Role of the interfacial binding domain in the oxidative susceptibility of lecithin:cholesterol acyltransferase

Kewei WANG* and Papasani V. SUBBAIAH*†[1]

*Department of Medicine, Rush Medical College, 1653 West Congress Parkway, Chicago, IL 60612-3833, U.S.A., and †Department of Biochemistry, Rush Medical College, 1653 West Congress Parkway, Chicago, IL 60612-3833, U.S.A.

We had previously shown that the cholesterol esterification activity of lecithin:cholesterol acyltransferase (LCAT) is destroyed by oxidation, but still it retains the ability to hydrolyse water-soluble substrates. This suggested that the inactivation of the enzyme is not due to its catalytic function, but due to a loss of its hydrophobic binding. Since recent studies have shown that a tryptophan residue in the putative interfacial domain (Trp^{61}) is critical for the activity, we determined the possible role of this residue in the oxidative susceptibility and substrate specificity of LCAT by site-directed mutagenesis. Deletion of Trp^{61} resulted in a 56% loss of cholesterol esterification (LCAT) activity, but the phospholipase A_2 (PLA_2) and the esterase activities of the enzyme were stimulated slightly. Replacing Trp^{61} with another aromatic residue [$Trp^{61} \rightarrow$ Tyr (W61Y)] resulted in an increase in all activities (14–157%), whereas replacing it with an aliphatic residue [$Trp^{61} \rightarrow$ Gly (W61G)] caused a dramatic loss of LCAT (−90%) and PLA_2 (−82%) activities, but not the esterase activity (−5%). W61Y was the most sensitive to oxidation, whereas W61G was the most resistant, with respect to the LCAT and PLA_2 activities. However, the activities which do not involve interfacial binding, namely the esterase activity and the transesterification of short-chain phospholipids, were more resistant to oxidation in all LCATs, indicating a selective loss of the interfacial binding by oxidation. Furthermore, replacing the two cysteines (Cys^{31} and Cys^{184}) in the Trp^{61} deletion mutant caused additional resistance of the enzyme to oxidizing agents, showing that both domains of the enzyme contribute independently to its oxidative susceptibility. Since the hydrolysis of truncated phospholipids, generated during the oxidation of low-density lipoproteins, does not require the interfacial-binding domain, our results suggest that LCAT may take part in the detoxification of these compounds even after the loss of its cholesterol esterification function.

Key words: cysteine, esterase, oxidized phospholipids, phospholipase A_2, tryptophan.

INTRODUCTION

Although the role of oxidatively modified low-density lipoproteins (LDL) in atherogenesis has been widely accepted [1], it is likely that the site of LDL oxidation is the arterial tissue rather than the plasma compartment, because of the presence of various anti-oxidant mechanisms in plasma. On the other hand, the modification of certain apoproteins and enzymes by oxidizing agents is more likely to occur in the plasma under physiological conditions, because they are not associated with antioxidants. One such sensitive enzyme is lecithin:cholesterol acyltransferase (LCAT), which is an essential component of the reverse cholesterol transport pathway, and is the main source of cholesteryl esters in plasma [2]. Many investigators have reported the extreme sensitivity of the enzyme to oxidative inactivation. Thus exposure of human plasma to cigarette smoke or 0.5 mM copper sulphate results in about 90% loss of the enzyme activity in 6 h [3]. Similarly, incubation of plasma with 33 mM 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH), a source of peroxy radicals, inhibits the enzyme activity by 85% in 4 h [4]. Even minimally oxidized LDL, containing very low concentration of thiobarbituric acid-reactive substances, strongly inhibits the enzyme [5], showing that the inhibition is not due to a loss of substrate phosphatidylcholine (PC) or apoprotein activators, but due to modification of the functional groups of the enzyme. The inhibition by cigarette smoke appears to be in part due to the derivatization of the thiol groups of the enzyme by the aldehydes present in the smoke [6], whereas the inhibition by the peroxy radicals is due to the formation of lipid peroxides, which in turn bind certain functional groups of the enzyme [3,4]. Studies from our laboratory showed that mild oxidation of whole plasma with Cu^{2+} or AAPH, which completely inhibited the cholesterol esterification by the enzyme, actually activated the transesterification of short-chain phospholipids produced by the lipid peroxidation [7]. Similarly, the oxidation of purified LCAT destroyed the ability of the enzyme to transesterify or hydrolyse long-chain PC, but had no effect on the transesterification of oxidized truncated PC or platelet-activating factor (PAF), indicating that the peroxy radicals did not damage the active site of the enzyme. Previous work [8] from our laboratory also showed that the presence of the two thiol groups in the vicinity of the active-site pocket is partly responsible for the high sensitivity of the enzyme to oxidative inactivation. We showed that the substitution of the two free cysteines (Cys^{31} and Cys^{184}) with glycines results in a significant protection of the enzyme against inactivation by the peroxy radicals. Furthermore, chicken LCAT, which contains only one free thiol group, was found to be more resistant to oxidation than human LCAT, which has two free

Abbreviations used: AAPH, 2,2′-azo-bis(2-amidinopropane) dihydrochloride; apo AI, apolipoprotein AI; CHO, Chinese-hamster ovary; C31G, $Cys^{31} \rightarrow$ Gly; NEFA, non-esterified fatty acid; HDL, high-density lipoproteins; LAT, lysolecithin acyltransferase; LCAT, lecithin:cholesterol acyltransferase; LDL, low-density lipoproteins; PAF, platelet-activating factor; PC, phosphatidylcholine; PLA_2, phospholipase A_2; PNPB, *p*-nitrophenyl butyrate; W61Y, $Trp^{61} \rightarrow$ Tyr; Trp^{61}del, LCAT in which Trp^{61} is deleted.

