and since there is no broadening of the line-widths of the lipid hydrocarbon protons in the native molecule, it appears that the protein exerts little constraint on the lipid.

These studies do not yield much information, however, concerning the effect of lipid on the structural mobility of lipoprotein protein. Delipidation of low or high density lipoproteins produces changes in circular dichroism and immunological reactions of the residual apolipoproteins. In this communication, we further study this question by examining the effect of lipid on the molecular mobility of nitroxide radical that is covalently bound to the protein moiety of high or low density lipoproteins.

Materials and Methods. The spin label, N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide (nitroxide radical I) was purchased from Varian, EPR product group, Palo Alto, Calif. LDL and HDL were isolated from normal, fasting donors by ultracentrifugal flotation between the densities of 1.019 to 1.063 gm/ml (LDL) and
1.063 to 1.21 gm/ml (HDL). These preparations were >99% pure by immunological criteria, based on the absence of other plasma proteins.

Sheep antisera to HDL (and apoHDL) and LDL (and apoLDL) were purified by chromatography on DEAE cellulose. The purified IgG fraction was used.

**Preparation of spin-labeled lipoproteins and apoproteins:** HDL and LDL were labeled in their protein moieties by adding nitroxide radical I to a solution of lipoprotein (10 mg protein per milliliter) in 20 mM sodium phosphate—0.01% EDTA, pH 8.0, and incubating for 3 hr at 25°. One mg of spin label was added per 50 mg of protein. The labeling and other manipulations involving the spin label were performed in the dark. Unbound spin label was separated from the reaction mixture by exhaustive dialysis at 4°. Spin-labeled lipoprotein derivatives were delipidated as previously described for succinyl-LDL except that LDL was dialyzed against distilled water containing EDTA (0.01%) adjusted to pH 7.0 with NaOH, was lyophilized, and the dry residue was extracted with ether: ethanol (3:1). The apoprotein of spin-labeled LDL was completely soluble in aqueous buffers. The apoprotein of spin-labeled LDL was readily solubilized, similar to apoLDL, by incubation at 4° for 2 hr with 100 mM sodium decyl sulfate, pH 8.0. After solubilization, the concentration of detergent could be reduced to 0.2 to 0.4 mM without loss of solubility. In other experiments, spin-labeled apoproteins were prepared by incubating apoHDL or apoLDL with nitroxide radical I (1 mg of spin label per 50 mg protein) in a similar procedure to that described above. Electron spin resonance spectra of spin-labeled apoproteins were qualitatively similar to those of delipidated spin-labeled lipoproteins. However, at the same ratio of spin label to protein, there was more extensive incorporation of the label into apolipoproteins than into the native lipoprotein.

Recombination of apoHDL with phospholipid was performed by the method of Scanu. The apoHDL-phospholipid complex was then spin labeled as described above for HDL and LDL. In other experiments spin-labeled apoHDL was recombined with phospholipid. Spin labeling did not appear to impede recombination and similar results were obtained with the two types of preparations.

Lipoproteins were reduced and alkylated (with iodoacetamide) as previously described. Details for immunochemical and circular dichroic measurements have been given. Protein was measured by the Lowry procedure.

Electron spin resonance spectra were obtained at 9500 MHz using a Varian V-4500-10 spectrometer at protein concentrations of 4–10 mg/ml. Correlation times (τc) were calculated from the ratios of the heights of the first-derivative lines using values for the Hamiltonian parameters described by Griffith et al.

**Results.** N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide derivatives of HDL, LDL, and their apoproteins: The maleimide group has been shown to react with the sulfhydryl and ε-amino groups of lysine in proteins. Nitroxide radical I has been found to label both of these groups in proteins. Since HDL does not contain free sulfhydryl groups, its esr spectra with nitroxide radical I are probably due to labeled amino groups. The extracted lipid contained small amounts of label, estimated at < 10 per cent of the total quantity associated with the labeled lipoprotein. However, since the ether-ethanol extract contained as much as 5 to 10 per cent of the protein, it is not certain that these signals were due to labeling of the lipid. The signals of the ether-ethanol extract were exclusively narrow and contained no broad component. LDL contains both ε-amino groups and free sulfhydryls, but the latter only to the extent of 1 mole/100,000 gm of protein.

At the low levels of spin label used in these experiments, no changes were observed in the solubilities, circular dichroism (Figs. 1 and 2), or immunochemical reactions (Fig. 3) of HDL, LDL, apoHDL, and apoLDL as a consequence of the labeling procedure.
Electron spin resonance characteristics of spin-labeled HDL, apoHDL, LDL, apoLDL, and recombined apoHDL-phospholipid: The native lipoproteins, both HDL and LDL, exhibited esr spectra characteristic of tightly bound, covalently linked spin label. At least two types of signals were present, a narrow one and a broad or strongly immobilized one (Figs. 4 to 6). The broad signal was relatively more prominent in HDL than in LDL (Fig. 6). Upon delipidation of spin-labeled HDL, the relative contribution of the broad component was markedly reduced (Fig. 4). Sodium decyl sulfate, in concentrations of 20-100 mM also reduced the contribution of the broad signal, although the effect was less
Fig. 4.—Electron spin resonance spectra of nitroxide radical I (maleimide) derivatives of HDL (upper tracing) and apoHDL (lower tracing). The protein concentrations were mg/ml.

Fig. 5.—Electron spin resonance spectrum of nitroxide radical I (maleimide) derivative of HDL to show peak-to-peak separation of the strongly immobilized component. High gain amplification is shown in the inset. The outermost peaks are indicated by the arrows.

