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The study of structural accessibility of free thiol groups in human low-density lipoproteins

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Abstract

The experimental evidence for the apolipoprotein B100 (apoB) domain structuring in low-density lipoprotein (LDL) was investigated focusing on the accessibility of free thiol groups. Three different spectroscopic methods were combined with the biochemical perturbations of LDL particle. The spectrophotometric method was adapted for LDL and the exposure of free thiols was analyzed in the native LDL and LDL exposed to sequential denaturation. The results indicate that 24-h denaturation does not expose all free thiols in LDL. Using thiol-specific spin labeling and electron paramagnetic resonance spectroscopy (EPR), different populations of labeled thiols were resolved. The comparison of the EPR spectra of native LDL and LDL with selectively blocked thiol groups revealed significant difference in the respective hyperfine splittings. The phenomenon can arise due to different polarity and/or mobility of the nitroxides in the microenvironments of spin label binding sites of these two LDL samples. The results indicate that nine thiol groups in apoB are distributed in different domains of LDL: two are more exposed, two are buried deeply in the lipid matrix of the particle and the rest are located in hydrophobic parts of this extremely complex protein–lipid assembly. These observations provide experimental support for the emerging theoretical models of apoB.

Keywords: LDL; Denaturation of apoB; EPR

1. Introduction

Low-density lipoproteins (LDLs) are the main cholesterol carriers in human plasma believed to be directly involved in the development of atherosclerosis [1]. They exhibit structural complexity with the surface monolayer organization of mainly phospholipid molecules surrounding the hydrophobic core composed of apolar lipids [2]. One assembly contains approximately 3000 lipids stabilized by only one amphiphatic apolipoprotein B100 (apoB) molecule [3]. The protein consists of 4536 amino acid residues [4,5] and, thus, represents one of the largest monomeric proteins known. Various biophysical methods have been employed to elucidate the structure of the LDL particle which is of central importance in the understanding of its function. The most important progress has been achieved by electron cryomicroscopy [6] but still the LDL structure at the atomic resolution has not been elucidated. At the same time, computational methods have proposed a pentapartite model which recognizes different structural domains in the apoB [7]. However, very little experimental evidence of their existence has been presented so far.

Therefore, in this study the domain organization of apoB was investigated with respect to the structural accessibility of free thiol groups. These are important as an antioxidant potential in LDL since thiol depletion occurs as a result of lipid peroxidation [8]. Based on the determination of the primary structure of apoB, 25 cysteinyl residues have been identified [4,5]. The characterization of apoB sulfhydryl groups from proteolytic cleavage of the protein indicated 16 cysteinyl residues forming disulfide bonds [9], which were recognized to guide the correct folding of apoB in the lumen of endoplasmic reticulum [10]. From these results and further studies based on the tryptic and peptic digestion of the protein, it has been concluded that nine cysteines bear free thiol groups in their side chains with two of them being more exposed at the surface of the native particle [9,11]. However, all nine thiol groups in apoB have never been confirmed

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directly. The attempts to deduce about thiol groups exclusively from the spectroscopic studies of native LDL particles failed to interpret their content correctly [8,12,13].

So, the aim of this study was to analyze the content of free thiols in the native LDL particle in order to resolve different observations cited in the literature [8,12,13]. In addition, further characterization of free thiol groups was attempted to experimentally verify their structural clustering. The approach relayed on three different spectroscopic methods combined with the controlled biochemical perturbations of LDL particles. The spectrophotometric assay introduced by Ellman [14] was adapted to provide free thiol determination in the lipoprotein particles. The exposure of free thiol groups in the native LDL was compared with their gradual exposure during the sequential denaturation of the particle. To confirm the local change in the properties of the environment during LDL denaturation, the intrinsic fluorescence emission was monitored. In order to further characterize free thiol groups in apoB, thiol-specific spin labeling [15] was performed and electron paramagnetic resonance spectroscopy (EPR) was applied. LDL with partially blocked thiol groups was studied, aiming to delineate different environments/dynamic status of labeled cysteines in apoB. Furthermore, the modulation of labeling stoichiometry was analyzed in order to possibly resolve different accessibility of free thiol populations in apoB to covalent modification with hydrophobic spin label. In this way, the content of free thiols detectable in the native LDL particle, which depends on the applied method, could be related to the total of nine cysteinyl residues in apoB, as well as their clustering in domains evidenced.

