Early stages of LDL oxidation: apolipoprotein B structural changes monitored by infrared spectroscopy

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Abstract Changes in the conformation of apolipoprotein B-100 in the early stages of copper-mediated low density lipoprotein oxidation have been monitored by infrared spectroscopy. During the lag phase no variation in structure is observed, indicating that copper binding to the protein does not significantly affect its structure. In the propagation phase, while hydroperoxides are formed but the protein is not modified, no changes in secondary structure are observed, but the thermal profile of the band corresponding to α-helix is displaced in frequency, indicating changes in tertiary structure associated with this conformation but not with β-sheet components. When aldehyde formation starts, a decrease of ~3% in the area of bands corresponding to α-helix and β-sheet is produced, concomitantly with an increase in β-turns and unordered structure. The two bands corresponding to β-turns vary as well under these conditions, indicating changes in these structures. Also at this stage the thermal profile shows variations in frequency for the bands corresponding to both α-helix and β-sheet. The results are consistent with the hypothesis that as soon as the polyunsaturated fatty acids from the particle core are modified, this change is reflected at the surface, in the α-helical components contacting the monolayer.—Chehin, R., D. Rengel, J. C. G. Milicua, F. M. Goñi, J. L. R. Arrondo, and G. Pifat. Early stages of LDL oxidation: apolipoprotein B structural changes monitored by infrared spectroscopy. J. Lipid Res. 2001. 42: 778–782.

Supplementary key words apolipoprotein B • secondary structure • thermal denaturation • copper-mediated oxidation • atherosclerosis

Human serum low density lipoprotein (LDL) is a major carrier of serum cholesterol in humans. It is described as a spherical particle containing a hydrophobic core of cholesterol esters and triglycerides, surrounded by an amphipathic monolayer of phospholipid and cholesterol in which a single molecule of apolipoprotein B-100 (apoB) is located. ApoB is a hydrophobic protein containing 4,536 residues. The structure of LDL has been studied previously by infrared spectroscopy (IR), showing that secondary structure at 37°C is 24% α-helix, 25% β-sheet, 6% β-turns, 24% unordered structure, and 24% β-strands. ApoB is affected by lipid transitions occurring in the particle lipid core (1) and contains a fraction resistant to proteolysis (2) that has been attributed to β-strands embedded in the monolayer (1). This fraction varies depending on the particle size (3).

Oxidatively modified LDL seems to play a significant role in the initiation and progression of atherosclerosis in vivo (4–6). Metal ions such as Cu²⁺ or Fe²⁺ are frequently used to initiate LDL oxidation in vitro, since the resulting ox-LDL has similar biological activities to that oxidized in vivo (7, 8). The kinetics of Cu²⁺-induced oxidation is well characterized. Hydroperoxides are produced from the polyunsaturated fatty acyl chains present in the core, which in turn give rise to aldehydes (9). These aldehydes form Schiff bases with the lysine residues of apoB.

In this article we have studied the changes occurring in apoB structure after mild oxidation with CuCl₂. Previous IR studies of apoB oxidation (10) were performed on particles in advanced stages of oxidation. The question addressed here is whether at the beginning of lipid oxidation initial changes in the core lipid can modify apoB conformation. Variations in apoB structure have been probed as previously (1) by monitoring the amide I IR band. This band arises mainly from C=O stretching vibrations and is sensitive to conformational changes. As in our former study, additional structural data have been obtained by examining conformational changes that accompany the protein thermal denaturation. In this case, however, an improved continuous heating procedure has been used instead of the previous temperature-step method. The results support the idea that, even in its early stages, lipid oxidation causes measurable changes in apoB conformation.

Abbreviations: LDL, low density lipoprotein; apoB, apolipoprotein B-100; IR, infrared spectroscopy; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; ESR, electron spin resonance.