[1] To whom correspondence should be sent at the Department of Chemistry address (e-mail psubbaia@rush.edu).

thiol groups. Introduction of an additional cysteine into the chicken enzyme, corresponding to that in the human LCAT led to increased inactivation of the activity compared with the wild-type chicken LCAT. In these experiments, however, complete protection of the enzyme was not achieved even after the substitution of the two cysteines of human LCAT with glycines, suggesting that additional domains of LCAT are involved in its oxidative inactivation. Furthermore, because of the retention of the activity against polar substrates, including the truncated PCs generated by the peroxidation of lipoprotein PC, we reasoned that the interfacial domain of the enzyme, which is responsible for its binding to the hydrophobic substrates, may be one of the targets of the oxidizing agents. Studies by Adimoolam and Jonas [9], and Peelman et al. [10] showed that the potential 'lid' domain of the enzyme, corresponding to residues 50–74, represents the interfacial binding domain, and is responsible for its binding to its lipoprotein substrates. Furthermore, the tryptophan residue at position 61 was found to be critical for its interaction with the lipoprotein substrates [10]. Since the tryptophan residue is highly susceptible to oxidizing agents [11], we investigated the possibility that the specific tryptophan in the interfacial domain may be an initial target of the oxidizing agents, in addition to the two cysteines near the active site. The results presented here show that deletion of the tryptophan residue in the interfacial domain or its substitution with an aliphatic residue is associated with a significant reduction in the oxidative susceptibility of LCAT, whereas its substitution with an aromatic residue results in increased susceptibility.

MATERIALS AND METHODS

Materials

[4-^{14}C]Cholesterol (specific activity of 33 mCi/mmol) was purchased from American Radiochemicals, whereas the labelled lyso PC (1-[1-^{14}C]-palmitoyl, 56.8 mCi/mmol) and 1-palmitoyl-2-[1-^{14}C] linoleoyl PC (50 mCi/mmol) were purchased from DuPont (New England Nuclear, Boston, MA, U.S.A.) and ($-^{35}$S-ATP) was obtained from Amersham. AAPH was obtained from Wako Chemical (Richmond, VA, U.S.A.), PAF and *p*-nitrophenyl butyrate (PNPB) were products of Sigma. Unlabelled lyso PC and PC were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Chinese-hamster ovary (CHO) cells were obtained from A.T.C.C. Apoprotein AI was prepared from delipidated human high-density lipoproteins 3(HDL_3), and LCAT was purified from human plasma (obtained from local blood bank) both as described previously [12].

Preparation of LCAT mutants

cDNA for human LCAT, covering the entire coding region, and cloned into *Hin*dIII/*Bgl*II sites of pSV2 was obtained from Dr Omar Francone (Pfizer Central Research, Groton, CT, U.S.A.). Site-directed mutagenesis of the LCAT was performed using the QuikChange mutagenesis kit from Stratagene (La Jolla, CA, U.S.A.) essentially according to the method of the manufacturer. The following oligonucleotide primers were used for the mutagenesis (with the mismatched bases underlined): For $Trp^{61} \rightarrow Tyr$ (W61Y) mutation: 5′-GACTTCTTCACCATCTACCTGGATCTCAACATG-3′; Trp^{61} deletion: 5′-GACTTCTTCACCATCCTGCTGGATCTCAACATG-3′; for $Trp^{61} \rightarrow Gly$ (W61G) mutation: 5′-GACTTCTTCACCATCGGGCTGGATCTCAACATG-3′. The triple mutant [$Cys^{31} \rightarrow Gly$ (C31G), C184G, Trp^{61}del)] was prepared by mutagenizing the double cysteine mutant (C31G, C184G) we prepared previously [8] with the primer used for Trp^{61} deletion as shown above. All sequences of the resultant cDNAs were verified by dideoxy chain termination method, using [γ-^{35}S]ATP (Amersham Corp). CHO cells were transfected with the mutant cDNAs using the calcium phosphate method, and the medium from the cells was used directly for the experiments without further purification. The concentration of LCAT protein in the medium was determined by solid-phase ELISA, employing chicken polyclonal antibody specific to human LCAT (provided by Dr John Parks, Wake Forest University, Winston-Salem, NC, U.S.A.), and purified human plasma LCAT as standard [8,13].

Assay of enzyme activities of LCAT

Five different reactions catalysed by LCAT were determined with all the LCAT preparations. (1) Cholesterol esterification (LCAT) activity was measured by the formation of labelled cholesteryl ester from labelled free cholesterol incorporated into proteoliposome substrate, prepared by cholate dialysis [14]. The substrate contained egg PC, 4-^{14}C cholesterol and apolipoprotein AI (apo AI) in the molar ratio of 300:15:1. The reaction mixture contained 50 μl of CHO cell medium containing the recombinant LCAT, 100 μl of the proteoliposome substrate, 2 mg of HSA, 5 mM mercaptoethanol and a buffer comprising 10 mM Tris/150 mM NaCl/1 mM EDTA (pH 7.4), in a final volume of 0.4 ml. Incubation was performed for 1 h at 37 °C, and stopped by the addition of 2 ml ethanol. The lipids were extracted with 2 ml of hexane (twice), and separated on silica gel TLC with the solvent system of petroleum ether/ethyl acetate (85:15, by vol.). The radioactivity in free and esterified cholesterol was determined in a liquid-scintillation counter, and the activity is expressed as nmol of cholesterol esterified/h per μg of LCAT protein. (2) Lysolecithin acyltransferase (LAT) activity was measured similarly, using proteoliposomes containing egg PC, [1-^{14}C]-palmitoyl lyso PC and apo AI in the molar ratio 300:15:1 [15]. After incubation with the enzyme for 1 h at 37 °C, the reaction mixture was extracted by the Bligh and Dyer procedure [16], and the lipids were separated by silica gel TLC with the solvent system of chloroform/methanol/water (65:25:4, by vol.). The spots corresponding to lyso PC and PC were scraped and their radioactivity determined in a liquid-scintillation counter after the addition of 0.5 ml of water and 5 ml of scintillation fluid. The enzyme activity was expressed as nmol of lyso PC esterified/h per μg of enzyme protein. (3) Transesterification of truncated PC (LAT II activity) was determined from the formation of labelled short-chain PC in the presence of ^{14}C-labelled lyso PC and unlabelled PAF as described previously [17]. (4) Phospholipase A_2 (PLA_2) activity was measured from the release of labelled non-esterified fatty acid (NEFA) from 16:0-[1-^{14}C]18:2 PC, which was incorporated into proteoliposomes containing the PC and apo AI in the molar ratio 300:1. The reaction mixture contained in addition to the enzyme preparation, 100 μl of labelled proteoliposomes, 2 mg of HSA and 5 mM mercaptoethanol in 0.4 ml final volume of 10 mM Tris/150 mM NaCl/1 mM EDTA (pH 7.4). Following incubation at 37 °C for 30 min, the reaction was terminated by the addition of 1 ml methanol, and the lipids extracted [16], and separated on silica gel TLC plate with the solvent system of chloroform/methanol/water (65:25:4, by vol.). The spots corresponding to NEFA and PC standards were scraped and their radioactivity determined in a liquid-scintillation counter. The enzyme activity was expressed