2. Materials and methods

2.1. Materials

(1-Oxyl-2,2,5,5-tetramethyl-Δ^3-pyrroline-3-methyl) MTS-SL (Reanal Budapest, Hungary), dithiothreitol (DTT, Boehringer Mannheim GmbH), urea (Merck), guanidine hydrochloride (GuHCl, Sigma), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma), sodium dodecyl sulfate (SDS, Merck), potassium iodide (KI, Merck), cesium chloride (CsCl, Boehringer Mannheim) were used. The phosphate-buffered saline (PBS-EDTA, pH 7.4) contained 10 mM potassium phosphate, 1 g/l EDTA and 0.15 M NaCl. For the free thiol group determination in apoB, 0.1 M phosphate buffer, pH 7.2, with 1 g/l EDTA was used.

2.2. Isolation of LDL and denaturation with GuHCl and urea

Human plasma was obtained from blood bank (pooled from three female donors) and lipoproteins were isolated as described previously [16,17]. To avoid the oxidation of lipoproteins, EDTA (1 g/l) was present in all steps of the preparation and all buffers were flushed with argon. The purity of the lipoprotein samples was checked by electrophoresis using Radiophor electrophoresis system with Lipidophor agar medium. The concentrations of lipoprotein samples were determined spectrophotometrically according to Lowry [18] and are expressed as the respective protein content in LDL.

The sequential denaturation of the LDL was performed using GuHCl and urea, which were directly solubilized in the native LDL samples. Final concentrations of GuHCl were 2.5, 4, 6 and 8 M and that of urea 4.5, 6, 8 and 10 M. Effects of these denaturants were followed within 24 h. Minimal concentrations of denaturants were chosen according to the sensitivity of the method.

2.3. Determination of free thiol groups in LDL

The content of thiol groups in apoB was determined using spectrophotometric method based on the procedure originally introduced by Ellman [14] and the measurements were performed on a UV–VIS SP8-100 Pye Unicam instrument at 298 K. The absorbance at 405 nm is indicative of the reaction between sulfhydryl groups and DTNB giving rise to the yellow colored 2-nitro-5-mercaptobenzoic acid anion. The molar extinction coefficient at 405 nm of 13 400 M^{-1} cm^{-1} was used. This approach offers the estimation of the number of thiol groups in LDL which are accessible to react with DTNB. Based on the results of the multiple measurements in the framework of this study, it was concluded that the estimation of free thiol groups according to Ellman and adapted for LDL is accurate within ± 1 thiol group.

In this study the analysis of thiol content in the native as well as in the pretreated LDL samples was performed at three different levels:

(a) The number of free thiols (NFT) was estimated from the number of thiol groups in apoB which are accessible to react with DTNB. In the framework of this study they are classified as exposed thiols.
(b) Determination of the total number of free thiols (TNFT) in the sample requires a disintegration of the LDL particle in order to expose also thiols from the hydrophobic part to react with DTNB. Therefore, SDS (10% aqueous solution) was mixed with LDL (2.3 μM) in a 1:1 volume ratio. The reaction was allowed for 10, 20 and 30 min and then, free thiol groups were determined. It should be emphasized that the reaction mixture has to be kept at room temperature and handled extremely gently.
(c) To estimate the total number of thiols (TNT) in apoB, DTT was used to reduce the disulfide bridges. DTT was prepared as 0.1 M aqueous solution and mixed with LDL (2.3 μM) in a 1:1 volume ratio. After 24 h of incubation followed by the exhaustive dialysis against PBS-EDTA, thiol groups in apoB were determined.