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**EXPERIMENTAL PROCEDURES**

**Preparation of lipoproteins**

Human plasma was obtained in the concentration gradient region of density 1.020–1.050 g/cm³ according to published procedures (1, 8, 11). To avoid oxidation, ethylenediaminetetraacetic acid (EDTA) was present in all steps of preparation and all buffers were flushed with nitrogen. The isolated LDL subfraction was concentrated by nitrogen pressure dialysis. The concentrated samples were dialysed in 0.1 M Tris, pH 7.4 with 1 g/l EDTA, sterilized by single-use Minisart (Sartorius) 0.45 µm microfilters and stored in the syringe with the microfilter at 4°C. The purity of the lipoprotein samples was checked by electrophoresis on Radiophor (Immuno AG). The lipoprotein concentrations in LDL solutions were determined by dry weight measurements, including the correction for the salt content of the buffer, or else according to the Lowry procedure (12).

**LDL peroxidation**

100 µl of LDL suspension (10 mg protein/ml) were incubated at 37°C with 26 µl of CuCl₂ at the appropriate concentrations to give final Cu²⁺ concentrations in the 0.5–5.5 µM range. The oxidation was stopped at different times with the addition of 5 µl of 0.40 M EDTA and butylated hydroxytoluene (BHT) up to 50 µM final concentration. The sample was dialysed against 100 mM HEPES pH 7.4. To transfer the lipoprotein to D₂O buffer, the aqueous solution was evaporated in a Speed-vac (Savant) evaporator and then dissolved in D₂O. Transfer to D₂O under these conditions does not alter the protein conformational features (1). Lipid oxidation was followed by measuring conjugated diene formation. Diene accumulation was calculated according to Gieseg and Esterbauer (13). Absorbance at 234 nm was recorded in a Varian Cary 45 spectrophotometer.

**Infrared studies**

The samples were recorded in a Nicolet Magna IR 550 spectrometer equipped with a MCT detector using a demountable liquid cell (Harrick Scientific, Ossining, NY) with calcium fluoride windows and 50-µm spacers. A tungsten-copper thermocouple was placed directly onto the window and the cell placed in a thermostatted cell mount. Thermal analysis was performed by heating continuously in the range of 15–75°C with a heating rate of 1°C/min. Spectra were collected by using a rapid scan software running under OMNIC (Nicolet). Typically, 610 interferograms were collected per spectrum and then referred to a background, the spectra being obtained with a nominal resolution of 2 cm⁻¹. Quantitative information on the amide I and its components at 37°C in D₂O and at the different incubation times studied. The parameters corresponding to these components are displayed in Table 1. The assignment of the lipoprotein bands has been discussed in a previous paper (1). The band at 1656 cm⁻¹ is assigned to

**RESULTS**

The susceptibility of LDL to copper-mediated oxidation can be followed by continuously monitoring the conjugated diene formation. Oxidation is dependent on protein concentration and on Cu²⁺/protein ratio. Different Cu²⁺ concentrations in the range 0.5–5.5 µM were assayed at 10 mg/ml protein. Figure 1 shows the oxidation profile corresponding to 0.5 and 4.5 µM Cu²⁺, the latter being the concentration used in our infrared studies, corresponding to 25 nmol Cu²⁺/mg protein. The three different stages in oxidation, namely the lag, propagation, and disruption phases, are clearly differentiated. The incubation times chosen for the infrared studies were 15, 110, and 180 min, corresponding to 0, 16.3, and 35.9 µM dienes in the sample.

**Fig. 1.** Diene formation corresponding to LDL oxidation produced by 0.5 and 4.5 µM Cu²⁺, the latter being the concentration used in the IR study and corresponding to 25 nmol Cu²⁺/mg protein. The oxidation times at which the samples were taken are shown by an arrow. The dotted lines divide the different oxidation regions (see text).

