Cysteine mutants of human apolipoprotein A-I: a study of secondary structural and functional properties

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Abstract **Apolipoprotein A-I$_{Milano}$ (A-I$_M$) (R173C), a natural mutant of human apolipoprotein A-I (apoA-I), and five other cysteine variants of apoA-I at residues 52 (S52C), 74 (N74C), 107 (K107C), 129 (G129C), and 195 (K195C) were generated. Cysteine residues were incorporated in each of the various helices at the same helical wheel position as for the substitution in A-I$_M$. The secondary structural properties of the monomeric mutants, their abilities to bind lipid and to promote cholesterol efflux from THP-1 macrophages, and the possibility of antiperoxidation were investigated. Results showed that the α helical contents of all of the cysteine mutants were similar to that of wild-type apoA-I (wtapoA-I). The cysteine variant of A-I$_M$ at residue 173 [A-I$_M$(R173C)] exhibited weakened structural stability, whereas A-I(G129C) a more stable structure than wtapoA-I. A-I(G129C) and A-I(K195C) exhibited significantly impaired capabilities to bind lipid compared with wtapoA-I. A-I(K107C) possessed a higher capacity to promote cholesterol efflux from macrophages than wtapoA-I, and A-I$_M$(R173C) and A-I(K195C) exhibited an impaired efflux capability. Neither A-I$_M$(R173C) nor any other cysteine mutant could resist oxidation against lipoxygenase. In summary, in spite of the similar mutant position on the helix, these variants exhibited different structural features or biological activities, suggesting the potential influence of the local environment of mutations on the whole polypeptide chain.**—Zhu, X., G. Wu, W. Zeng, H. Xue, and B. Chen. **Cysteine mutants of human apolipoprotein A-I: a study of secondary structural and functional properties.** ***J. Lipid Res.*** **2005.** 46: **1303–1311.**

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Plasma levels of HDL and its apolipoprotein A-I (apoA-I) were demonstrated to be inversely correlated with the incidence of cardiovascular disease (1). This cardiovascular protective effect was attributed mainly to their involvement in the process of reverse cholesterol transport, through which HDL or apoA-I delivered cholesterol from peripheral tissues to the liver.

Mature apoA-I contains eight tandem repeat units of 22 amino acids, forming an amphipathic α helix, which allows for the main biological activities of apoA-I (2). Studies have revealed that the N-terminal region of apoA-I plays an important role in initiating lipid binding and the formation of nascent HDL (3). The central region (residues 144–186) was the key domain for the activation of LCAT (4, 5) and was also important for the maturation and stability of HDL (6). The C-terminal domain (residues 190–243) was critical in mediating apoA-I association with phospholipid (7, 8) and promoting cellular cholesterol efflux (9, 10).

Recently, more and more studies have focused on apolipoprotein A-I$_{Milano}$ (A-I$_M$), a natural mutant of apoA-I characterized by Arg-173 being substituted by cysteine, resulting in the formation of a disulfide-linked homodimer (A-I$_M$/A-I$_M$) or heterodimer with apoA-II (11). Plasma lipoprotein spectra of its carriers exhibited a very low level of HDL-cholesterol and moderate hypertriglyceridemia (12), but none of the carriers exhibited an increased risk of cardiovascular disease (13). In agreement with this finding, in vivo injection of recombinant A-I$_M$ into experimental animals prevented the development of atherosclerosis (14–16). Furthermore, a clinical trial revealed that administration of recombinant A-I$_M$/phospholipid complex (ETC-216) produced significant regression of coronary atherosclerosis (17). The abnormal coexistence of an increased risk of plasma lipoprotein spectra and a decreased risk of vascular disease was observed in another cysteine variant, apoA-I$_{Paris}$, with Arg-151 substituted by cysteine (18, 19). All of these data suggest that the introduction of cysteine into apoA-I endows the mutants with

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Abbreviations: A-I$_M$, apolipoprotein A-I$_{Milano}$; A-I(S52C), A-I(N74C), A-I(K107C), A-I(G129C), A-I$_M$(R173C), and A-I(K195C), the cysteine variants of apolipoprotein A-I at residues Ser-52, Asn-74, Lys-107, Gly-129, Arg-173, and Lys-195, respectively; apoA-I, apolipoprotein A-I; BCA, bicinchoninic acid; CD, circular dichroism; DMPC, 1,2-dimyristoyl-*sn*-glycerol-3-phosphatidylcholine; ΔG_D^0, free energy of unfolding; PLPC, 1-palmitoyl-2-linoleoylphosphatidycholine; wtapoA-I, recombinant wild-type human apolipoprotein A-I.

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some particular properties, resulting in increased protection from cardiovascular risk. Both of the cysteine substitutions occurred at the polar-nonpolar interface on the α helix, exactly 22 amino acids apart, as shown by the Edmundson wheels (20) (**Fig. 1**). In view of this fact, we speculate that the unique protective behavior of the cysteine mutants may result largely from the formation of more suitable secondary or tertiary structures or more efficient interactions between proteins or between proteins and lipids.

Because of the unique mutant positions of A-I_M [named A-I_M(R173C) in this study] and apoA-I_{Paris}, we designed and constructed five other cysteine mutants, with every cysteine residue located in each of the various helical domains at the same helical wheel position as for the substitution in A-I_M: A-I(S52C), A-I(N74C), A-I(K107C), A-I(G129C), and A-I(K195C) (Fig. 1). It was known that A-I_M was present in plasma mainly in three forms: monomer, homodimer, or heterodimer (21). In this study, the monomers of the recombinant mutants were used as objects, and their secondary structural features, their abilities to associate with phospholipids and to promote cellular cholesterol efflux, and their antiperoxidation effects were investigated. Our aim is to explore how the replacement of cysteines at specific sites would influence the structure and biological functions of apoA-I.