as nmol of labelled fatty acid released/h per μg of enzyme protein. (5) Esterase activity was determined by the hydrolysis of a water-soluble substrate PNPB as described by Bonelli and Jonas [18]. The reaction mixture contained the enzyme preparation and 15 μl of 100 mM PNPB in acetonitrile (final concentration of acetonitrile was 3 %) and buffer [10 mM Tris/150 mM NaCl (pH 7.4)] in a final volume of 3 ml. The formation of *p*-nitrophenoxide was monitored in a recording spectrophotometer at 400 nm and the enzyme activity was calculated as described by Bonelli and Jonas [18].

Oxidation of the enzyme and substrate preparations

The medium from CHO cells was subjected to oxidation in the presence of increasing concentrations of copper sulphate or AAPH at 37 °C for 30 min. The oxidation was stopped by the addition of 2 mM EDTA in the case of Cu^{2+} oxidation, and by dialysis at 4 °C in the case of AAPH. Proteoliposome containing the appropriate labelled substrate was then added, incubation performed for 1–3 h, and the products analysed as described above. In some experiments, the oxidation of the substrate was similarly performed in the presence of either Cu^{2+} or AAPH, and enzyme activities were tested in the presence of unoxidized enzymes.

Analytical

Protein was estimated by the modified Lowry procedure [19], using BSA as standard. Lipid phosphorus was determined by the modified Bartlett procedure [20].

RESULTS

Effect of substitution of Trp^{61} residue on the various activities of LCAT

In addition to cholesterol esterification (LCAT) reaction, LCAT protein catalyses the hydrolysis of long-chain PC (PLA_2 reaction) [21], and the transesterification of lyso PC with either a long-chain PC (LAT I reaction) [15] or short-chain PC (LAT II reaction) [17], as well as the hydrolysis of water-soluble esters (esterase activity) [18] and truncated phospholipids [22]. Whereas the LCAT, PLA_2 and LAT I activities are dependent on the interfacial binding of the enzyme to the substrate particle, the LAT II and esterase reactions involve water-soluble substrates, and are not dependent on the interfacial binding or apo AI activation [17,18]. To determine the role of the interfacial binding domain, and specifically that of Trp^{61} in the different reactions performed by the enzyme, we prepared site-directed mutants of human LCAT in which Trp^{61} was deleted (Trp^{61}del), or replaced

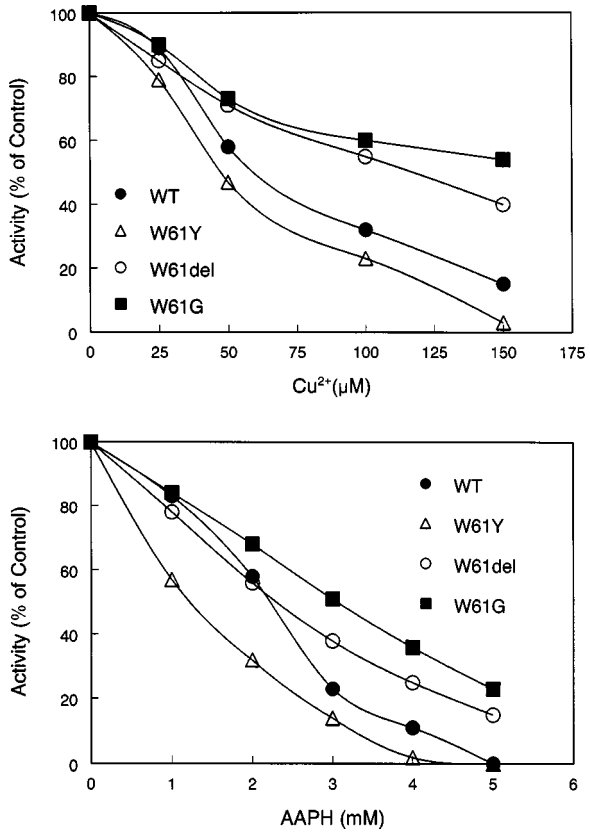

Figure 1 Effect of oxidation with Cu^{2+} or AAPH on the cholesterol esterification activity of LCAT

Medium (150 μl) from the CHO cells expressing the various mutant LCATs was treated with the indicated concentrations of the oxidizing agent for 30 min at 37 °C. Oxidation was terminated by the addition of 2 mM EDTA (in the case of Cu^{2+} oxidation) or by dialysis against the buffer Tris/NaCl/EDTA, pH 7.4 (in the case of AAPH oxidation). The samples were then incubated with proteoliposome substrate containing egg PC, ^{14}C cholesterol and apo AI in the molar ratio 300:15:1 for 3 h at 37 °C, and the formation of labelled cholesteryl ester was determined as described in the text. The enzyme activities were calculated as percentage of the activity obtained by the corresponding unoxidized enzyme. The values shown are averages of duplicate estimations from a single experiment.