Protein structure can be studied by IR through decomposition of the original amide I band located between 1700 and 1600 cm⁻¹. This band arises mainly from the stretching vibration of the peptide bond C=O. Figure 2 shows the decomposition of apoB amide I envelope into its components at 37°C in D₂O and at the different incubation times studied. The parameters corresponding to these components are displayed in Table 1. The assignment of the lipoprotein bands has been discussed in a previous paper (1). The band at 1656 cm⁻¹ is assigned to
α-helix and the one around 1630 cm\(^{-1}\) to β-sheet. Bands around 1670 and 1680 cm\(^{-1}\) arise from turns, and the band at 1643 cm\(^{-1}\) is due to unordered structure. The bands at 1618 and 1694, which are characteristic of apoB, were assigned to β-strands, extended structures penetrating the monolayer and establishing hydrophobic interactions at levels deeper than the outer charged shell. This assignment is consistent with a later study by cryoelectron microscopy that shows protein superfdomains connected by a density that, in some cases, crosses the projectional interiors of the LDL particles (16).

Band-fitting of the LDL amide I spectrum shows that the relative proportions of secondary structure of apoB do not change either in the lag (15-min oxidation) or the propagation (110-min oxidation) phase (Table 1). The α-helical component represented by the band at around 1656 cm\(^{-1}\) is 21% of the amide I in all the three spectra, whereas the β-sheet component associated to the band at 1631 cm\(^{-1}\) is also around 21%. The β-strands represented by the bands at 1618 and 1694 cm\(^{-1}\) in the control spectrum remain at 24%, as do β-turns, with two components that together account for 8%, and the unordered structure that accounts for 27% of the amide I band. However, after 180 min of oxidation, at the beginning of the decomposition phase, changes in the percent distribution of the apoB amide I band components can be detected. Thus, the α-helix and β-sheet band components decrease by ~3% when compared with the control, with a corresponding increase in β-turns and unordered structure. Moreover, in the case of β-turns, of the two bands associated to this structure, the component around 1680 cm\(^{-1}\) is lost and the one at 1670 cm\(^{-1}\) increases from around 5% up to 12% (see Fig. 2C and D), indicating a reorganization in the β-turns. It must be noted that the β-strand component, attributed to extended chains penetrating the monolayer, does not vary upon oxidation, at least before the decomposition phase. However, in LDL particles subjected to very long oxidative periods, this band is also affected (data not shown).

Changes in apoB conformation can also be monitored by looking at the effect of temperature on the protein in its native state or after modification by oxidation. The significance of studying temperature-induced protein conformational changes by IR has been previously reported for membrane (17) and soluble (18) proteins, as well as for lipoproteins (1). In Fig. 3 the band positions obtained from the deconvolved spectra of control and oxidized LDL, and corresponding to α-helix (≈1654 cm\(^{-1}\)), β-sheet (≈1630 cm\(^{-1}\)), and β-strands (≈1617 cm\(^{-1}\)), have been plotted against temperature using a continuous heating method. In all three bands, the profiles corresponding to control and 15-min oxidized LDL particles are virtually indistinguishable. However, after 180-min oxidation, when a maximum concentration of dienes is reached, the temperature profile corresponding to the α-helix and β-sheet has been displaced to lower wave numbers, indicating variations in tertiary but not in secondary structure (14, 19). The band at 1617 cm\(^{-1}\) that is attributed to structures embedded in the lipid monolayer is not affected after 180-min oxidation. At 110-min oxidation, the situation is intermediate between the two described above, i.e., the temperature profile of the 1630 cm\(^{-1}\) band (β-sheet) is not affected by oxidation, whereas the band cor-

<table>
<thead>
<tr>
<th>Secondary Structure</th>
<th>Oxidation Time (min)</th>
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<tbody>
<tr>
<td>Control</td>
<td>15</td>
</tr>
<tr>
<td>α-Helix</td>
<td>21</td>
</tr>
<tr>
<td>β-Sheet</td>
<td>21</td>
</tr>
<tr>
<td>β-Strands</td>
<td>24</td>
</tr>
<tr>
<td>β-Turns</td>
<td>8</td>
</tr>
<tr>
<td>Unordered</td>
<td>27</td>
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</tbody>
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The values are taken from experiments such as the one shown in Fig. 2. The assignments are discussed in the text.