MATERIALS AND METHODS

Materials

1,2-Dimyristoyl-*sn*-glycerol-3-phosphatidylcholine (DMPC), 1-palmitoyl-2-linoleoylphosphatidycholine (PLPC), PMA, fatty-acid-free BSA, soybean lipoxygenase, and guanidine-HCl (ultrapure) were purchased from Sigma. TRIzol reagent was purchased from Gibco-BRL. [1α, 2α(n)-^{3}H]cholesterol was obtained from Amersham UK. Reagents for the PCR and restriction enzymes were from Takara. Cell culture reagents were obtained from Hyclon. His binding resin was purchased from Novagen. All other reagents were of analytical grade. pET30b(+) expression vector and BL21 (DE3) bacterial strain were purchased from Novagen. Human THP-1 monocytes were obtained from the Cell Culture Center of Peking Union Medical College. The reverse transcription system was purchased from Promega, and the site-directed mutagenesis kit was obtained from Invitrogen. The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce. The thiol and sulfide quantification kit was obtained from Molecular Probes.

Construction of expression plasmids of wild type and cysteine mutants of apoA-I

Using liver total RNA as template, RT-PCR was carried out to generate the apoA-I cDNA encoding the prepropeptide form of human apoA-I (22) with oligonucleotides 5′-ATGAAAGCTGCGGTGCTGACC-3′ as forward primer and 5′-TCACTGGGTGTTGAGCTTCTTAG-3′ as reverse primer. The cDNA fragment obtained was then subcloned into T Easy vector (Promega). This plasmid was used as template to construct expression plasmids of wild-type and cysteine mutants of apoA-I. To increase the level of expression, silent mutations were introduced to the first codons of apoA-I (23, 24). To simplify purification, a 6×His tag was added to the C terminus. Briefly, two completely complementary oligonucleotides for silent mutations were synthesized with flanking restriction sites of *Nde*I and *Nco*I: 5′-GGGAATTCCATATGGACGAGCCACCGCAGAGTCCATGGCATG-3′ (sense) and 5′- CATGCCATGGACTCTGCGGTGGCTCGTCCATATGGAATTCCC-3′ (antisense). Primers 5′-CATGCCATGGGATCGAGTGAAGGACC-3′ (sense) and 5′-ACGCGTCGACCTGGGTGTTGAGCTTCTTAG-3′ (antisense) were used to amplify the cDNA fragment coding 9–243 residues with PCR. Then, the PCR product and double-stranded complementary oligonucleotides were digested with *Sal*I and *Nde*I, respectively. The expression plasmid of pET30b(+) was digested with *Nde*I and *Xho*I. These triple-digested products were ligated to obtain a large linear DNA fragment, and the latter was then digested with *Nco*I to obtain a fragment with *Nco*I protruding cohesive termini at both sides. After self-ligation and transformation, the recombinant expression plasmid of the wild-type human apolipoprotein A-I (wtapoA-I) [pET30b(+)- wtapoA-I] was obtained.

Using the plasmid of pET30b(+)-wtapoA-I as template, PCR-based site-directed mutagenesis (Stratagene) was used to create the cysteine mutants A-I(S52C), A-I(N74C), A-I(K107C), and A-I(G129C), and Mega-primer PCR (25) was performed to create A-I_M(R173C) and A-I(K195C). For A-I_M(R173C), the forward long primer was 5′-GCCACCGCAGAGTCCATGGGATCG-3′, the middle mutagenic primer was 5′-GCTGCGCCAGTGCTTGGCCGC-3′, and the reverse short primer was 5′-GAGCTTAGTGTAC-3′; for A-I(K195C), the middle mutagenic primer was 5′-AGTACCACGCCTGCGCCACCGAGCATC-3′, and the sequences of the forward and reverse primers were identical to those of A-I_M(R173C).

All expression plasmids were identified by DNA sequencing.

Expression and purification of recombinant apolipoproteins

All expression plasmids were transformed into BL21 (DE3) host cells. Isopropyl-β-D-thiogalactopyranoside (final concentration, 1 mM) was used to induce protein expression after the cell density (A_{600}) reached 0.5–0.7, and the cells continued to be incubated for 3 h.

The cells were harvested and resuspended in binding buffer (5 mM imidazole, 0.5 mM NaCl, and 20 mM Tris-HCl, pH 7.9) and then lysed by sonication. After centrifugation, the supernatant was directly loaded into a His-affinity column that had been charged previously with $NiSO_4$. After washing with binding buffer and wash buffer (90 mM imidazole, 0.5 mM NaCl, and 20 mM Tris-HCl, pH 7.9), the apolipoproteins were eluted with strip buffer (100 mM EDTA, 0.5 mM NaCl, and 20 mM Tris-HCl, pH 7.9). The factions containing high concentration of apolipoproteins were collected and dialyzed thoroughly against 0.01 M PBS (pH 7.4) supplemented with 1 mM DTT to reduce disulfide bonds. After dialysis against 0.02% ammonium bicarbonate, the proteins were lyophilized and stored at −70°C. These lyophilized proteins were dissolved in the proper solutions and used in the assays described below.