Table 1 Specific activity of various reactions catalysed by LCAT mutants

All values are means ± S.D. for three separate experiments, except for LAT I and LAT II activities, which are averages for two experiments.

Specific activity (nmol of substrate converted/h per μg of enzyme protein)						Ratios	
	LCAT	PLA_2	LAT†	LAT II‡	Esterase	LCAT/PLA_2	LCAT/esterase
Wild-type	3.61 ± 0.13	1.20 ± 0.06	0.529	0.11	13.08 ± 1.13	2.64	0.24
Trp^{61}del	1.72 ± 0.08§	1.27 ± 0.08	0.44	0.097	14.59 ± 1.48	1.35	0.12
W61Y	4.19 ± 0.10§	3.09 ± 0.06§	0.96	0.126	15.77 ± 1.50	1.36	0.27
W61G	0.33 ± 0.06§	0.21 ± 0.03§	0.072	0.097	12.37 ± 1.26	1.57	0.03

† LAT, transesterification of lyso PC with a long-chain PC.
‡ LAT II, transesterification of lyso PC with PAF.
§ $P < 0.005$ compared with wild-type.

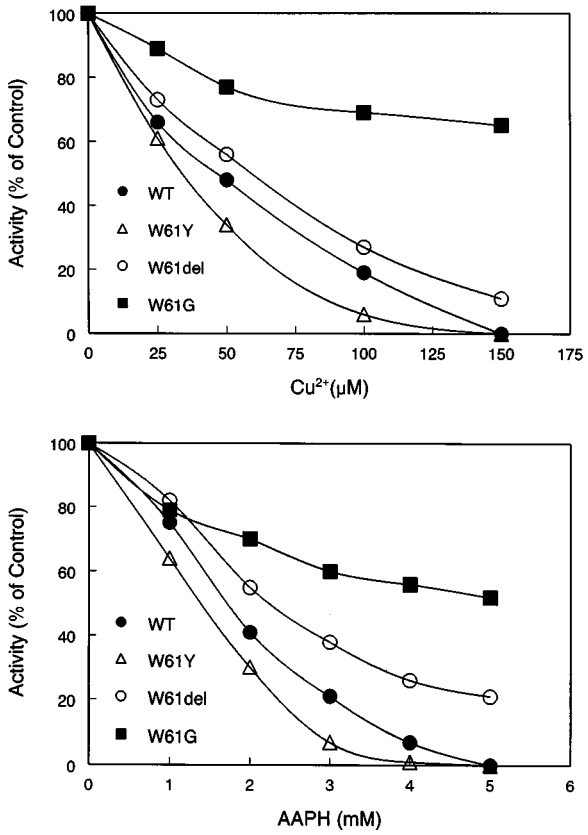

Figure 2 Effect of oxidation on the PLA_2 activity

Oxidation of the enzyme preparations was performed as described in Figure 1. The PLA_2 activity was assayed in the presence of propteoliposome substrate containing 16:0-[1-^{14}C]18:2 PC and apo AI in the molar ratio 300:1 for 3 h at 37 °C. TLC determined the amount of labelled NEFA released, as described in the text. All activities are expressed as percentage of activity obtained in the absence of oxidizing agent. All values are averages of duplicate determinations from a single experiment.

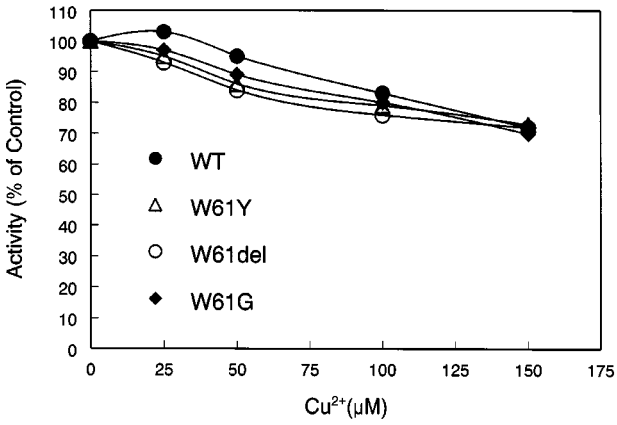

Figure 3 Effect of oxidation of the LCAT mutants on the LAT II activity

The oxidation of the various preparations of the enzyme was performed in the presence of the indicated concentrations of Cu^{2+} for 30 min at 37 °C, and the oxidation was terminated by the addition of 2 mM EDTA. The LAT II activity was determined by the formation of labelled PC in the presence of labelled lyso PC and unlabelled PAF, as described in the text. The enzyme activity is expressed as the percentage of activity obtained in the presence of un-oxidized enzyme (averages of duplicate values from one experiment).

with either an aromatic residue (W61Y) or an aliphatic residue (W61G), and determined the various activities employing appropriate substrates as described in the Materials and methods section. As shown in Table 1, the deletion of Trp^{61} decreased the cholesterol esterification activity significantly (−52%), but slightly stimulated the PLA_2 (+6%) and esterase (+11%) activities, and modestly decreased the LAT I and LAT II activities (−17% and −12% respectively). Substitution of Trp^{61} with another aromatic residue, namely tyrosine (W61Y), stimulated all the reactions, the PLA_2 activity being stimulated the most, and the LAT II and esterase activities being affected the least. However, the replacement of Trp^{61} with glycine resulted in a dramatic loss of the three activities of the enzyme which require the interfacial binding, namely LCAT (−90%), PLA_2 (−82%) and LAT I (−86%). However, the two activities LAT II (−12%) and esterase (−5%), which are not dependent on interfacial binding, were affected only modestly. These results show the importance of tryptophan in the surface-loop region in interaction with lipophilic substrates and support the results of Peelman et al. [10], who first reported the importance of Trp^{61} in cholesterol esterification. They also showed a significant increase in PLA_2 activity by the W61Y mutation, as reported here. However, they determined PLA_2 activity with a monomeric substrate, which should be considered an esterase activity, whereas we used a long-chain PC in proteoliposome, similar to the one used for the cholesterol esterification (LCAT) reaction. Initially, we have also determined the enzyme activities in the medium from control (untransfected) CHO cells, and found no LCAT or LAT activity. Therefore these controls were not included in the later experiments.

