Effect of Probucol on the Physical Properties of Low-Density Lipoproteins Oxidized by Copper

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ABSTRACT: Human plasma low-density lipoproteins (LDL) were incubated with 10 μM probucol for 1 h at 37 °C. Probucol incorporation into the LDL was complete as judged by filtration through a 0.2-μm filter, ultracentrifugation, and gel filtration. LDL with and without probucol were incubated for up to 24 h with 5 μM Cu^{2+} at 37 °C. Copper oxidation increased the content of random structure in the LDL protein from 30% to 36% at the expense of β-structure (which decreased from 22% to 16%) without a change in α-helical content as measured by circular dichroism spectroscopy. This loss of β-structure was prevented by the presence of probucol in the LDL during the copper incubation. Probucol reduced the rate of increase of fluorescence during copper oxidation at 37 °C. After 6 h, the fluorescence intensity at 360-nm excitation and 430-nm emission was 30% less in probucol-containing samples. Probucol had no effect on the circular dichroic spectrum of LDL and only minimal effects (<5%) on the fluorescence emission spectrum at wavelengths below 500 nm. Two fluorescence peaks, with emission at 420 nm and excitation at 340 and 360 nm, are resolved in three-dimensional fluorescence spectra of oxidized LDL. Probucol reduces the intensity of both peaks equally. The binding of a highly reactive heparin (HRH) fraction to LDL was measured by titration of LDL with HRH in the presence of fluoresceinamine-labeled HRH. The decrease in fluorescence anisotropy of the labeled HRH is proportional to the concentration of bound HRH. Copper oxidation of LDL reduced the number of binding sites for HRH from 3.5 to 0.8 and increased the K_d of binding from 1.2 to 9.9 μM. Probucol completely protected the LDL so that incubation with Cu^{2+} for 24 h at 37 °C had no effect on HRH binding to the probucol-LDL. Similarly, probucol completely prevented the 40% decrease in reactive amino groups on the surface of LDL observed after 24 h of incubation with Cu^{2+}. These data support the hypothesis that probucol reduces the rate of lipid oxidation and prevents lipoprotein surface modifications associated with oxidation of LDL.

Probucol, 4,4′-(isopropylidenedithio)bis(2,6-di-*tert*-butylphenol), an antioxidant that significantly reduces plasma cholesterol concentrations in several animal species, was discovered during a search for nontoxic hypocholesterolemic agents (Barnhart et al., 1970). Recently, probucol has been shown to be effective in diminishing plasma cholesterol concentrations in patients with hypercholesterolemia (Cortese et al., 1982; Miettinen et al., 1986; Fellin et al., 1986; Dachet et al., 1985), in preventing the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits (Kita et al., 1987; Carew et al., 1987), and in reducing Achilles tendon xanthomata in patients with homozygous familial hypercholesteroemia (Baker et al., 1982; Yamamoto et al., 1986).

The mechanism of action of probucol appears, at least in part, to be due to its properties as an antioxidant. It has been proposed that probucol limits oxidative modification of low-density lipoproteins (LDL)[1] and thus minimizes uptake of modified LDL by macrophages (Naruszewicz et al., 1984). Many of the lipid-loaded "foam cells" that accumulate in the subendothelial spaces during atherogenesis appear to be derived from monocyte/macrophages that enter by penetration of the endothelial lining (Fowler et al., 1979). Oxidation of LDL has been proposed as a mechanism by which the macrophage accumulates lipid [reviewed in Brown and Goldstein (1983)]. If this oxidative modification hypothesis is correct, then treatment with antioxidants that block modification of LDL may reduce the formation of fatty streak lesions, in which macrophage-derived foam cells contain most of the stored lipid (Fowler et al., 1979). Carew et al. (1987) have shown that the rate of degradation of LDL in the macrophage-rich fatty streak lesions of the LDL receptor deficient rabbit is reduced by about half in rabbits fed probucol in the diet.

Binding of LDL to macrophages is promoted by modification of the LDL surface by acetylation (Brown & Goldstein, 1983) and by treatment with the lipid peroxidation product malonaldehyde (Fogelman et al., 1980; Haberland et al., 1982). These modifications reduce the number of reactive amino groups and increase the net negative charge on the surface of LDL. Similar changes in surface charge and macrophage uptake are observed following incubation of LDL with endothelial cells or Cu^{2+} (Henrikson et al., 1981, 1983; Morel et al., 1984; Heinecke et al., 1984; Steinbrecher et al., 1984; Steinbrecher, 1987). Hydrolysis of protein and phosphatidylcholines in LDL is also observed in lipoproteins incubated under oxidizing conditions (Schuh et al., 1978; Steinbrecher et al., 1984; Fong et al., 1987).

Chelation of copper with EDTA (ethylenediaminetetraacetic acid) or preincubation of LDL with BHT (butylated hydroxytoluene) or α-tocopherol reduces the extent of the oxidative changes observed in LDL (Schuh et al., 1978; Steinbrecher et al., 1984; Morel et al., 1984). Probucol-containing LDL obtained by incubation of LDL with probucol, or from patients taking probucol to reduce plasma cholesterol levels, do not show the increase in thiobarbituric acid reactive sub-

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[1] Abbreviations: LDL, low-density lipoproteins; HRH, heparin with high reactivity to LDL; TNBS, 2,4,6-trinitrobenzenesulfonic acid: CD, circular dichroism.