Table 1
The comparative analysis of thiol content in LDL particle

Method	Total number of cysteinyl residues per LDL particle	Number of free thiol groups detected per LDL particle
Ellman procedure adapted for LDL[a]	25	9
apoB primary structure determination	25[b], 25[c]	1–3[b]
Proteolytic digestion of apoB[d]	25	7
EPR spectroscopy of spin labeled LDL[e]		5–6
EPR spectroscopy of spin labeled LDL[a] (MTS-SL/LDL=10:1)		6–7
Fluorescence spectroscopy of LDL[f]		3

[a] This study, the accuracy is within ± 1 thiol group.
[b] Ref. [4].
[c] Ref. [5].
[d] Refs. [9,11],—these authors are aware of the total number of 9 free thiols in apoB but two of them could not be confirmed directly.
[e] Refs. [8,13,24].
[f] Ref. [12].

2.4. Fluorescence spectroscopy

In order to detect the change in the local environment of the fluorophores in the presence of denaturants, the intrinsic fluorescence measurements of the native LDL and LDL denatured for 24 h with urea (10 M) and GuHCl (8 M) were performed using a Varian Cary Eclipse spectrofluorimeter. Steady state fluorescence spectra were recorded with excitation at 280 nm and with sampling data interval of 0.5 nm. The intrinsic fluorescence spectra of native LDL are characterized with a single band centered at 332 ± 3 nm, assigned to Trp residues in apoB [19]. Fluorescence quenching (excitation at 280 nm, emission at 332 nm) was induced with KI, prepared as 5 M aqueous solution containing 0.1 M $Na_2S_2O_3 \times 5\ H_2O$ [20] and with CsCl, prepared as 5 M aqueous solution. The measurements were performed at 298 K.

2.5. EPR spectroscopy and analysis

Thiol-specific spin labeling of apoB was performed using MTS-SL as described previously [15]. In brief: the labeling performed at 298 K for 10 min was stopped by passing the sample through a column of Sephadex G-25 and immediate exhaustive dialysis. Special attention was paid to avoid the MTS-SL biradical formation at pH=7.4. This effect drastically restricts the time duration of the spin labeling procedure as elaborated in Ref. [15]. Thus, the level of spin labeling has to be controlled with the amount of the spin label available for thiol modification. The residual EPR signal in dialysates, detected in several experiments, refers to the signal measured after the gel filtration step. It is indicative of the nonspecific partitioning of the spin label in the LDL particle. The level of cysteine modification was checked in each experiment as TNFT remaining in the spin-labeled LDL after treatment with SDS.

In order to selectively block exposed thiols of apoB, DTNB was allowed to react with free thiols for 30 min in a concentration ratio of LDL/DTNB = 1:19. The excess of the reagent was removed by gel filtration and the remaining free thiols were labeled with MTS-SL.

Stoichiometric titration of the potential thiol labeling sites in apoB was performed for the native LDL and LDL denatured for 24 h either by urea (10 M) or GuHCl (8 M).

The experiments were performed in glass capillaries (1-mm inner diameter) on an X-band Varian E-109 EPR spectrometer. The acquisition of the data was based on the supplied software [21]. Spectral simulations were performed according to the method described by Steinhoff [22]. In order to improve the comparison of different local environments of the spin labels in differently prepared LDL samples, the common approach in the EPR spectroscopy was applied [23]. Namely, the EPR spectra were measured at 260 K to slow down the molecular motion. Relaying on the reported data that it is possible to study frozen, hydrated LDL particles [6], it was assumed that their structural integrity was preserved in the EPR experiments.

3. Results

3.1. Denaturation of apoB

The analysis of the sulfhydryl group content in LDL particle is presented in Table 1 and Fig. 1. In this study, the total of nine free thiol groups (i.e. TNFT) could be detected only after the treatment of the particle with a detergent (e.g.