It is of interest to note that all mutations affected the cholesterol esterification more than the PLA_2 activity, as shown by the decrease in the ratios of LCAT/PLA_2 (Table 1). There was approximately a 50% decrease in this ratio from the wild-type enzyme, regardless of the mutation. On the other hand, the ratio of LCAT/esterase was affected unequally by different mutations, with the W61Y showing no change (both cholesterol esterification and hydrolysis of water-soluble substrate stimulated to the same extent), and W61G showing a disproportionate loss of cholesterol esterification activity.

Effect of oxidation on the enzyme activities

The medium from the CHO cells expressing the various mutant LCATs was subjected to oxidation in the presence of various concentrations of Cu^{2+} or AAPH, and the enzyme activities were determined employing the unoxidized substrates. Figure 1 shows the effect of oxidation on the LCAT (cholesterol esterification) activity. Compared with wild-type enzyme, W61Y was found to be more sensitive to oxidation, whereas Trp^{61}del and W61G were more resistant to both of the oxidizing agents. Although the basal activity (unoxidized enzyme) was the lowest in W61G, this mutation conferred the greatest resistance to oxidation. Thus at 150 μM Cu^{2+}, W61Y lost 97% of the activity, whereas Trp^{61}del lost 60% activity and W61G lost 46% of the activity. Similarly, at 3 mM AAPH, the wild-type enzyme lost 77% activity, whereas W61Y lost 86%, and Trp^{61}del lost 62% and W61G lost only 49%. Therefore the presence of an aromatic residue (tryptophan or tyrosine) at position 61 results not only in increased activity, but also in increased sensitivity to oxidation. Similar results were

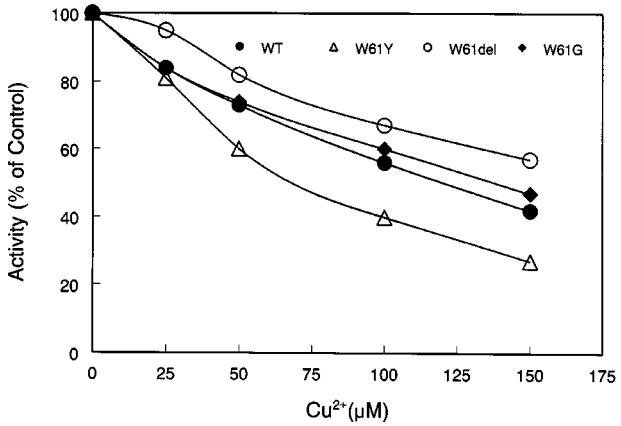

Figure 4 Effect of oxidation on the esterase activity of LCAT

Oxidation of the enzyme in the presence of the indicated concentrations of Cu^{2+} was performed for 30 min at 37 °C, and the oxidation terminated by the addition of 2 mM EDTA. The esterase activity of the enzyme was determined from the hydrolysis of PNPB (20 min at 37 °C) as described in the text. All activities were then calculated as percentage of the esterase activity obtained in the presence of the corresponding unoxidized enzyme. The values shown are from one typical experiment (averages of duplicate determinations).

obtained when potassium ferricyanide was used as the oxidizing agent (results not shown).

Figure 2 shows the effects of oxidation on the PLA_2 activity. The loss of PLA_2 activity followed a similar pattern as the LCAT activity, although this reaction was in general more sensitive to oxidation. One exception was W61G mutation, which appeared to confer more resistance to the hydrolytic activity than for cholesterol esterification. The LAT reaction (transfer of a fatty acid from PC to lyso PC) followed similar pattern as the LCAT and PLA_2 activities (results not shown).

The effect of Cu^{2+} oxidation was then tested by two reactions, which do not require interfacial binding. Figure 3 shows the effect of oxidation on LAT II reaction, which was assayed by the esterification of labelled lyso PC in the presence of PAF as the acyl donor [17]. At a Cu^{2+} concentration, which inhibited over 80% of cholesterol esterification activity in wild-type LCAT (150 μM), the LAT II activity was only inhibited by about 28%. Furthermore, all the mutants LCATs, including W61Y, exhibited more resistance of LAT II activity to oxidation compared with the LCAT and PLA_2 activities. These results show that the transesterification of lyso PC with short-chain PCs, such as those formed by oxidation of lipoprotein PC [23–25], is unaffected by the oxidation of the enzyme, especially in the interfacial domain. In the previous studies, we showed that the LAT II activity in whole plasma is stimulated following oxidation of the plasma, apparently because of the formation of the short-chain PCs [7].

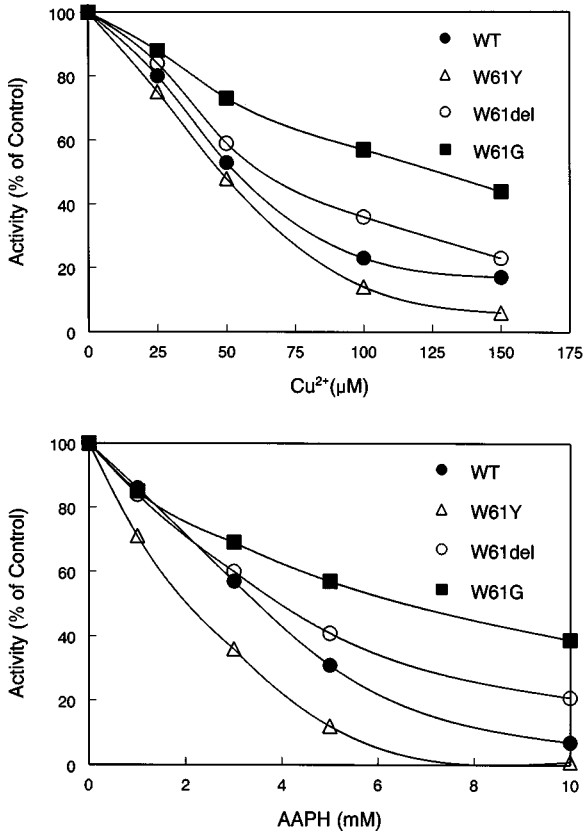

Figure 5 Effect of oxidized PC substrate on the cholesterol esterifying activity of LCAT mutants