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stances (primarily lipid oxidation products) nor the increase in macrophage uptake characteristic of oxidized LDL when incubated for up to 24 h with Cu^{2+} (Parthasarathy et al., 1986).

A major unresolved question is the effect of probucol on the structure of LDL during incubation under oxidizing conditions. Since probucol minimizes LDL binding to macrophages after incubation with Cu^{2+} (Parthasarathy et al., 1986), copper incubation was chosen as a model for LDL oxidation with particular relevance to the development of atherosclerosis. The concentration of probucol (10 μM) chosen for the experiments is similar to that used by Parthasarathy et al. (1986) and is significantly less than the 25–100 μM concentration of probucol in the serum of patients treated with probucol (Fellin et al., 1986; Dachet et al., 1985). The effectiveness of probucol in the prevention of oxidative damage to LDL secondary structural elements and the production of fluorescent products of oxidation were examined. The surface of LDL was probed by measurement of reactive amino groups and the binding of heparin purified by affinity chromatography to normal LDL (Hirose et al., 1987; Cardin et al., 1987). The data suggest that the effectiveness of probucol in inhibiting macrophage uptake by LDL incubated with Cu^{2+} is related to protection of surface lysine resiudes and maintenance of specific heparin binding.

Materials and Methods

Isolation of LDL. Human plasma was obtained by plasmapheresis of fasting donors. Sodium azide (0.01%) and EDTA (1 mM) were added to the plasma. LDL were isolated by ultracentrifugation in salt solutions of KBr between densities of 1.019 and 1.063 g/mL. LDL were then dialyzed against Tris-buffered saline (0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.01% sodium azide, 1 mM EDTA) and stored at 4 °C for up to 1 month. Prior to each experiment, LDL were dialyzed against phosphate-buffered saline (0.15 M NaCl, 20 mM sodium phosphate, pH 7.4) and filtered through a Gelman Acrodisc 13 (0.2-μm) filter.

Incorporation of Probucol into and Oxidation of LDL. LDL were diluted to a concentration of 0.2 mg of protein/mL in degassed, phosphate-buffered saline. Degassing of buffers was achieved by stirring under vacuum at 50–70 °C for >2 h. Probucol (26 μL of a 0.4 mg/mL solution in ethanol) was added while the solution was vortexed to attain a final concentration of 10 μM in a 2-mL volume. The same amount of ethanol was added to a second aliquot of LDL to serve as a control. Samples were capped under a N_2 atmosphere. The samples were then incubated for 30 min at 37 °C. Following incubation, the samples were immersed in an ice–water bath for 30–45 min. Samples were dialyzed in the dark at 4 °C against degassed phosphate-buffered saline immediately prior to measurements and copper oxidation (LDL incubated with probucol is referred to as probucol-LDL). For copper oxidation of LDL, 0.02 mL of 0.5 mM $CuSO_4$ was added to 2 mL of freshly dialyzed LDL (0.2 mg/mL) to attain a concentration of 5 μM Cu^{2+}. Then, the test tubes containing LDL were capped under an air atmosphere and were incubated at 37 °C for up to 24 h.

Column Chromatography. Probucol, labeled with ^{14}C in the phenol ring (11 mCi/mL), was a kind gift of E. R. Wagner (Dow Chemical Co., Midland, MI). LDL (0.2 mg/mL) were incubated for 1 h at 37 °C with 10 μM [^{14}C]probucol, added in ethanol (0.4 mg of probucol/mL). The sample was filtered through a Gelman Acrodisc 13 (0.2-μm) filter, and 0.5 mL was applied to a Superose 6 (Pharmacia) column. The column was eluted with phosphate-buffered saline solution at a flow rate of 0.5 mL/min. Absorbance at 275 nm was monitored continuously on an LKB detector. Fractions of 1.3 mL were collected and assayed for protein. The concentration of probucol was measured by counting a 0.1-mL aliquot in 10 mL of BudgetSolve (Research Products International) by liquid scintillation.

Circular Dichroism Spectra. CD spectra were recorded at 25 °C from 300 to 190 nm on a Jasco J-500A spectropolarimeter with 2-nm slit width. The CD of buffer was subtracted from the CD of the sample after each scan. A total of nine scans were averaged. The data were transferred to a computer, and the data from 250 to 200 nm at 1-nm intervals were fit by nonlinear regression analysis (Marquardt, 1963) with a set of four reference spectra (Bolotina et al., 1980; Yang et al., 1986) as described (McLean et al., 1986). The data were fit with both constrained (to 100% total structures) and unconstrained (sum of structures can take on any value) sums of α, β, turn, and random structures.