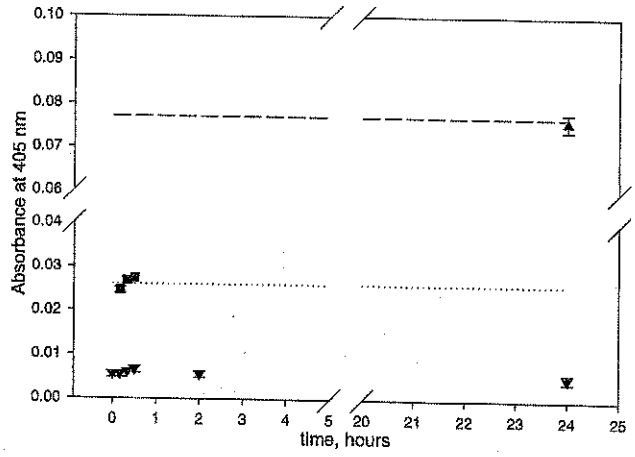

Fig. 1. The time dependence of the absorbance due to the reaction of free thiols in LDL with DTNB in the presence of the detergent and disulfide linkage reducing agent. The following symbols are used: native LDL (▼), LDL exposed to SDS (■) and LDL exposed to DTT (▲). The dotted (dashed) line represents theoretically predicted absorbance (± 0.004) for nine (25) free thiol groups expected in the sample. Experiments with SDS and DTT are described in Section 2.3. The presented data are normalized to the LDL concentration of 2.3 μM.

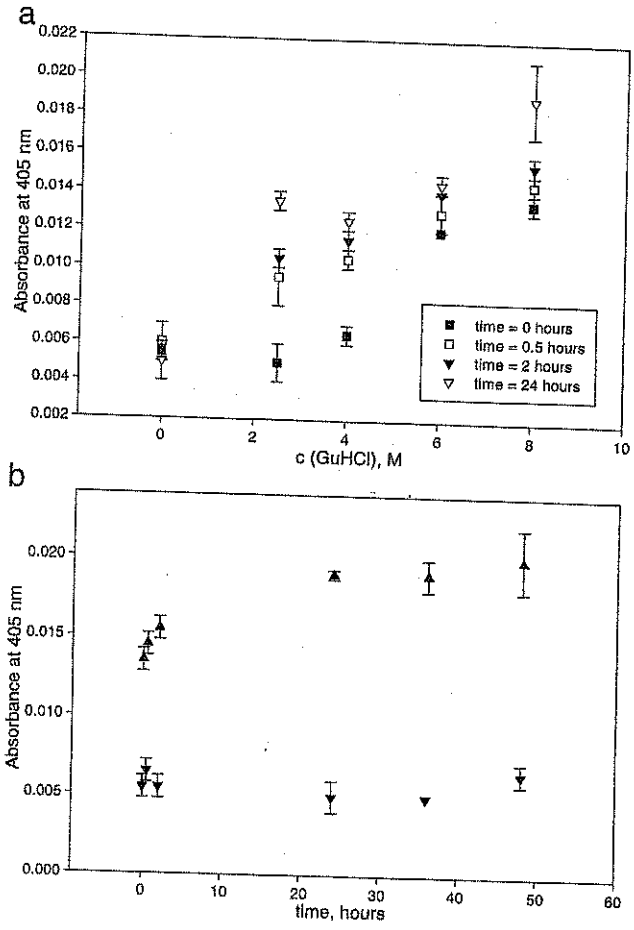

Fig. 2. The change in the absorbance due to the reaction of free thiols in LDL with DTNB. Different time duration of the exposure of LDL (2.3 μM) to GuHCl is indicated in the legend. In the insert the time course of the experiment is shown for the selected concentration of denaturant. The following symbols are used: native LDL (▼) and LDL exposed to 8 M GuHCl (▲).