Proteoliposome substrate containing egg PC, labelled cholesterol and apo AI in the molar ratio 300:15:1 was first oxidized with the indicated concentrations of the oxidizing agent for 40 min at 37 °C, and oxidation terminated either by the addition of 2 mM EDTA (in the case of Cu^{2+} oxidation) or by dialysis at 4 °C (in case of AAPH oxidation). The oxidized substrate of 100 μl was then used for the determination of cholesterol esterification (LCAT) activity of the unoxidized enzyme preparations. The enzyme activities are expressed as percentage of the activity obtained in the presence of unoxidized substrate. All values are averages of duplicate determinations from one experiment.

Higher resistance to oxidation was also found for another reaction of LCAT not requiring the interfacial binding, namely the esterase reaction, although the loss of activity was more than that in the case of the LAT II reaction (Figure 4). At the highest Cu^{2+} concentration used (150 μM), the wild-type enzyme retained 42% activity, whereas Trp^{61}del retained 57% and W61Y retained 27% activity. It should be pointed out that the initial esterase activities of the enzyme preparations are much higher

Table 2 IC_{50} values

	Cholesterol esterification		PLA_2 activity		Esterase activity	
	Cu^{2+} (μM)	AAPH (mM)	Cu^{2+} (μM)	AAPH (mM)	Cu^{2+} (μM)	AAPH (mM)
Wild-type	60.9	2.02	43.8	1.67	121.6	3.24
W61Y	48.7	1.30	30.6	1.26	71.6	2.47
Trp^{61}del	121.3	2.36	55.1	2.17	195.1	5.85
W61G	220.3	3.04	> 250	5.89	139.4	7.99

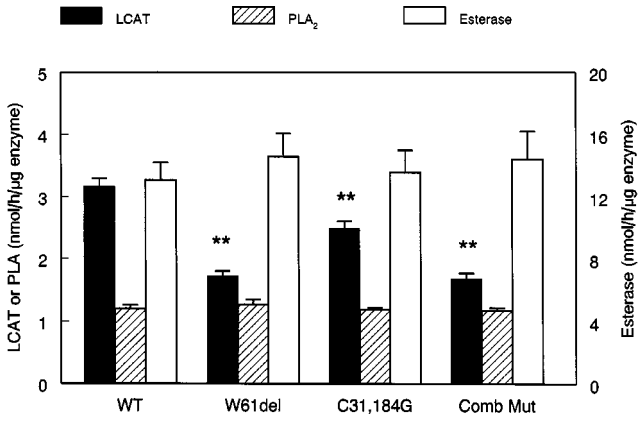

Figure 6 Effect of cysteine mutations in Trp[61]del mutant

The double cysteine mutant (C31G, C184G) was prepared as described previously [8] and the triple mutant (Comb Mut) was prepared by deleting Trp[61] from this LCAT. The enzyme activities were described in the medium from CHO cells expressing the various mutant enzymes, as described in the text. The values shown are means ± S.D. for three separate determinations. $^{**}P < 0.005$, compared with wild-type.

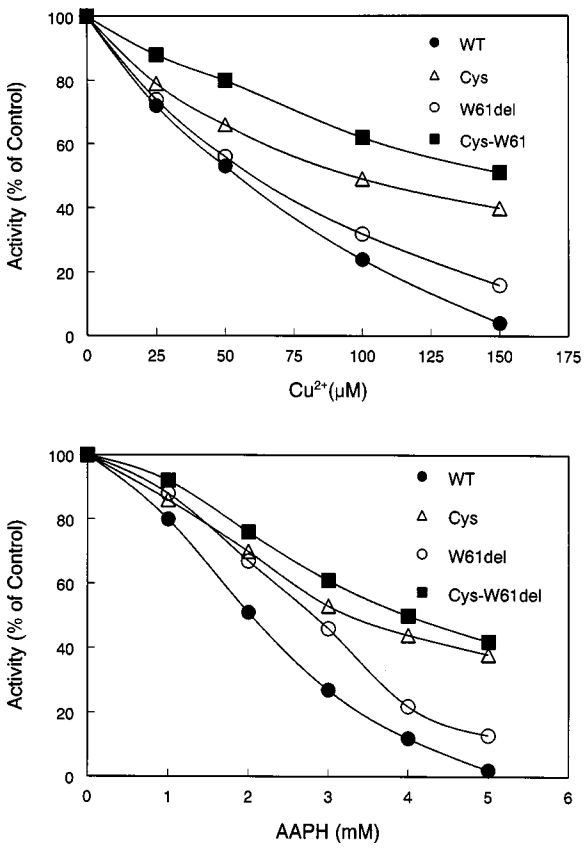

Figure 7 Effect of oxidation of the combined mutant on cholesterol esterification

The LCATs with the cysteine mutations alone (Cys), deletion of Trp[61] alone (Trp[61]del; 'W61del'), or the combination of the two mutations (Comb Mut; 'Cys-W61del') were oxidized for 30 min at 37 °C in the presence of the indicated concentrations of Cu^{2+} or AAPH. The oxidation was terminated by the addition of 2 mM EDTA or dialysis, and the cholesterol esterification activity was determined as described in the text. The enzyme activities are expressed as percentage of the activity obtained in the presence of unoxidized enzyme (averages of duplicate determinations from one experiment).

than the LAT II activities (Table 1), and the residual activities after oxidation are also higher.