Fluorescence Spectra. Fluorescence spectra were recorded on an SLM 4800 spectrofluorometer under a N_2 atmosphere at 25 °C. The samples were gently stirred to prevent settling of the lipoprotein. For three-dimensional spectra, emission spectra were recorded with excitation wavelengths from 310 to 500 nm in 10-nm intervals. Data were collected in the emission scan from 390 to 600 nm in 5-nm intervals and truncated for emission wavelengths less than 30 nm greater than the excitation wavelength to remove the light scattering peak. The data files were converted to ASCII format and transferred to an RS-1 (BBN Research Systems, Cambridge, MA) table. The THREED graphics display program in RS-1 was used to generate the three-dimensional figures.

Heparin Binding. Fluoresceinamine-labeled high-reactive heparin (F-HRH) and unlabeled HRH were kind gifts of Dr. A. D. Cardin (Cincinnati, OH) and were prepared as described by Hirose et al. (1987). LDL (0.4 mg) were mixed with 0.02 mg of F-HRH in 2 mL of 10 mL Tris-HCl, pH 7.4. The decrease in polarization of the fluorescent heparin on addition of unlabeled HRH was followed on an SLM 4800 spectrofluorometer operating at 25 °C. Samples were excited with 465-nm light from the excitation monochromater with 8-nm slits; emission was observed through an Ealing 35-3557 filter (center 510 nm, bandwidth 7.7 nm). Light scattering contributions of LDL to the emission signal were subtracted prior to calculation of the anisotropy. Reactant concentrations were corrected for dilution; molecular weights of 13 000 for HRH (Cardin et al., 1987) and 512 937 for LDL apoB-100 (Chen et al., 1986) were used. The correction factor for the detection system ($G = I_{HV}/I_{HH}$) was measured with the excitation polarizer oriented horizontally and the emission polarizer oriented vertically (I_{HV}) or horizontally (I_{HH}); the anisotropy $r = (I_{VV} - GI_{VH})/(I_{VV} + 2I_{VH})$, where I_{VH} and I_{VV} are the fluorescence intensities measured with the excitation polarizer oriented vertically and the emission polarizer oriented horizontally or vertically, respectively.

The fraction of bound F-HRH was calculated from $B = (r - r_{min})/r_{max} - r_{min})$, where r is the measured anisotropy in the presence of LDL, r_{min} is the anisotropy of F-HRH in aqueous solution, and r_{max} is the maximum anisotropy of F-HRH bound to LDL determined by extrapolation of r vs [F-HRH] to zero concentration in a titration of unmodified LDL. The anisotropy data as a function of added HRH, at concentration $[HRH]_t$, were fit to an equilibrium binding model for a single noninteracting binding site. Here, $K_d = n[LDL][HRH]_f/[HRH]_b$, where K_d is the dissociation constant for the binding, n is the number of binding sites per LDL particle, [LDL] is

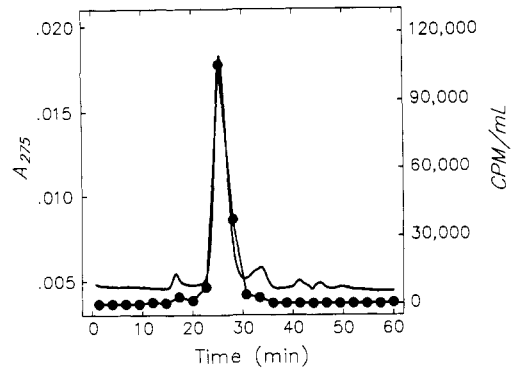

FIGURE 1: Association of [^{14}C]probucol with LDL. [^{14}C]Probucol-LDL was passed over a Superose 6 gel filtration column. Fractions of 1.3 mL were collected. The elution of LDL was observed at 275 nm with an LKB UV detector (—), and [^{14}C]probucol was measured by liquid scintillation counting of a 0.1-mL aliquot of each fraction (●).

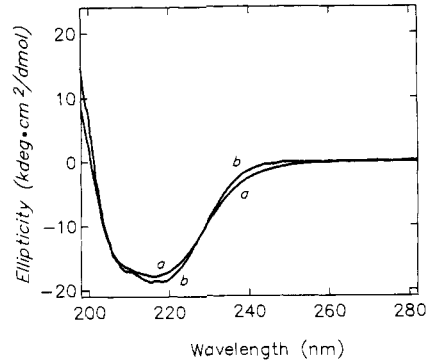

FIGURE 2: CD spectra of (a) untreated LDL and (b) probucol-LDL incubated in the presence of 5 μM Cu^{2+} for 24 h at 37 °C. Spectra were recorded at 25 °C in phosphate-buffered saline. Data were collected every 0.2 nm, and buffer blanks were subtracted. Each spectrum is the average of nine scans.

the concentration of LDL, and the concentrations of free and bound HRH are $[HRH]_f$ and $[HRH]_b$, respectively. Substitution of $[HRH]_f = [HRH]_t - [HRH]_b$ results in the quadratic expression $[HRH]_b^2 - [HRH]_b(K_d + [HRH]_t + n[LDL]) + n[LDL][HRH]_t$. The smaller root of this equation gives an expression for $[HRH]_b$ in terms of r with physically meaningful values of K_d and n. Since LDL concentrations vary somewhat during the titration with HRH, the LDL concentrations were indexed to the volume of HRH added. The data were fit by nonlinear regression (Marquardt et al., 1963) in a FORTRAN program (McLean & Jackson, 1985) with fixed r_{min} and r_{max}.