SDS). The total number of 25 cysteinyl residues (i.e. TNT) in apoB was determined after the exposure of the system to DTT. These results indirectly suggest the existence of eight disulfide bridges in apoB. The comparison with the data from the literature is also given in Table 1.

In the native LDL particle, two free thiols (i.e. NFT) out of nine are detected to be accessible to DTNB. During the sequential denaturation of LDL induced by the increasing concentrations of GuHCl or urea, gradual increase in the absorbance of the product obtained from the reaction of DTNB with accessible free thiols is presented in Figs. 2 and 3. The derived NFT in the LDL samples exposed for 24 h to maximal concentration of denaturants reveals six to seven thiols accessible to DTNB in the presence of either GuHCl or urea. It should be noted that in the presence of denaturants, there exist thiol groups in apoB which cannot be brought into contact with DTNB even under the intensive denaturation conditions.

To independently verify the induced change in the local environment of the intrinsic fluorophores in apoB in the presence of GuHCl or urea, the steady state fluorescence of LDL was observed. Since no shift in the fluorescence maximal emission was detected in the presence of denaturants, the quenching of fluorescence by negatively charged iodide was investigated (Fig. 4). The results indicate that denatured LDL samples exhibit larger fluorescence quenching than the native ones. In the framework of the chosen concentrations of denaturants, GuHCl had somewhat stronger impact on apoB than urea. Quenching of the Trp fluorescence in apoB by positively charged cesium ions appeared ineffective in both native and denatured LDL (data not shown). This result can be a consequence of a local density of positive charges in the neighborhood of the intrinsic fluorophores. The inspection of the primary structure of apoB reveals that ca. 30% tryptophyl residues are located in the close proximity of positively charged amino acids (lysines, arginines). In addition, the lipid content of the surface monolayer in LDL is predominantly represented by zwitterionic phosphatidylcholines, which might locally contribute to the electrostatic barrier towards positively charged quencher which persists even in the denatured state of the particle.

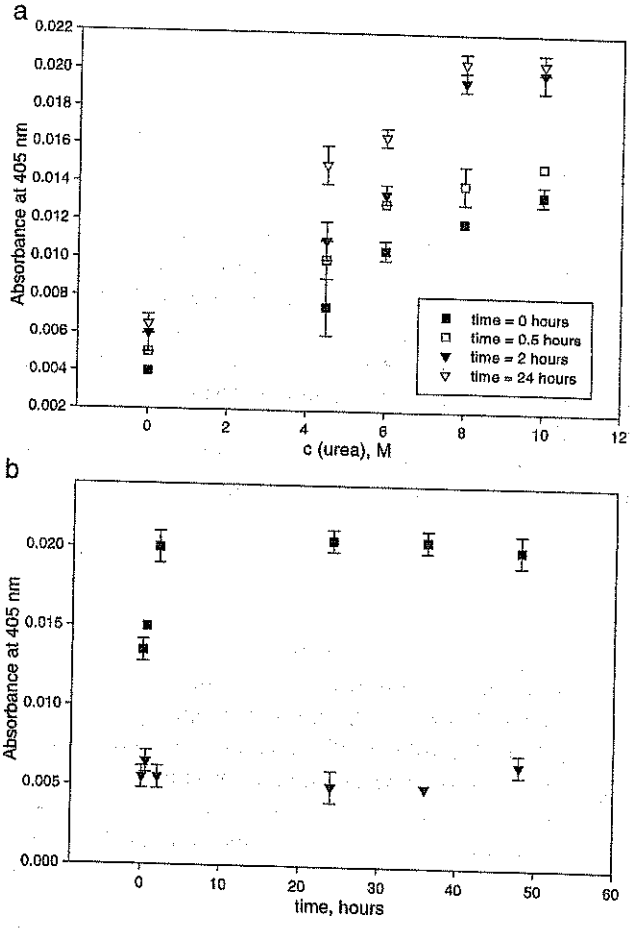

Fig. 3. The change in the absorbance due to the reaction of free thiols in LDL with DTNB. Different time duration of the exposure of LDL (2.3 μM) to urea is indicated in the legend. In the insert the time course of the experiment is shown for the selected concentration of denaturant. The following symbols are used: native LDL (▼) and LDL exposed to 10 M urea (■).