Reduced and nonreduced SDS-PAGE (with and without DTT in the SDS sample loading buffer, respectively) were used to investigate the recombinant proteins. Free thiols in the mutants were quantified according to the thiol and sulfide quantification kit's description.

Circular dichroism

WtapoA-I or the mutants were dissolved in 0.1 M phosphate buffer (pH 8.0) and maintained at 0.06–0.1 mg/ml to prevent protein self-association. The circular dichroism (CD) spectra were recorded with a Jasco J-715 spectropolarimeter (Jasco Corp., Tokyo, Japan) at room temperature using a 0.2 cm quartz cu-

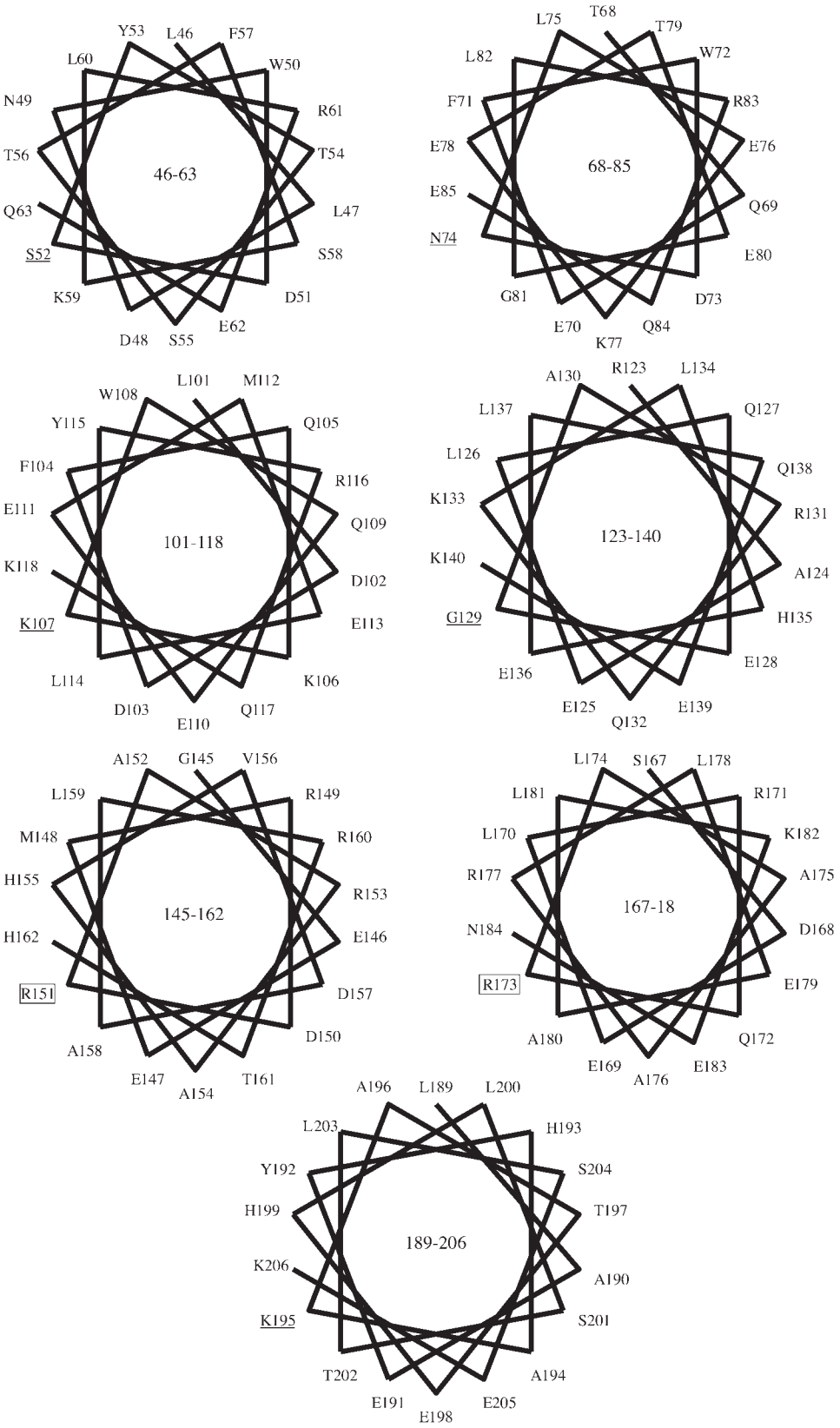

Fig. 1. Edmundson wheel representations of apolipoprotein A-I (apoA-I) α helices (helices 1–7) (20). Arg-151 (helix 5) and Arg-173 (helix 6) were the mutant sites of apolipoprotein A-I_{Milano} (A-I_M) and apoA-I_{Paris}, respectively. The underlined residues were mutagenized into cysteines in this study.

vette. The spectra were read over wavelengths of 250–190 nm. Three scans of the same solution were averaged for each protein. The CD signal was converted to molar mean residue ellipticity ([θ]) expressed as degrees·cm^2·dmol^{-1}, which was calculated as $[\theta] = \theta_{obs}\cdot w/(10\cdot l\cdot c)$, where θ_{obs} is the observed ellipticity at 222 nm in degrees, w is the mean residue molecular mass of the protein, l is the optical path length in centimeters, and c is the protein concentration in grams per milliliter. In this study, the mean molecular masses used were as follows: 116.1 Da for wtapoA-I, 116.1 Da for A-I(S52C), 116 Da for A-I(N74C), 116.2 Da for A-I(G129C), 115.8 Da for A-I$_M$(R173C), and 116 Da for A-I(K195C). The α helical content was calculated from the formula $[\theta_{222}] = -30{,}300f_H - 2{,}340$, where f_H is the fraction of α helix (26).