For the complete table, please refer to the original source.
responding to α-helix at 1654 cm\(^{-1}\) is displaced almost as much as after 180-min oxidation.

**DISCUSSION**

The present study deals with changes in LDL apolipoprotein structure in the early stages of oxidation, before disruption of the lipoprotein particle. The oxidation process induced by copper ions has been well characterized (8), and it has also been shown that similar results can be obtained at lower copper concentrations with increasing incubation times (9,13). We have used a copper concentration equivalent to 25 nmol/mg protein, which produces a degree of oxidation measured as diene formation equivalent to those described by other authors. This copper/protein ratio is similar to the one used previously in X-ray scattering (20) or DSC studies (21) but is lower than in previous infrared studies (10).

Besides the secondary structure features usually present in proteins, i.e., α-helix, β-sheet, and β-turns, LDL contains a unique extended structure characterized by bands at 1617 and 1693 cm\(^{-1}\) (1, 3). In the lag phase, while the natural antioxidants are still present and no lipid peroxidation is produced, no changes are detected in the secondary structure of apoB, as monitored by spectral decomposition of the amide I band, or in the thermal profile of the bands corresponding to α-helix, β-sheet, or β-strands. An involvement of apoB in the initiation of lipid peroxidation through binding of copper ions has been postulated (13). However, this binding does not affect apoB conformation. In the propagation phase, when hydroperoxides are formed, the secondary structure of apoB does not change either, in agreement with the idea that the main structural features of the protein are still preserved but the thermal profile for the α-helix band is modified. The observed shift in band position can be assigned to a variation in either the helix parameters or the environment surrounding the structure (14) and can imply a change in the tertiary structure of the protein. This result suggests that changes in the lipid core are transmitted to the protein-lipid interface, in agreement with the previously published effect of lipid core phase changes on apoB structure (1, 20). Our observation is also compatible with the calorimetric data in the early stages of oxidation (21) according to which a decrease in the denaturation temperature concomitant with a reduction of about 55–60% of the calorimetric enthalpy is produced. Further oxidation also affects the thermal profile corresponding to β-sheet.

ApoB structure has been postulated to contain amphipathic α-helices (22) that would be inserted in the monolayer and first affected by the core changes. Interestingly, the β-strands embedded in the lipid moiety, resistant to proteolysis and associated to the band at 1617 cm\(^{-1}\), are not affected in these early oxidation stages when the particle is not yet decomposed. If these strands were related to β-hairpins similar to those found in lecithins (18), these structures would be very stable, with a high denaturation temperature and presumably resistant to these small oxidative changes. Then, some of the α-helical structures in contact with the phospholipid monolayer in a hydrophobic environment, where it has been shown by spin-labeling that the oxidative damage occurs first (23), would be affected. Later the structural change would propagate to the β-sheet structures inducing changes in secondary structure, with a decrease in regular structures and a concomitant increase in unordered structure and β-turns. The maintenance of the lipid organization in the samples under study is corroborated by the fact that the bands corresponding to the lipid moiety of LDL are not affected in our samples after 180-min oxidation (data not shown). Longer oxidation periods that produce particle decomposition and changes in the lipid bands also produce larger variations in the secondary structure of the protein, including the β-strands (data not shown). These results are in agreement with calorimetric findings showing that the core lipids that constitute the bulk of the particle are affected after extensive oxidation (21).

In summary, the results presented here show that even in the early oxidation stages of LDL, in the absence of changes in the secondary structure or physical state of the particle, apoB undergoes changes in tertiary structure, primarily in the α-helical and then in the β-structure component, that could be associated with changes on the particle surface. Such an altered surface would then promote the binding of LDL to putative receptors and/or accumulation of cholesterol.

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