Analytical Methods. Protein concentrations were measured by the micro-BCA (bicinchoninic acid) technique (Pierce Chemical Co.). Heparin concentrations were calculated from uronic acid concentrations measured by the carbazole technique (Bitter & Muir, 1962). The concentration of reactive amine groups in a sample of LDL was measured by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method as described by Steinbrecher (1987).

RESULTS

Incorporation of Probucol into LDL. The concentration of Cu^{2+} and the conditions of incubation of LDL with copper were designed to duplicate the conditions of Parthasarathy et al. (1986), who studied the effect of oxidation of LDL on binding to macrophages. Although probucol is relatively insoluble in aqueous buffers, it is important to establish that probucol added in ethanol is entirely within the lipoprotein. LDL were incubated with [^{14}C]probucol added in ethanol for 30 min at 37 °C and then at 4 °C for an additional 30 min. After incubation, the LDL were filtered through a 0.2-μm Gelman filter. When [^{14}C]probucol was added to buffer alone and filtered, the [^{14}C]probucol was quantitatively trapped on the filter; <0.1% of the ^{14}C was observed in the filtrate. By contrast, >90% of the ^{14}C and LDL protein was recovered in the filtration when LDL were included in the incubation. No decrease in probucol/protein ratio was observed in the filtrate on the basis of recovery of LDL protein. When [^{14}C]probucol-labeled LDL were centrifuged in a KBr gradient, the ^{14}C counts were observed only in fractions containing the LDL protein. LDL labeled with [^{14}C]probucol were also chromatographed on a Superose 6 column (Figure 1). The [^{14}C]-probucol and the LDL elution patterns coincided; no peak of ^{14}C was observed that was not associated with LDL protein, indicating that all of the probucol is in the lipoprotein and not in the aqueous phase.

Table I: Effect of Copper Oxidation on Structural Elements of LDL with and without Probucol[a]

sample Θ_{220}	α	β	turn	random
LDL	36.2 ± 0.4	22.3 ± 0.8	11.8 ± 0.7	29.7 ± 1.0
probucol-LDL	37.4 ± 0.5	23.1 ± 1.0	10.6 ± 0.8	28.9 ± 1.3
LDL	35.4 ± 0.9	15.9 ± 1.6[b]	12.5 ± 1.4	36.3 ± 2.1[b]
probucol-LDL oxidized	34.5 ± 0.4	21.6 ± 0.7	13.0 ± 0.6	30.9 ± 0.9

[a] The CD spectra of LDL at 25 °C were analyzed for four secondary structural components (Bolotina et al., 1981) with the constrained, normalized technique described under Materials and Methods. [b] Significantly different from LDL at $p < 0.05$.

Secondary Structural Analysis of LDL. An estimate of the secondary structural components of the LDL protein was made by fitting CD spectra to a set of standard spectra for each element. In Figure 2, the CD spectra of LDL and probucol-LDL after incubation with 5 μM Cu^{2+} for 24 h at 37 °C are shown. The CD spectra for LDL and probucol-LDL before copper incubation are not distinguishably different from line b in the figure. In samples incubated with copper, but without probucol, Θ_{220} is increased by 1100 deg cm^2/dmol and Θ_{240} is decreased by 1200 deg cm^2/dmol. These changes are significantly greater than the error in the measurements (SD of <200 at 220 nm and <100 at 240 nm). The data were fit by a constrained technique in which the sum of the four fitted structures must equal 100% and by an unconstrained technique in which the sum of the structures may take on any value. The unconstrained results were then normalized to 100% structure. The advantage of the unconstrained, normalized technique is that the estimates of secondary structure are less affected by absorption flattening due to light scattering and inaccuracies in the protein concentration measurement (Wallace & Teeters, 1987). A check of the accuracy of the fitted data is to compare the constrained and unconstrained results. In all cases the unconstrained sum of structures did not differ by more than 10–20% from 100%. In addition, both fitting techniques gave qualitatively similar results. Probucol had no significant effect on the structural elements of LDL (Table I). Oxidation reduced the β content of LDL in the absence of probucol but not in the presence of probucol. The reduction in β content of LDL upon oxidation was mirrored by an increase in the random structure of the lipoprotein. Higher concentrations of Cu^{2+} [up to 100 μM as in Herzyk et al. (1987)] had no further effect on the CD spectrum. Oxidation did not have a significant effect on the α-helical content or the content of β-turn structure in the lipoprotein as measured by CD.

Fluorescence Spectra. The rate of increase of fluorescent products of copper oxidation in LDL at 37 °C is shown in

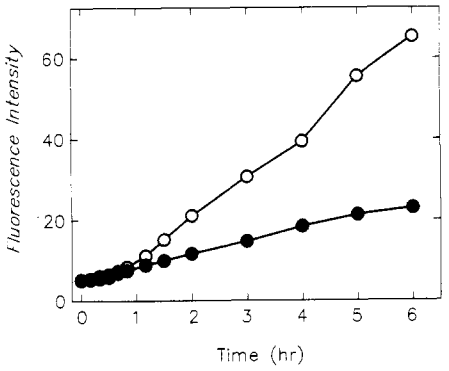

FIGURE 3: Fluorescence intensity change during incubation of LDL with and without probucol in the presence of 5 μM Cu^{2+} at 37 °C. Fluorescence spectra were recorded with excitation at 360 nm and emission at 430 nm. Untreated LDL (○); probucol-LDL (●).