For guanidine-HCl denaturation studies, wtapoA-I or mutants (final concentration, 0.06–0.1 mg/ml) were diluted into the indicated concentrations of guanidine-HCl (final concentration, 0.25–6.0 M) and stored at 4°C for 24 h to equilibrate. Free energy of unfolding (ΔG_D^0) was used to assess secondary structural stability (27).

DMPC clearance assay

The kinetics of association with lipid was determined according to the method described by Cho, Durbin, and Jonas (28) with slight modifications. Dry DMPC was dispersed in 0.01 M PBS (pH 7.4) and vortexed for 3–5 min to form multilamellar liposomes. An aliquot of the DMPC liposomes was added to each protein solution with a weight ratio of 2:1 to give a final protein concentration of 0.1 mg/ml. The measurements were initiated after the addition of DMPC and monitored at 325 nm for 1 h at intervals of 2 min, maintaining the reaction temperature at 24 ± 0.1°C during the entire monitoring period. A plot of absorbance at 325 nm versus time in minutes [A(t)] was fitted by the exponential decay function $A(t) = ae^{-t/k}$. The rate constant $k_{1/2}$ ($= 1/t_{1/2}$) was determined for each protein (with $t_{1/2}$, i.e., half-time, corresponding to a 50% decrease in absorbance). In addition, to investigate the influence of minor dimmers in the mutant proteins on the ability to bind lipid, especially for A-I(G129C) and A-I(K195C), the protein samples were reduced by incubation with 1 mM DTT for 3 h, and then the DMPC clearance assay was performed with 1 mM DTT in the reaction system.

Cellular cholesterol efflux

The cellular cholesterol efflux assay was carried out mainly according to the methods described by Burgess et al. (9). Human THP-1 monocytes were maintained in complete RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol, and 2 mM glutamine. To differentiate these cells into macrophages, the monocytes were seeded onto 12-well plates at a density of 1×10^6 cells/well in the same medium containing 100 nM PMA and incubated for 4 days. The differentiated macrophages were cholesterol-loaded and labeled for 48 h in 1 ml of RPMI 1640 supplemented with 10% FBS and 1.5 μCi/ml [^{3}H]cholesterol that had been preincubated for 30 min at 37°C with 75 μg/ml (final concentration) acetylated LDL. After a 2 day loading, the cells were incubated in 1 ml of RPMI 1640 containing 2 mg/ml fatty acid-free BSA (referred to as RPMI-BSA) for 24 h to allow equilibration of the label. For the efflux experiments, the cells were washed three times with RPMI 1640, and the lipid-free apolipoprotein (1.7 μM final concentration) in RPMI-BSA was added to initiate cholesterol efflux. After 24 h, the medium was collected and centrifuged at 1,000 *g* for 10 min to remove debris. The amount of [^{3}H]cholesterol in the media was then measured by liquid scintillation counting. Percentage of efflux was expressed as $CPM_{medium}/(CPM_{medium} + CPM_{cells}) \times 100$.

Assessment of antioxidant activity

The oxidation system consisted of a micelle substrate composed of PLPC (3 mM) dispersed in borate (pH 9.0) and deoxycholate (6 mM) as described (29–31). Soybean lipoxygenase (3 U/μl) was used to initiate lipid peroxidation immediately after the addition of recombinant apolipoproteins to the phospholipid micelles. The reactive concentrations of recombinant A-I$_M$(R173C) were from 25 to 100 μg/ml, and other recombinant apolipoproteins were used at 50 μg/ml in the oxidative system. Increases in conjugated dienes (lipid peroxidation) were monitored by ultraviolet absorption spectroscopy (234 nm) at 25°C for 10 min after the initiation of the reaction. The mass of phospholipid hydroperoxides was calculated using the molar absorptivity coefficient (ε = 29,500 l·mol^{-1}·cm^{-1}) of conjugated dienes. Increasing rates of lipoxygenase-catalyzed lipid hydroperoxidation were quantified from the linear portion of the oxidation curves.

Other methods

The acetylated LDLs was prepared according to previous reports (32). The protein concentrations were determined by BCA protein assay (Pierce). All experiments were repeated at least two times, and all data are expressed as means ± SD. Differences between groups were examined for significance using Student's two-tailed *t*-test, and $P < 0.05$ was considered statistically significant.

RESULTS

Expression and purification of recombinant wtapoA-I and cysteine mutants

The recombinant apolipoproteins were expressed mainly in soluble form in BL21 (DE3) cells, with a yield of more than 10 mg of purified protein from 1 liter of bacterial culture. All of the purified proteins were present in monomeric form under reducing condition (**Fig. 2A**). Under nonreducing conditions, the degrees of dimerization and the migration rates of the dimmers were variable depending on the sites of cysteine substitution (Fig. 2B). Of the mutants, A-I(G129C) migrated slowest, and the migration rate increased with the mutant sites moving to the N- or C-terminal regions. This phenomenon was attributed to the different forms of cross produced by interchain disulfide bonds. Thiol quantitation assay indicated the existence of free cysteine in the purified mutants, and there was no significant difference for the contents of free thiol between A-I$_M$(R173C) and the other five mutants ($P < 0.05$) (**Table 1**). According to the nonreduced SDS-PAGE and thiol quantitation assay, we concluded that our purified cysteine mutants were composed mainly of monomers (>85%). The proteins, therefore, were used in subsequent examinations without further purification.