Fig. 6.—Electron spin resonance spectra of nitroxide radical I (maleimide) derivatives of HDL (upper tracing) and LDL (lower tracing). Identical spectra were obtained in the presence of antisera to HDL or LDL at ratios of antigen to antibody corresponding to antibody excess as determined in separate experiments.

pronounced than with delipidation. The esr spectrum of labeled apoHDL was indistinguishable from that of labeled apoHDL after recombination with phospholipid (Fig. 7).

Delipidation of spin-labeled LDL similarly decreased the constrained signal (Fig. 8), but the results were less reproducible than with HDL, possibly due to variation in extent of aggregation and to the requirement of small amounts of detergent for solubility. Although not measured quantitatively, the intensity of the narrow signal was generally increased by reduction and alkylation of the lipoprotein prior to spin labeling (Fig. 8). When nitroxide radical I was added to a protein-free extract of lipoprotein lipids (dispersed by sonication) narrow signals were obtained comparable to those exhibited when the spin label was dissolved in aqueous buffer. Binding of antibody by spin labeled HDL, LDL, and their apoproteins did not alter their esr spectra. These experiments were performed at ratios of antigen to antibody shown to represent antibody excess and to form precipitin lines on Ouchterlony plates. The resulting spectra were superimpos-
FIG. 7.—Electron spin resonance spectra of nitroxide radical I (maleimide) derivatives of apoHDL (upper tracing) and reconstituted apoHDL-phospholipid complex (lower tracing). The protein concentration in each sample was 4 mg/ml. An identical spectrum was obtained in the presence of antiserum to apoHDL at ratios of antigen to antibody corresponding to antibody excess as determined in separate experiments.

FIG. 8.—Electron spin resonance spectra of nitroxide radical I derivatives of (A) LDL (upper tracing); (B) reduced, alkylated LDL (middle tracing); and (C) reduced, alkylated apoLDL (lower tracing). (B) was prepared by reduction and alkylation of LDL prior to spin labeling. (C) was prepared by delipidation of (B). The protein concentration in each sample was 4 mg/ml.

able to those shown in Figures 6 and 7. Heterogeneity of signals precluded calculation of correlation times for labeled lipoproteins. The calculated value of \( \tau \) for spin-labeled apoHDL was \( 9.5 \times 10^{-10} \) seconds, which is longer by approximately an order of magnitude than the value for the unbound spin label in aqueous solution.

Discussion. The two types of signals contained in the esr spectra of spin-labeled HDL and LDL most likely represent different sites at which the label is covalently attached to the protein. Our results are comparable to those obtained with spin-labeled albumin or with erythrocyte membranes labeled with nitroxide radical I in that the spin label attaches to both weakly and strongly immobilized sites. Peak-to-peak separation, estimated from the distance between the outermost lines, of labeled HDL is 60 to 65 gauss (Fig. 5), which is very close to the value found for similar radicals in rigid glass at low temperature. That these two kinds of signals might represent gross contamination of the lipoprotein preparations was excluded by the immunochemical homogeneity of the HDL and LDL. It seems unlikely that the strongly constrained signal is a consequence of structural alterations produced by the spin-labeling procedure since
neither circular dichroic nor immunochemical changes are detected. Denaturation, or conversion of the lipoprotein to a disordered structure, would have contributed to the narrow signal rather than to the strongly immobilized signal. While aggregation of protein can result in the broadening of a signal, this does not seem a plausible explanation for the strongly constrained signals of the labeled lipoproteins, since the delipidated preparations, which are subject to extensive aggregation, have relatively little broad component. Detergent, which resembles delipidation in its effect, has been shown both to alter the conformation and partially to delipidate lipoproteins.26

It seems likely that the strongly immobilized signal is due to local steric factors rather than to a generalized change in the structural properties of the molecule, since the formation of antigen-antibody complexes with the labeled lipoproteins and apoproteins does not lead to broadening of the signals. These results are consonant with the observation that extensive modification (>90%) of the free ε-amino groups of lipoproteins and apoproteins by amidination does not produce immunological changes and with the conclusion that these groups do not contribute significantly to the antigenic properties of the molecule.7

The strongly immobilized signal cannot be accounted for by a partial distribution of the label within the lipid sphere of lipoproteins. Firstly, there is no appreciable constraint on the molecular motion of the lipids1−2 and secondly, the signals are not broadened in the presence of sonically dispersed lipids. The strongly constrained signal is not regained when apoHDL recombines with phospholipid, a procedure which has been reported to restore the circular dichroic spectrum to one resembling that of the native lipoprotein9 and to restore the ability to activate adipose tissue lipoprotein lipase.13 In a study of spin-labeled proteins (cytochrome C, lysozyme, poly-L-lysine) mixed with phospholipid either in isooctane or sonicated aqueous dispersions, it was inferred that phospholipid molecules decreased the molecular motion of the spin label, the constraint being much greater in isooctane.27 However, more consistent with our findings is the report that nuclear magnetic resonance spectra indicate a looser structure in the recombined complex of apoHDL and phospholipid than in HDL.3 Furthermore, the recombined complex more closely resembles apoHDL than HDL in the temperature dependence of circular dichroism.28

We conclude that the strongly immobilized signal is likely due to a specific type of lipid-protein interaction that is present in the native lipoprotein and is not reformed in a reconstituted apoHDL-phospholipid complex. Removal of lipoprotein lipid greatly reduces the contribution of this strongly constrained signal and increases the relative intensity of the narrow signal by about twofold (Fig. 4). The magnitude of this effect is very similar to that observed when a solution of spin-labeled albumin is acidified.23

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