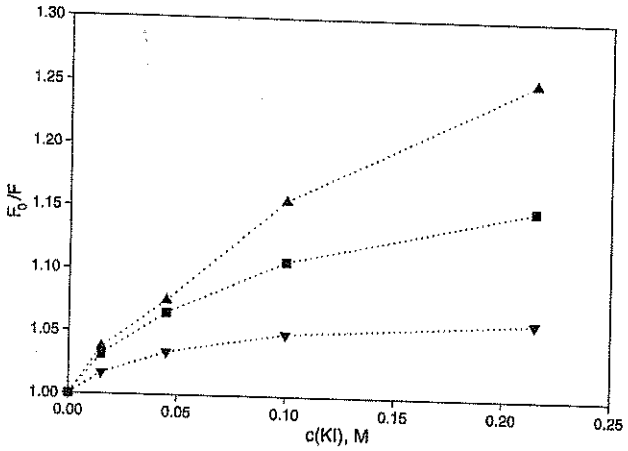

Fig. 4. Quenching of the steady state intrinsic fluorescence of apoB by KI (excitation at 280 nm, emission at 332 nm) after 24-h exposure to denaturants. The intensity in the absence (presence) of the quencher is denoted by F_0 (F). The following symbols are used: native LDL (▼), LDL exposed to urea (■) and LDL exposed to GuHCl (▲).

3.2. EPR spectra of the spin-labeled apoB with partially blocked thiol groups

In order to resolve different populations of thiols, the apoB was labeled with MTS-SL. In comparison with DTNB, MTS-SL is a smaller molecule with the preferential partitioning in the apolar environment. Since it can covalently modify sulfhydryl groups, it can be used to probe those thiol groups in the native LDL which are otherwise sterically inaccessible to DTNB. Therefore, two different spin-labeled LDL samples were prepared, i.e. LDL was spin labeled in the native state as well as after selectively blocking thiol groups. In the later experiment, two exposed cysteinyl residues of the native LDL (i.e. NFT) were covalently modified with DTNB, thus leaving the other seven potential binding sites for the reaction with MTS-SL. The EPR spectra of the spin labeled native LDL and LDL with blocked thiol groups were measured at low temperature to slow down molecular motion (Fig. 5). The maximal hyperfine splittings were estimated directly from the experimental data, $2A_{max}$(exp), as well as from simulated spectra, $2A_{max}$(sim). For the spin-labeled native LDL, $2A_{max}(exp) = 2A_{max}(sim) = 6.58$ mT, whereas for the LDL with partially blocked thiols the respective values are $2A_{max}(exp) = 6.36$ mT and $2A_{max}(sim) = 6.34$ mT. Despite the rather low signal-to-noise ratio of the observed EPR spectra, different hyperfine splittings, characteristic for these two different LDL samples, were experimentally reproducible.

3.3. Titration of the MTS-SL potential labeling sites in apoB

The analysis of the stoichiometry of the covalent modification of free thiol groups in apoB by MTS-SL was performed in order to probe the accessibility of the potential labeling sites with respect to the hydrophobic spin label.