CD

As indicated in **Table 2**, the α helical content of wtapoA-I was 54 ± 4%, consistent with other reports (33, 34). Cysteine substitutions did not generate significant effects on the α helical content of apoA-I (Table 2). Chemical denaturation CD (**Fig. 3**) revealed that ΔG_D^0 of lipid-free wtapoA-I was 2.42 ± 0.04 kcal/mol (Table 2), being close to that described previously (27, 33). ΔG_D^0 of A-I$_M$(R173C) was lower than that of wtapoA-I, indicating

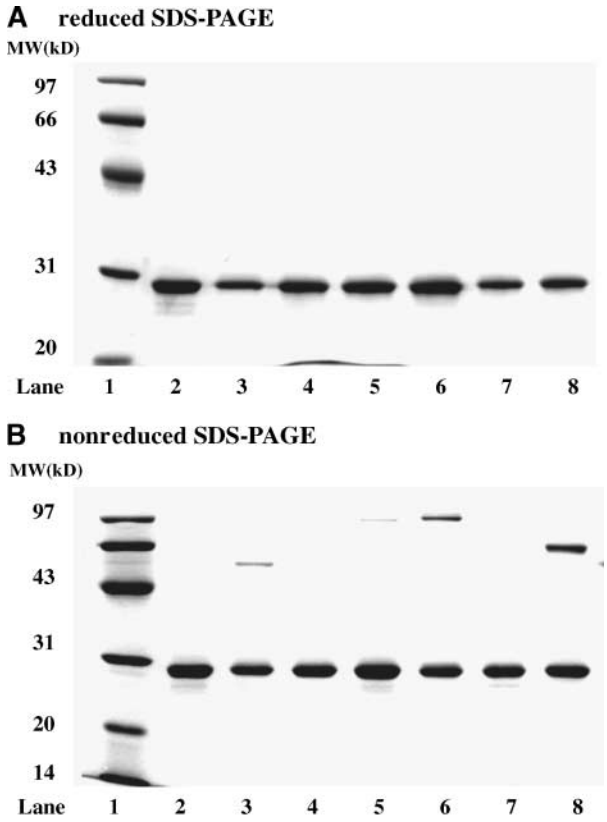

Fig. 2. Recombinant purified wild-type and cysteine mutants of apoA-I were examined by 12% SDS-PAGE. A: Under reducing conditions (100 mmol/l DTT), cysteine mutants migrated as single bands of the same molecular mass as the recombinant wild-type human apolipoprotein A-I (wtapoA-I). B: Under nonreducing conditions, cysteine mutants were covalently linked dimers. Each gel was loaded with ~2 μg of protein per lane. Lanes 1 to 8 represent protein low molecular mass marker, wtapoA-I, and the cysteine variants of apolipoprotein A-I at residue 52 [A-I(S52C)], A-I(N74C), A-I(K107C), A-I(G129C), A-I_M(R173C), and A-I(K195C). MW, molecular mass.

that these mutations weakened the stability of secondary structures of apoA-I (Table 2). In contrast, A-I(G129C) presented a higher value of ΔG_D^0 (2.80 ± 0.10 kcal/mol), suggesting a more stable structure. While no statistical differences were observed for the structural stability of the other variants compared with wtapoA-I (Table 2).

TABLE 1. Contents of free cysteine in purified apoA-I mutants

Mutant	Free Cysteine
	mol/mol
A-I(S52C)	0.93 ± 0.01
A-I(N74C)	0.95 ± 0.01
A-I(K107C)	0.94 ± 0.01
A-I(G129C)	0.89 ± 0.02
A-I_M(R173C)	0.92 ± 0.03
A-I(K195C)	0.88 ± 0.03

A-I(S52C), A-I(N74C), A-I(K107C), A-I(G129C), A-I_M(R173C), and A-I(K195C), the cysteine variants of apolipoprotein A-I at residues Ser-52, Asn-74, Lys-107, Gly-129, Arg-173, and Lys-195, respectively; apoA-I, apolipoprotein A-I. The amounts of free cysteine in apoA-I mutants were determined by the thiol and sulfide quantitation assay. Values represent means ± SD of two independent observations.

TABLE 2. α helical content and ΔG_D^0 of wtapoA-I and cysteine mutants in monomeric form

ApoA-I	α Helical Content	ΔG_D^0
	%	*kcal/mol*
wtapoA-I	54 ± 4	2.42 ± 0.04
A-I(S52C)	49 ± 4	2.20 ± 0.14
A-I(N74C)	50 ± 2	2.34 ± 0.09
A-I(K107C)	51 ± 6	2.29 ± 0.03
A-I(G129C)	56 ± 4	2.80 ± 0.10[a]
A-I_M(R173C)	52 ± 3	2.18 ± 0.11[a]
A-I(K195C)	54 ± 1	2.20 ± 0.11

ΔG_D^0, free energy of unfolding; wtapoA-I, recombinant wild-type human apolipoprotein A-I. α helical content was determined from molar ellipticity at 222 nm obtained from circular dichroism (CD) spectra. Values represent means ± SD of two independent observations. ΔG_D^0 is the standard change in free energy of denaturation of the α-helical segments monitored by CD at 222 nm in response to increasing concentrations of guanidine hydrochloride.

[a] $P < 0.05$ versus wtapoA-I.