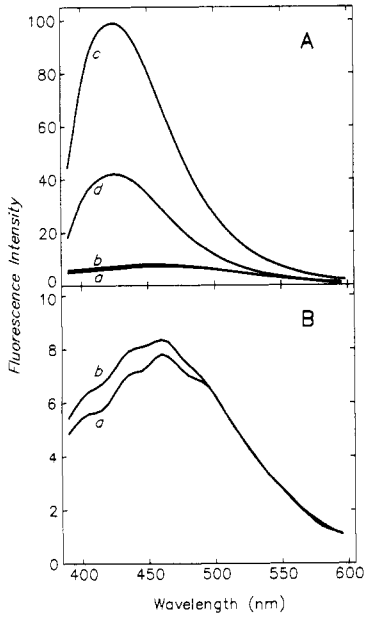

FIGURE 4: (A) Comparison of fluorescence emission spectra (excitation 360 nm) of untreated LDL (a) and probucol-LDL (b) and the same samples after 24 h of incubation with 5 μM Cu^{2+} at 37 °C: oxidized LDL (c) and oxidized probucol-LDL (d). (B) The same emission spectra of LDL (a) and probucol-LDL (b) on a different scale.

Figure 3. Probucol in LDL reduces the rate of increase of fluorescent products over the first 6 h by 3-fold. After 6 h of incubation, the rate of increase of fluorescent products is slowed and appears to be near equilibrium after 24 h; the spectra at 48 and at 24 h are similar (data not shown). After 24 h of incubation at 37 °C, the fluorescence of LDL measured with excitation at 360 nm and emission at 430 nm is ~10-fold enhanced over that of LDL incubated without copper (Figure 4). The fluorescent products (EX 360/EM 430) in probucol-LDL samples incubated with copper are ~40% of those of normal LDL incubated with copper (Figure 4A).

Probucol has only a minimal effect on the fluorescence of LDL (Figure 4B). A weak, broad fluorescence peak is observed with excitation at 360 nm in the range of 400–550 nm. Oxidation increases the fluorescence emission in this region, shifting the peak emission wavelength from 460 to 420 nm in the presence and absence of probucol. Two peaks at excitation wavelengths of 340 and 360 nm are resolved in the three-dimensional spectrum (Figure 5) with emission at 420 nm. The 360-nm excitation peak is not well resolved from the peak expected for the Raman band of water at 410 nm, but the Raman band contribution to this emission band is minimal; LDL before oxidation show only a small Raman band at 420 nm after subtraction of the buffer blank, and there is no visible change in the turbidity of the LDL samples after oxidation. The emission maxima of the major peaks in oxidized samples correspond to the two peaks observed by Jurgens et al. (1986, 1987) in hydroxynonenal-treated LDL. The shapes of the spectra of LDL and probucol-LDL incubated with copper are similar. The principal difference in copper-incubated LDL is that probucol reduces the intensity, and presumably concentration, of fluorescent products produced.

Table II: Effect of Copper Oxidation of Heparin Binding to LDL and Amino Group Reactivity with and without Probucol[a]

sample	heparin binding		reactive amines (mol/mol of apoB)
	n	K_d (μM)	
LDL	3.5 ± 0.3	1.2 ± 0.2	290 ± 10
probucol-LDL	4.3 ± 0.3	1.4 ± 0.2	270 ± 10
LDL oxidized	0.8 ± 0.4[b]	9.9 ± 0.5[b]	180 ± 10[b]
procubol-LDL oxidized	3.2 ± 0.1	1.4 ± 0.1	290 ± 20

[a] Heparin binding was measured by displacement of fluoresceinamine-labeled heparin of high reactivity of LDL (HRH) by unlabeled HRH. Binding constants were obtained from analysis of the fluorescence anisotropy of fluorescent HRH as a function of added HRH with a single noninteracting site model. Reactive amines were measured on LDL samples with TNBS as descrubed under Materials and Methods. [b] Significantly different from LDL at $p < 0.05$.