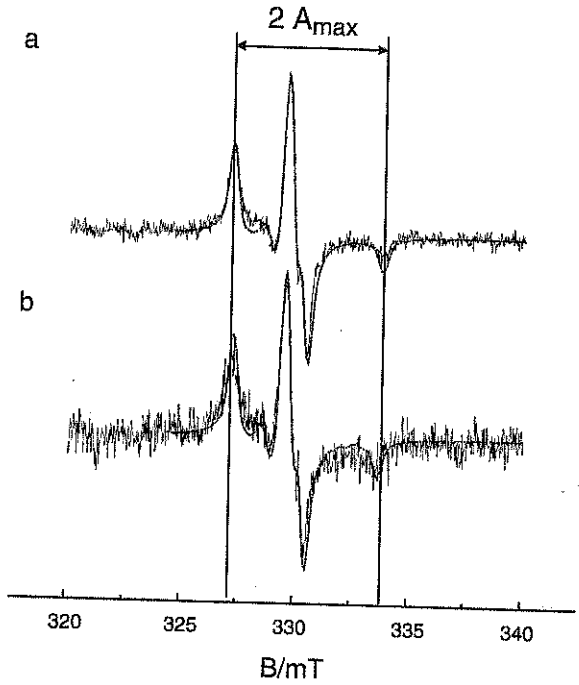

Fig. 5. EPR spectra of the spin-labeled native LDL (a) and LDL with partially blocked thiol groups (b). The spectra were recorded at 260 K with 50-mW microwave power, 0.1-mT modulation amplitude and 100-kHz modulation frequency. Full lines represent the experimental spectra and dotted lines the theoretically fitted ones. The spectra are normalized to the same maximum amplitude and the maximal hyperfine splittings, $2A_{max}$, are indicated. Both LDL samples were spin-labeled using concentration ratio MTS-SL/LDL>25:1.

Native and denatured LDL were labeled in concentration ratios MTS-SL/LDL = 10:1 and 25:1, which should meet the criteria for labeling all nine free thiols in apoB assuming their equal probability to encounter MTS-SL molecules. After spin labeling under lower concentration ratio, the native LDL sample contained still two thiol groups (i.e. TNFT) despite unreacted spin label detected in the dialysate by EPR spectroscopy (Table 2). Similar result was observed when the LDL sample was first exposed to 10 M urea for 24 h and then spin-labeled. On the other hand, LDL denatured with 8 M GuHCl prior to spin labeling exhibited neither free thiol groups nor free MTS-SL in the dialysate. When the

Table 2
Estimation of the number of free thiol groups in apoB prior and after spin labeling using a concentration ratio MTS-SL/LDL = 10:1

Sample	Number of free thiols exposed to DTNB prior to the spin labeling (NFT)	Total number of free thiols left after the spin labeling (TNFT)	Residual EPR signal in dialysate
LDL	2	2	+
LDL + 10 M urea after 24 h	7	2	+
LDL + 8 M GuHCl after 24 h	6	0	–

The method of Ellman [14] was adapted for LDL with the accuracy of ± 1 thiol group.

higher concentration ratio of MTS-SL to LDL was applied, complete thiol labeling was achieved in all LDL samples.

4. Discussion

In this study the experimental evidence for the existence of domain structuring in apoB was investigated. Free thiol groups were studied in the native LDL and LDL exposed to sequential denaturation by GuHCl and urea.

The total number of 25 cysteinyl residues in apoB was detected in accordance with the data reported for the primary structure of apoB [4,5]. Furthermore, all nine free thiol groups in LDL were directly experimentally confirmed. The results indicate that in the native LDL only two free thiols out of nine react with DTNB which can be correlated with the thiols suggested to be exposed at the surface of the particle [9,11]. It should be emphasized that DTNB, besides its chemical affinity to sulfhydryl groups, can react only with a fraction of thiol groups in apoB which are accessible within the boundary of sterically allowed diffusion of the reagent. However, when the structural constraints are removed in the presence of denaturants, additional thiols become accessible to react with DTNB. Interestingly, denaturation for 24 h in the presence of high concentrations of GuHCl and urea still left ca. two to three free thiols inaccessible to DTNB. It can be proposed that these sulfhydryl groups reside in the domains of apoB which are buried in the hydrophobic regions of LDL, e.g. where the protein sequence is anchored in the lipid matrix, and, thus, cannot be brought to the surface of LDL by the hydrophilic denaturants. This reasoning can be supported by the experimental evidence that only in the LDL particle pretreated with a detergent, like SDS, can all free thiol groups be exposed to react with DTNB.