DMPC clearance assay

DMPC clearance assay was used to assess the abilities of apoA-I and its mutants to associate with lipid. The ability to clear the lipid turbidity (determined by rate constant $k_{1/2}$) was decreased by 83% and 76% for A-I(G129C) and A-I(K195C), respectively, relative to wtapoA-I ($P < 0.05$), whereas the other mutations did not exert significant influences on the values of $k_{1/2}$ (**Fig. 4**, **Table 3**). In addition, no statistical differences were observed for the $k_{1/2}$ between the reduced and nonreduced protein samples (data

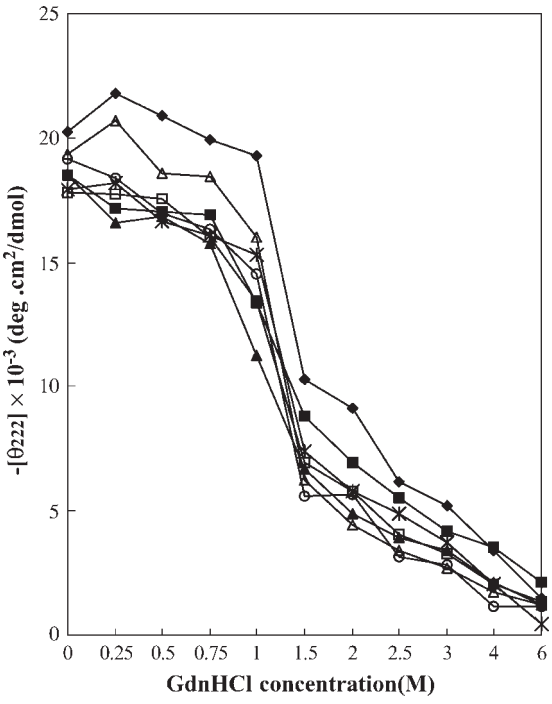

Fig. 3. Effects of guanidine hydrochloride (GdnHCl) concentration on the molar ellipticity at 222 nm [θ_{222}] of lipid-free recombinant wtapoA-I and monomeric cysteine mutants. Open triangles, wtapoA-I; asterisks, A-I(S52C); open squares, A-I(N74C); open circles, A-I(K107C); closed diamonds, A-I(G129C); closed triangles, A-I_M(R173C); closed squares, A-I(K195C).

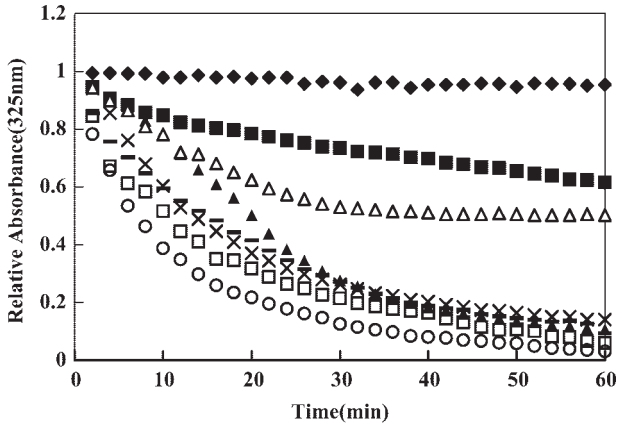

Fig. 4. Kinetics of interaction of lipid-free apolipoproteins with 1,2-dimyristoyl-*sn*-glycerol-3-phosphatidylcholine multilamellar liposomes. The change in turbidity was monitored by the change in absorbance at 325 nm at 2 min intervals for the initial 60 min and plotted as a function of time. Closed diamonds, negative; open squares, wtapoA-I; bars, A-I(S52C); open circles, A-I(N74C); closed triangles, A-I(K107C); closed squares, A-I(G129C); crosses, A-I_M(R173C); open triangles, A-I(K195C).

not shown), especially for A-I(G129C) ($k_{1/2}$ = 0.014 ± 0.002 vs. 0.010 ± 0.001) and A-I(K195C) ($k_{1/2}$ = 0.021 ± 0.006 vs. 0.014 ± 0.001) ($P > 0.05$), which excluded disturbance of the minor dimers.

Cholesterol efflux from cholesterol-loaded THP-1 macrophage

To analyze the influences of cysteine mutations on the ability of apoA-I to promote cellular cholesterol efflux, we examined cholesterol efflux mediated by apoA-I and cysteine mutants using differentiated human THP-1 macrophages as a cell model. When cholesterol-loaded THP-1 cells were incubated with different lipid-free apolipoproteins, only A-I(K107C) exhibited a significantly enhanced ability to promote cholesterol efflux, with a 21.46% increase in its ability to remove cholesterol from macrophages after a 24 h incubation compared with wtapoA-I (**Table 4**). Conversely, the mutations occurring in A-I_M(R173C) and A-I(K195C) conferred a weakened ability to extract cholesterol from cells, whereas the other mutants possessed efflux abilities similar to those of wtapoA-I (Table 4).

TABLE 3. Rate constants for the decrease of turbidity after mixing wtapoA-I or cysteine mutants with 1,2-dimyristoyl-*sn*-glycerol-3-phosphatidylcholine

ApoA-I	$k_{1/2}$
	min^{-1}
wtapoA-I	0.059 ± 0.002
A-I(S52C)	0.052 ± 0.009
A-I(N74C)	0.079 ± 0.023
A-I(K107C)	0.061 ± 0.012
A-I(G129C)	0.010 ± 0.001[a]
A-I_M(R173C)	0.046 ± 0.005
A-I(K195C)	0.014 ± 0.001[a]

$k_{1/2}$ indicates the rate constant, calculated by $k_{1/2} = 1/t_{1/2}$, where $t_{1/2}$ is the time required for a 50% decrease in relative turbidity. Each value represents the mean ± SD of at least three independent determinations.