High-Reactive Heparin Binding. High-reactive heparin is a heparin preparation purified by affinity chromatography on a column of LDL–Affi-Gel (Hirose et al., 1987). Two high-affinity binding sites with K_d of 1 μM and several low-affinity binding sites of K_d = 10 μM have been reported for this heparin (Cardin et al., 1987). Binding of a fluoresceinamine-labeled HRH to LDL results in an increase in anisotropy of the fluorescent reporter group due to the decrease in rotational motion of the HRH–LDL complex compared to HRH in solution. This change in anisotropy was used by Cardin et al. (1987) to define the high-affinity binding sites of LDL for HRH. A similar technique is employed here, except that F-HRH bound to LDL is displaced by titration with unlabeled HRH. This technique obviates the need for correction of the data for viscosity effects that arise in titrations of F-HRH with LDL. The limiting anisotropy at high concentrations of HRH approaches the anisotropy of F-HRH in aqueous solution (r_{min} = 0.077). Somewhat higher values for n (Table II) are observed in low ionic strength buffer (10 mM Tris-HCl) than in 50 mM HEPES and 0.1 M NaCl (Cardin et al., 1987). Probucol has no effect on the concentration of HRH required to displace the F-HRH (Figure 6) and does not change the number of binding sites or their affinity significantly (Table II). After incubation of the LDL with 5 μM Cu^{2+}, F-HRH does not bind effectively to LDL in the absence of probucol even at the low ionic strength (no NaCl and 10 mM Tris-HCl) of the present experiments. The small amount of F-HRH that binds (<10%) to the oxidized LDL is nearly quantitatively displaced by the first addition of HRH. Oxidation of LDL in the absence of probucol results in a 10-fold increase in K_d and a reduction of the number of sites to <1 (Table II). By contrast, probucol-LDL incubated under the same conditions, for 24 h with 5 μM Cu^{2+}, binds F-HRH as effectively as unoxidized LDL or probucol-LDL, and the concentration of HRH required to displace the F-HRH is not changed (Figure 6) nor is n or K_d decreased.

Reactivity of LDL Amino Groups. Recently a decrease in reactivity of LDL to TNBS following oxidation with Cu^{2+} was reported (Steinbrecher, 1987). The decrease in reactivity was shown to be due primarily to decreased lysine content in LDL

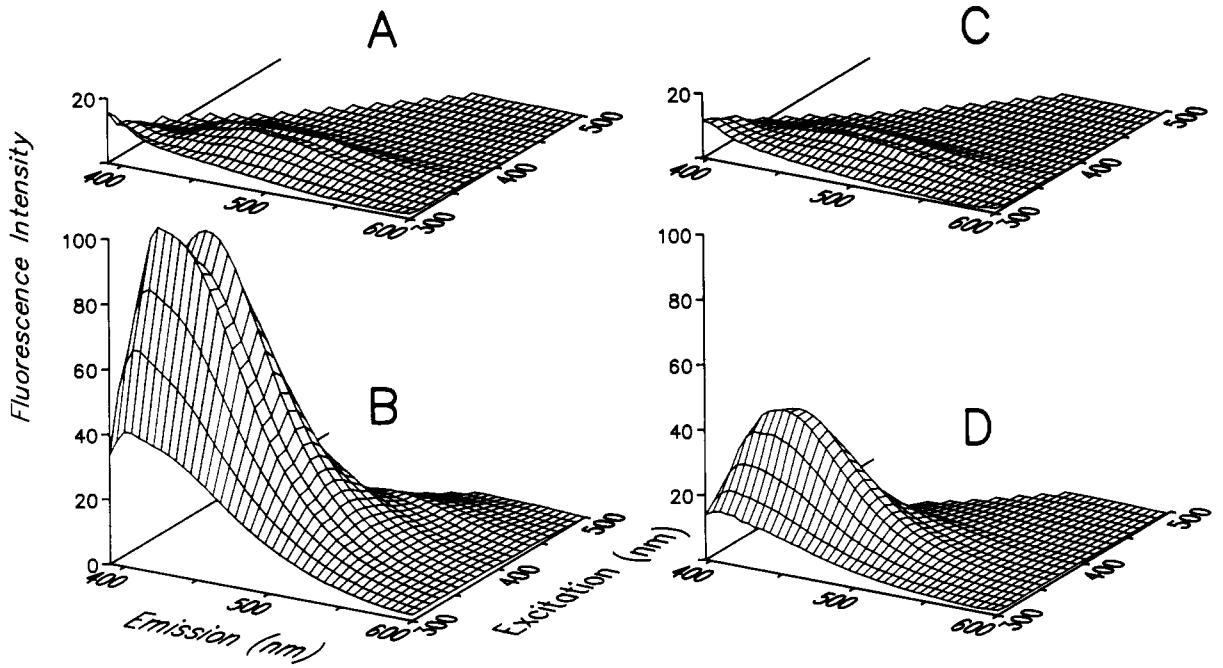

FIGURE 5: Three-dimensional spectra of LDL before (A) and after (B) incubation and of probucol-LDL before (C) and after (D) incubation for 24 h with Cu^{2+}. Excitation was varied from 310 to 500 nm in 10-nm intervals and emission from 390 to 600 nm in 5-nm intervals. The data were truncated at emission wavelengths <30 nm greater than the excitation wavelength.

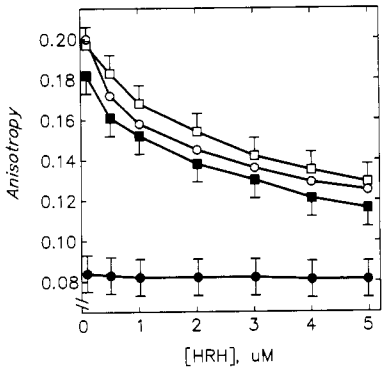

FIGURE 6: Displacement of F-HRH bound to LDL by unlabeled HRH. The decrease in fluorescence anisotropy of fluoresceinamine-labeled high-reactive heparin bound to LDL (○), probucol-LDL (□), 24-h Cu^{2+}-oxidized LDL (●), and oxidized probucol-LDL (■) was measured during a titration with unlabeled HRH.

although part (<10%) of the decrease in TNBS reactivity may be due to modification of phosphatidylethanolamine and phosphatidylserine on the surface of the lipoprotein. A significant decrease in the reactivity of LDL amino groups was observed following oxidation of LDL with 5 μM Cu^{2+} (Table II). This decrease in TNBS reactivity parallels the increase in K_d and the decrease in n for heparin binding. The presence of probucol in LDL completely prevents the decrease in reactive amino groups of LDL following incubation with copper.