IC_{50} values

The IC_{50} values (concentration of the oxidizing agent required for 50 % inhibition) were calculated for all the activities in the presence of Cu^{2+} and AAPH, to determine the relative susceptibilities. As shown in Table 2, the lowest IC_{50} values were found for W61Y in all cases showing that the aromatic residue in the interfacial region is particularly sensitive to oxidation. Conversely, the highest IC_{50} values were found for W61G, whereas Trp[61]del was intermediate between the wild-type LCAT and W61G. As expected, the esterase activity showed a much higher IC_{50} value than the LCAT and PLA_2 activities, showing that this activity is least affected by oxidation.

Effect of oxidation of the substrate

Previous studies from our laboratory [7,8], as well as others [26], showed that oxidized PC is an inhibitor of the LCAT reaction. In order to determine the possible role of the interfacial domain in the inactivation by the oxidized substrates, we first oxidized the standard proteoliposome substrate (egg PC, labelled FC and apo AI in the molar ratio 300:15:1) with increasing concentrations of either Cu^{2+} or AAPH, and determined the cholesterol esterification activity of the various mutant LCAT in the presence of the oxidized substrate. The oxidizing agent was either neutralized with EDTA (in case of Cu^{2+}) or dialysed out (in case of AAPH) before adding to the reaction mixture. As shown in Figure 5, the substrate oxidized with either of the oxidizing agents was inhibitory for all the LCAT preparations. The pattern of inhibition was similar to that obtained when the enzyme was directly oxidized and the activity was assayed with unoxidized substrate. The most sensitive mutant was again W61Y, and the least sensitive one was W61G. These results show that the effect of oxidized substrates is similar to the oxidizing agents.

Relative roles of cysteine residues and the interfacial aromatic residues

Since our previous studies had shown that the two cysteine residues near the active-site pocket are also targets of the oxidizing agents [8], we determined whether the effects on the cysteines and Trp[61] are additive. For this purpose, we generated the triple mutant in which both the cysteines are replaced by glycines, in addition to the deletion of Trp[61]. We did not use the W61G mutation for these studies, although it was the most resistant to oxidation because it has very low initial activity. Figure 6 shows the initial specific activities of the various mutant LCATs expressed in CHO cells. As shown above, the Trp[61]del mutation resulted in a significant loss (−46 %) of LCAT activity, whereas the replacement of the two cysteines with glycines resulted in a modest loss (−21 %) in agreement with our previous results [8]. However, the combination of the two mutations did not result in any more loss than with Trp[61] deletion alone. Neither the PLA_2 activity nor the esterase activity was affected by individual mutations or the combination of mutations.

The effect of oxidation on LCAT activity is shown in Figure 7. The wild-type enzyme lost almost all LCAT activity at 150 μM

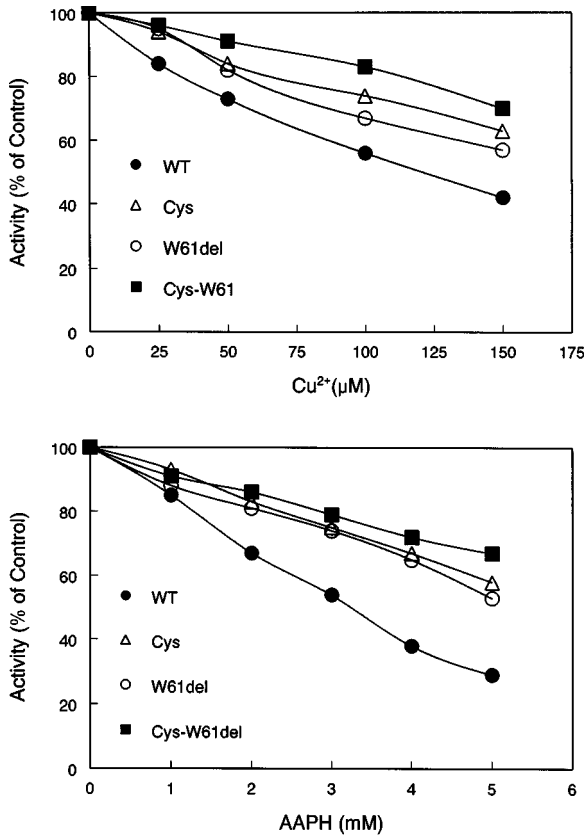

Figure 8 Effect of oxidation of the combined mutant on esterase activity

Mutant LCATs were oxidized in the presence of the indicated concentrations of the oxidizing agent for 30 min at 37 °C, and the oxidation terminated by the addition of EDTA (for Cu^{2+}) or by dialysis (for AAPH). The esterase activity was determined from the hydrolysis of PNPB, as described in the text. The enzyme activities are expressed as percentage of the activity obtained with the unoxidized enzymes. The values shown are from one typical experiment (averages of duplicate determinations).

Cu^{2+} concentration, whereas the double cysteine mutant retained about 40% activity, and the Trp^{61}del retained only 16%. The combination of the two mutations, however, retained 51% of activity, showing that the effect of oxidation on the two domains is additive. The IC_{50} values were 54.7, 96.8, 58.8 and 166.3 μM for the wild-type, double cysteine mutant, Trp^{61}del and the combined mutant respectively. Similar results were obtained in presence of AAPH, where, at the highest concentration of the oxidant used, the wild-type enzyme retained only 2% of the activity, Trp^{61}del alone retained 12% of the activity, and the double cysteine mutant retained 38%, but the combination of mutations resulted in a retention of 43% of the activity. Note that this preparation of Trp^{61}del was more sensitive to oxidation than the one shown in Figure 1.

The effect of oxidizing agents on the esterase activities of the combined mutants is shown in Figure 8. In the presence of 150 μM Cu^{2+} the wild-type LCAT retained about 42% of the activity, whereas the cysteine mutant retained 63% and the Trp^{61}del mutant retained 57%. The combined mutant retained about 70% of the activity, showing that the effects of cysteines and Trp^{61} are additive for the esterase activity also, although the loss of activity was much lower than that of LCAT activity. Similar additive effects were seen when the oxidation was performed in the presence of AAPH (Figure 8, lower panel).