[a] $P < 0.05$ versus wtapoA-I.

TABLE 4. Cholesterol efflux from human THP-1 macrophages in the presence of wtapoA-I or the mutants in lipid-free states

Acceptor	Fractional Efflux
	%
Negative	7.77 ± 0.23[a]
wtapoA-I	16.68 ± 1.77[b]
A-I(S52C)	17.49 ± 2.18[b]
A-I(N74C)	15.51 ± 2.16[b]
A-I(K107C)	20.26 ± 0.18[a,b]
A-I(G129C)	15.09 ± 1.16[b]
A-I_M(R173C)	13.6 ± 0.99[a,b]
A-I(K195C)	12.8 ± 0.93[a,b]

Fractional cholesterol efflux was calculated as $CPM_{medium}/(CPM_{medium} + CPM_{cells}) \times 100\%$. Values represent means ± SD of two independent experiments (each with three wells).

[a] $P < 0.05$ versus negative.

[b] $P < 0.05$ versus wtapoA-I.

Assessment of antioxidant activity

Bielicki and colleagues (29, 30) reported that cysteine mutation conferred on A-I_M protection against peroxidation, which was thought to result from the unique thiol group in the monomeric molecule. In our study, we used the oxidative system described by Bielicki et al. to assess whether our cysteine mutants could suppress the peroxidation initiated by soybean lipoxygenase. However, remarkably distinct from theirs, our results (**Table 5**) showed no evident dose-dependent decrease in the production rate of conjugated dienes (which was used to express the extent of peroxidation) when increasing concentrations of A-I_M(R173C) (final concentration of A-I_M varying from 25 to 100 μg/ml) were added to the reaction system ($P > 0.05$). Furthermore, results from the other five oxidative systems (each containing an individual cysteine mutant) revealed no significant differences between wtapoA-I and the mutants in the rate of oxidation ($P > 0.05$) (**Table 6**). Furthermore, addition of apolipoproteins into the oxidative system before or after the initiation of peroxidation did not exert significant influence on the results (data not shown). Our findings suggested that the monomeric cysteine mutants failed to inhibit the de-

TABLE 5. Rates of lipoxygenase-mediated oxidation of PLPC using A-I_M(R173C) as the antioxidant at different concentrations

A-I_M(R173C) Concentration	Rate of Conjugated Dienes
μg/ml	*nmol/min/ml*
0	4.23 ± 0.43
25	4.06 ± 0.66
50	4.42 ± 0.33
75	4.23 ± 0.48
100	3.66 ± 0.63

PLPC, 1-palmitoyl-2-linoleoylphosphatidylcholine. Values of conjugated dienes are means ± SD of at least three independent determinations.

TABLE 6. Comparison of the rates of lipoxygenase-mediated oxidation of PLPC coincubated with wtapoA-I or cysteine mutants

ApoA-I	Rate of Conjugated Dienes
	nmol/min/ml
No apolipoprotein	4.11 ± 0.09
wtapoA-I	4.02 ± 0.65
A-I(S52C)	3.72 ± 0.46
A-I(N74C)	3.82 ± 0.28
A-I(K107C)	3.68 ± 0.30
A-I(G129C)	3.69 ± 0.46
$A\text{-}I_M$(R173C)	4.14 ± 0.39
A-I(K195C)	3.79 ± 0.46

Values of conjugated dienes are means ± SD of at least three independent determinations, and the concentration of each apolipoprotein was kept at 50 μg/ml in every reaction system.

velopment of peroxidation induced by the soybean lipoxygenase.

DISCUSSION

In this study, we constructed six cysteine mutants of apoA-I: A-I(S52C), A-I(N74C), A-I(K107C), A-I(G129C), $A\text{-}I_M$(R173C), and A-I(K195C). Each cysteine was incorporated in an individual helix of 22 amino acids at the same helical position as that of $A\text{-}I_M$. The incorporation of cysteine failed to significantly alter the α helical content of monomeric apoA-I, whereas incorporation at residues 129 and 173 influenced the secondary structural stability of apoA-I. The displacement of cysteine at residues 129 and 195 significantly impaired the lipid binding abilities of apoA-I. A-I(K107C) exhibited more efficient cholesterol efflux ability than wtapoA-I, whereas $A\text{-}I_M$(R173C) and A-I(K195C) exhibited weakened efflux abilities. To our knowledge, this is the first study that systemically assesses the influence of cysteine substitutions at different helices on the structure and functions of apoA-I.

In our CD assay, the α helical content of wtapoA-I was ~54%, consistent with other reports (33, 34). The cysteine displacements had no significant influence on the α helical content. Similar to our results, Suurkuusk and Hallen (35) observed that monomeric $A\text{-}I_M$ possessed a similar α helical content to wtapoA-I, but when the interchain disulfide bridge was introduced in $A\text{-}I_M/A\text{-}I_M$, α helical content increased greatly, indicating a more folded structure (wtapoA-I, 44%; monomer $A\text{-}I_M$, 43%; $A\text{-}I_M/A\text{-}I_M$, 59%). In addition, a study of two other cysteine mutants (A124C and A232C) revealed that the fraction of α helix in the monomers was slightly lower than that of plasma apoA-I; unlike $A\text{-}I_M/A\text{-}I_M$, the formation of homodimer failed to remarkably alter the α helical content (36). Our study, combined with these previous reports, indicate that single cysteine residue substitution exerted minor effects on the α helical content of monomers. In the chemical unfolding CD assay, the ΔG_D^0 of $A\text{-}I_M$(R173C) was lower than that of wtapoA-I, indicating impaired structural stability, whereas A-I(G129C) exhibited a more stable structure than wtapoA-I. Indeed, the displacement of cysteine in $A\text{-}I_M$ disrupted the salt bridge between Arg-173 and Glu-169, which was assumed to stabilize the polypeptide (37). The disrupted salt bridges may partially explain the decrease of structural stability that we observed.