These conclusions can be supported by the results of the experiments performed to resolve different populations of the labeled thiols in apoB. The existence of the domains which might be sterically or structurally detached from the surface of apoB was independently verified in the experiments with differently spin-labeled LDL. The comparison of the low temperature EPR spectra of spin-labeled LDL with blocked sulfhydryl groups with those of the native LDL revealed a significant difference in the respective hyperfine splittings. This phenomenon can arise due to different polarity and/or mobility of the nitroxides in the micro-environments of spin label binding sites of these two LDL samples. For a completely immobilized spin label in a polar or apolar environment one would expect $2A_{max} = 7.32$ or 6.72 mT, respectively [23,25]. The value obtained for LDL bearing partially blocked thiols ($2A_{max}(exp) = 6.36$ mT, $2A_{max}(sim) = 6.34 \pm 0.06$ mT) is close to the nonpolar limit. Thus, it can be anticipated that in this LDL sample, thiol groups modified with MTS-SL do reside in the hydrophobic part of the LDL particle. In addition, residual motion of the spin label side chains must be responsible for the remaining deviation from the rigid limit value. The hyperfine splitting of the other LDL sample is larger ($2A_{max}(exp) = 2A_{max}(sim) = 6.58 \pm 0.04$ mT). A reasonable explanation for this behavior is that the additionally bound spin labels reside in a polar environment shifting the average effective hyperfine splitting to higher values. In order to distinguish between the polarity and mobility effects on the hyperfine tensor, further experiments at lower temperatures have to be performed. Nevertheless, a clear difference between these two LDL samples indicates different populations of the spin-labeled thiols in apoB.

In order to support the above reasoning, the titration of the potential labeling sites in LDL was studied. The results of these experiments undoubtedly pointed out that two out of nine thiol groups are beyond diffusion limited reaction with MTS-SL when the approximately equal molarity of the spin label versus sulfhydryl groups is applied. Furthermore, two thiol groups could not be brought to react with MTS-SL even after the exhaustive denaturation with urea. Only when the intensive perturbation of the electrostatic interactions in LDL was forced by GuHCl, the full exposure of thiol groups to MTS-SL was achieved. This observation can be assigned to different mechanisms of interaction of GuHCl and urea with the biological system as extensively discussed in the literature [26,27]. The phenomenon can also be supported with the results of the experiments with steady state fluorescence quenching by KI. Namely, LDL denatured with GuHCl exhibited larger quenching of Trp fluorescence than LDL denatured with urea, implying lower hydrophobic barrier in the former case. Although it is not possible to provide experimental evidence that always the very same two thiol groups were inaccessible to either DTNB or MTS-SL, the results strongly indicate that two thiols should reside in a separate domain, or in a part of a domain in irreversible interaction with the lipid phase.

5. Conclusion

The results presented here indicate that nine thiol groups in apoB are distributed in different domains of LDL: two are more exposed, two are buried deeply in the lipid matrix of the particle and the rest located in hydrophobic parts of this extremely complex protein–lipid assembly. In the literature [5,7,9,28] one consistently finds Cys^{3734} and Cys^{3890} located in the trypsin releasable fragments of apoB which are part of the receptor binding domain. They can be assigned, in the framework of this study, to the cysteinyl residues bearing free thiol groups accessible to DTNB. Cys^{2906} and Cys^{4190} are reported to reside in the trypsin nonreleasable segments of apoB and these may be the candidates for the thiols deeply buried in the hydrophobic part of LDL as evidenced in this work. From the remaining five thiols in apoB, four should be situated in one domain (Cys^{1085}, Cys^{1395}, Cys^{1478}, Cys^{1635}) and, according to the

results from this investigation, all of them could be exposed in the presence of GuHCl or urea. Thus, here presented material provides experimental support for the proposed model of the apoB [7,9] which suggests clustering of free cysteinyl residues in different domains of the protein.

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