DISCUSSION

LCAT is an interfacial enzyme, acting primarily on the surface of the lipoproteins, and therefore, its binding to the hydrophobic lipoprotein substrate is a prerequisite for its activity. All interfacial enzymes have in common an amphipathic domain on their surface, which is required for their interaction with lipid surface. In the case of lipoprotein lipase, e.g. a C-terminal amphipathic domain that is enriched in tryptophan residues (Trp^{390}, Trp^{393}, Trp^{394}), has been identified as the domain responsible for the binding to the lipid–water interface [27]. Mutation of these tryptophan residues results in loss of activity against lipid emulsions, but not against water-soluble substrates (esterase activity). Similarly, the C-terminal domain of hepatic lipase contains four tryptophan clusters, which appears to have similar function in the lipid–enzyme interaction. In pancreatic lipase, Trp^{252} may perform similar function. Based on the threading alignment studies, the residues 50–74 in LCAT have been designated as the 'lid' domain responsible for the interfacial interactions [9,28]. Similar to the lipases, deletion of this domain results in complete loss of activity against long-chain hydrophobic substrates, but only partial loss of esterase activity [9]. Out of 12 tryptophan residues in LCAT, only one is present in the putative surface-loop domain (Trp^{61}), and this is highly conserved among various species. Peelman et al. [10] showed that mutation of this critical tryptophan results in profound changes in enzyme specificity and activity. Thus replacing it with another aromatic residue (tyrosine or phenylalanine) retains the activity against HDL, but not against LDL. Replacing it with an aliphatic residue (glycine or leucine) results in loss of activity against both lipoproteins, showing that the presence of an aromatic residue is essential at this position. Because of its amphipathic nature, and its flat rigid shape, tryptophan appears to be ideally suited to mediate the interaction of lipolytic enzymes with their lipid substrates. In fact, it has the highest affinity for the phospholipid interface [29]. However, tryptophan is also one of the most susceptible residues to oxidation [11]. In LDL, the oxidation of tryptophan precedes the oxidation of lipids, and the formation of tryptophanine radicals may indeed be critical for lipid peroxidation to occur in the presence of Cu^{2+} [11,30]. Our results show that Trp^{61} of LCAT is one of the primary targets of oxidizing agents, supporting the hypothesis that it is exposed on the enzyme surface. We propose that the oxidation of this residue results in the loss of hydrophobic interaction with the phospholipid surface, but not its catalytic function. This is supported by the fact that the esterase activity is not affected by either mutation of this residue or its oxidation in the wild-type enzyme. Furthermore, the presence of an aromatic residue at this position makes the enzyme vulnerable to oxidative inactivation, as evident from the sensitivity of wild-type and W61Y LCATs to oxidizing agents. Deletion of this residue, or its substitution with an aliphatic residue (W61G) renders the enzyme resistant to oxidation, although the basal activity is lower because of decrease in interfacial interaction. It is possible that the tryptophan residues in the interfacial domain of other lipases are similarly vulnerable for oxidative damage, although no direct studies have been performed.

In the previous studies, we showed the importance of two cysteine residues of human LCAT for its sensitivity to oxidizing agents [8]. However, unlike the Trp^{61} residue, the role of the cysteines in the enzyme function is unknown. The inactivation of the enzyme following derivatization of cysteines by oxidation products is apparently due to steric hindrance at the active site, whereas the loss of activity following oxidation of Trp^{61} is due to a loss of interfacial binding. Therefore the effect of oxidation on

the two domains of the enzyme should be additive, as indeed found in the present study with the triple mutant LCAT (C31G, C184G, Trp^{61}del). Unlike its cholesterol esterification and phospholipase activities, the esterase and LAT II activities of LCAT are neither dependent on interfacial binding, nor affected by derivatization of cysteines [17,18] and, therefore, these activities are significantly retained even after extensive oxidation of the enzyme or the substrate.

We previously proposed the possible importance of LCAT in the detoxification of oxidized phospholipids, based on its ability to hydrolyse and transesterify these compounds *in vitro* in the presence of a specific inhibitor for PAF acetylhydrolase [22]. The hydrolysis of oxidized PC by LCAT was also directly demonstrated *in vitro* [22,31]. Plasma from patients with familial deficiency of PAF acetylhydrolase can partially perform the hydrolysis [32], suggesting a role for LCAT in the metabolism of oxidized phospholipids. Furthermore, LCAT-deficient patients accumulate oxidized PC in plasma and glomerular lesions [33]. The retention of this activity following oxidation of LCAT may therefore be physiologically significant, especially since the other known enzymes (PAF acetylhydrolase and paraoxonase) which hydrolyse these compounds are known to be inactivated under oxidizing conditions [34–36]. The effect of oxidized PC substrate on the various LCAT mutants followed the same pattern as found with direct oxidation of the enzyme (Figure 5), showing that the oxidized lipids affect the same domains of LCAT as the oxidizing agents such as Cu^{2+} and AAPH. It is known that the oxidation of HDL renders it an ineffective substrate for cholesterol esterification [6,37] and that lipid peroxides are inhibitory to LCAT in the isolated system [26]. The hydrolysis of the oxidized phospholipids by the esterase activity of LCAT, which is operative under these conditions, would prevent the harmful effects of these lipids. It is of interest to note that 'inactive' immunoreactive LCAT protein has been found in association with LDL by several investigators [38–40]. It is possible that this LCAT protein represents the oxidized form of the enzyme, which lost its ability to esterify cholesterol, but is capable of metabolizing the oxidized PC products formed during LDL oxidation.

This research was supported by grant no. HL 52597 from the National Institutes of Health.

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Received 10 January 2002/16 April 2002; accepted 19 April 2002
Published as BJ Immediate Publication 19 April 2002, DOI 10.1042/BJ20020064