Discussion

In cell culture experiments, oxidation of LDL in the presence of Cu^{2+} results in an increase in LDL uptake by macrophage receptors (Steinbrecher et al., 1984). Probucol reduces this oxidative damage to the LDL, minimizing the uptake of LDL by the macrophage receptor and the production of thiobarbituric acid reactive substances (Parthasarathy et al., 1986). The present paper focuses on structural modifications of the lipoprotein and the effect of probucol on reducing structural damage following incubation under oxidizing conditions. Probucol prevents oxidative changes in LDL secondary structure, reduces the rate of formation of fluorescent products of oxidation, and protects the surface of LDL from oxidative damage. Probucol completely inhibits the loss of reactive amino groups on the LDL surface that occurs during oxidation. In addition, it prevents the loss of specific heparin binding to the LDL, which occurs after 24 h of incubation with Cu^{2+}.

Oxidation of LDL with Cu^{2+} increases somewhat the random structure of the LDL protein at the expense of β-structure. In Fourier transform infrared studies of Cu^{2+} oxidation of LDL (Herzyk et al., 1987) an increase in random structure was also observed. However, the infrared data suggested a loss of β-turn structure in the LDL in contrast to the loss of β-structure apparent in the CD. Some shifts in bands that can be assigned to β-structure (1630.5 and 1682.5 cm^{-1}) are also observed in the IR, but the major β-structure band at 1620 cm^{-1} is unaffected by oxidation. The differences do not appear to be due to the higher Cu^{2+} concentration used in the IR studies as the CD data are not further altered by increasing the concentration of copper during the incubation. The content of β-structure (22%) observed in the CD is similar to that predicted from the secondary structure (21%) and is somewhat higher than that found by Cardin et al. (1982), who used a three-component analysis of their CD data. One plausible mechanism for the loss of β-structure on oxidation is the release of small peptides from the LDL, which have a reduced β-structure (Cardin & Jackson, 1986) and which were not separated from the intact LDL particle. This mechanism is consistent with the protective effects of probucol on degradation of apoB-100 in LDL incubated with Cu^{2+} (S. Busch, R. Barnhart, and R. L. Jackson, unpublished results). Alternatively, oxidized lipid may alter the lipid–protein interactions in the lipoprotein, resulting in an altered secondary structure that is protected in the presence of probucol.

Oxidation increases the concentration of fluorescent products of oxidation associated with LDL (Jurgens et al., 1986, 1987). Since some increase in fluorescent products in LDL is observed in the presence of probucol and probucol does not completely prevent the increase in thiobarbituric acid reactive products during incubation with Cu^{2+}, some oxidation of LDL occurs

even in the presence of probucol. One of the major oxidation products that is formed during oxidation of LDL is malonaldehyde, but most of the malonaldehyde does not remain associated with LDL. By contrast, 4-hydroxynonenal, another oxidation product, remains associated with the LDL particle (Esterbauer et al., 1987). The increase in fluorescence of LDL with excitation at 360 nm and emission at 430 nm is attributed to reaction of apoB with 4-hydroxynonenal and similar aldehydes but not with malonaldehyde, which increases fluorescence intensity at an excitation wavelength of 400 nm and an emission wavelength of 470 nm (Jurgens et al., 1986). In the three-dimensional fluorescence spectra of LDL incubated with Cu^{2+} in the presence or absence of probucol, the latter fluorescence intensity is minimal. Probucol does not alter the distribution of fluorescent products but simply reduces the intensity of the fluorescent products produced by oxidation.

Although probucol only reduces the concentration of fluorescent products produced during Cu^{2+} oxidation of LDL, it prevents modification of reactive amino groups on the surface of LDL. On the basis of amino acid analysis, the majority of the decrease in reactive amino groups on LDL oxidized with Cu^{2+} is lysine residues (Steinbrecher, 1987); derivatization of lysine groups on LDL was the result of adducts of lipid peroxidation products that reacted with the lysine amino groups on the surface of LDL. Since probucol does not completely inhibit the production of fluorescent oxidation products but completely inhibits the decrease in reactive amino groups on the surface of LDL, these data suggest that probucol prevents a secondary oxidation event, which results in modification of lysine groups on the surface of the LDL.

Probucol protects lysine residues on the surface of LDL which are critical to the binding of high-reactive heparin (HRH). Acetylation of LDL increases the binding of LDL to macrophages (Brown & Goldstein, 1983) and inhibits binding of HRH to LDL (Cardin et al., 1987). Probucol is effective both in inhibiting LDL uptake by the macrophage receptor (Parthasarathy et al., 1986) and in preventing the loss of HRH binding to LDL incubated under oxidizing conditions. The fluorescent products that are formed during incubation of probucol-LDL with Cu^{2+} do not interfere with HRH binding. One explanation for this is that the products formed are fluorescent lipids and adducts with amino acid residues not critical to HRH binding, such as Gly, His, Arg, and Lys residues embedded in the core of the lipoprotein.

Several mechanisms have been proposed for the effectiveness of probucol in reducing the incidence of atheromatous lesions in experimental animals, including alterations in catabolism of LDL (Nestel & Billington, 1981; Kesaniemi & Grundy, 1984), decreased hepatic synthesis of lipoproteins (Li et al., 1980; Balasubramanian et al., 1981), and interference with the assembly of proteins and lipids (McLean & Hagaman, 1988). The reduction of macrophage uptake of LDL by probucol appears to be primarily mediated by the antioxidant effect of probucol on LDL structure. Since macrophage uptake of surface-modified LDL may be one of the early events in atherogenesis, leading to lipid deposition in the arterial wall, probucol may be particularly effective in preventing atherosclerosis in humans. The present data suggest that the effectiveness of probucol in inhibiting macrophage uptake by LDL incubated under oxidizing conditions is related to protection of surface lysine residues. We propose that probucol reduces the concentration of lipid peroxides produced during oxidation and prevents the reaction of lipid peroxides with lysine residues on the surface of LDL. This mechanism predicts that specific macrophage binding sites on the surface of LDL are protected by probucol.

Registry No. Probucol, 23288-49-5; heparin, 9005-49-6; L-lysine, 56-87-1.

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tRNA and the Guanosinetriphosphatase Activity of Elongation Factor Tu†

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ABSTRACT: Different sites of the tRNA molecule influence the activity of the elongation factor Tu (EF-Tu) center for GTP hydrolysis [Parlato, G., Pizzano, R., Picone, D., Guesnet, J., Fasano, O., & Parmeggiani, A. (1983) *J. Biol. Chem. 258*, 995–1000]. Continuing these studies, we have investigated some aspects of (a) the effect of different $tRNA^{Phe}$ species, including Ac-Phe-$tRNA^{Phe}$ and 3′-truncated $tRNA_{-CCA}$ in the presence and absence of codon–anticodon interaction, and (b) the effect of occupation of the ribosomal P-site by different $tRNA^{Phe}$ species. Surprisingly, we have found that 3′-truncated tRNA can enhance the GTPase activity in the presence of poly(U), in contrast to its inhibitory effect in the absence of codon–anticodon interaction. Moreover, Ac-Phe-$tRNA^{Phe}$ was found to have some stimulatory effect on the ribosome EF-Tu GTPase in the presence of poly(U). These results indicate that under specific conditions the 3′-terminal end and a free terminal α-NH_2 group are not essential for the stimulation of the catalytic center of EF-Tu; therefore, the same structure of the tRNA molecule can act as a stimulator or an inhibitor of EF-Tu functions, depending on the presence of codon–anticodon interaction and on the concentration of monovalent and divalent cations. EF-Tu·GTP does not recognize a free ribosomal P-site from a P-site occupied by the different $tRNA^{Phe}$ species. When EF-Tu acts as a component of the ternary complex formed with GTP and aa-tRNA, the presence of tRNA in the P-site strongly increases the GTPase activity. In this case, the extent of the stimulation displays variations dependent on the tRNA species. Ac-Phe-$tRNA^{Phe}$ enhances the GTPase activity more than Phe-$tRNA^{Phe}$ and $tRNA_{OH}$. Whether these differential effects are a consequence of a direct or indirect interaction between EF-Tu and the tRNA species on the ribosome remains to be verified. In conclusion, our data show that the interaction between tRNA and mRNA is pivotal for the specific binding of the ternary complex favoring the activation of the EF-Tu center for GTP hydrolysis by the ribosome.

The study of the GTPase reaction uncoupled from protein synthesis and catalyzed by elongation factor Tu (EF-Tu)[1] has yielded helpful information for an understanding of the mechanism of the coupled one. The hydrolysis of GTP is one of the key reactions for the proper functioning of the factor: formation of the complex with GTP allows EF-Tu to adopt the active conformation needed for a productive interaction with aa-tRNA and the ribosome, while hydrolysis of GTP to GDP induces a conformational transition resulting in dissociation from the ribosome [for reviews, see Kaziro (1978), Parmeggiani and Sander (1981), Bosch et al. (1983), and

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[1] Abbreviations: EF-Tu, elongation factor Tu; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; P-site, ribosomal donor or peptidyl site; A-site, ribosomal acceptor site; tRNA, general name indicating several transfer RNA species; $tRNA_{OH}$, unacylated transfer RNA; aa-tRNA, aminoacyl-tRNA; $tRNA^{Phe}$, purified phenylalanine-accepting tRNA; $tRNA^{Val}$, purified valine-accepting tRNA; $tRNA_{-CCA}$, tRNA lacking the 3′-terminal CCA sequence; Ac-aa-tRNA, (*N*-acetylamino)acyl-tRNA.

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