One of the most important biological activities of apoA-I is to bind lipid and form lipoprotein. In this regard, the influence of cysteine incorporation in apoA-I on the kinetics of its lipid binding was assessed. We found that A-I(G129C) and A-I(K195C) presented remarkably impaired abilities to disrupt the liposome compared with wtapoA-I. Residue Gly-129, located at the putative hinge domain, was assumed to be important for maintaining the efficiency of lipid binding and the stability of lipoprotein particles (6, 38). The increased secondary structural stability of A-I(G129C) shown in the CD assay, which indicated a tighter or more folded structure, might influence the flexibility of this region. This decreasing flexibility was not favorable to the interaction between apoA-I and lipid and might partially account for the decreased ability to bind lipid for A-I(G129C). The C-terminal region was critical for apoA-I to associate with lipid and to form HDL (7, 8). Based on this finding, we speculated that the cysteine displacement in A-I(K195C) interfered with the interaction between the C-terminal region and lipid, leading to decreased lipid binding ability. These data also support the idea that the central and C-terminal regions were indeed critical for apoA-I when associating with lipid (6–8, 38).

In recent years, human THP-1 monocytes have been used to study lipid metabolism in macrophages because of its more complete differentiation and of more loyal to properties of human macrophages (39). In our study, A-I(K107C) exhibited a greater efficiency in recruiting cholesterol from THP-1 macrophages, whereas $A\text{-}I_M$(R173C) and A-I(K195C) showed decreased efflux abilities compared with wtapoA-I. The decreased efflux capacity of $A\text{-}I_M$(R173C) was also observed by other research groups (40, 41). Conversely, $A\text{-}I_M/A\text{-}I_M$, distinct from its monomer, was a more efficient acceptor of cellular cholesterols after mixing with phospholipid (42). It is known that the ability of apoA-I to promote cholesterol efflux was closely correlated with its ability to bind lipid (43, 44). In our investigations, without significant alteration in secondary structure, A-I(K195C) exhibited a remarkably impaired lipid binding ability, which might bear some responsibility for its weakened efficiency at extracting cholesterol from macrophages. However, the lipid binding capacity could not explain the increased efflux efficiency that A-I(K107C) displayed. A specific arrangement of acidic residues on the α helices was required to mediate cholesterol efflux (45). As a matter of fact, the mutation in A-I(K107C) disrupted the salt bridges between Lys-107 and Asp-103, which might lead to a rearrangement of the amino acids to facilitate efflux. How this mutation facilitates cholesterol efflux requires further investigation. At present, there are two known mechanisms of cellular cholesterol efflux: the nonspecific diffusion pathway (46) and the special receptor-mediated mechanism. ABCA1 was considered the principal candidate for the latter mechanism, which was observed to notably recruit cholesterol from macro-

phages via direct association with apoA-I helices (47, 48). In addition, ABCA1 exhibited a high level of expression on the surface of macrophages (49), indicating an important role in mediating cholesterol efflux from macrophages or foam cells. To date, the mechanism of apoA-I in promoting cholesterol efflux is still unclear. Do the cysteine mutations influence the efflux ability of apoA-I through ABCA1? The answer to this question would help us explain the efflux effects that cysteine mutants have exhibited in our research and the work of others.

We knew that the protection of apoA-I or HDL against atherosclerosis was attributed mainly to the key activities in the process of reverse cholesterol transport. Likewise, HDL was observed to directly protect LDL from oxidation (50), which was believed to be attributable to paraoxonase-1. This raises an interesting question: can A-I_M protect against peroxidation? By coincubating monomeric A-I_M or apoA-I_{Paris} with the lipoxygenase-mediated oxidation system, Bielicki and Oda (29) concluded that free cysteine residue in the monomers of the mutants endowed the mutants with protection against peroxidation, and A-I_M was twice as effective as apoA-I_{Paris} in preventing the oxidation of phospholipids. Almost at the same time, Bielicki et al. (30) observed that the synthesized peptides with a single cysteine, based on the sequences of A-I_M and apoA-I_{Paris}, exhibited antioxidation activity like that of full-length proteins. In our study, the oxidative system that this laboratory described was used to detect our recombinant monomeric cysteine mutants. We observed that production rates of conjugated dienes in the control group and the wtapoA-I group were consistent with their reports (29). However, distinct from their findings, recombinant A-I_M [i.e., A-I_M(R173C)] failed to restrain the peroxidation of phospholipid. The production of conjugated dienes did not decrease with the increasing dose of A-I_M(R173C) in the reaction system. Negative results were also obtained when we tested the other monomeric cysteine mutants. What, then, accounts for this difference? First, we excluded the interference of the His tag; in the experiment of Bielicki and Oda (29), their A-I_M was expressed and purified using the pET system and Ni^{2+} affinity chromatography, respectively, with 6×His at the N terminus. Second, the thiol quantitation assay proved the existence of free cysteine in our mutants (Table 1). Therefore, whether A-I_M could inhibit peroxidation by its cysteine residue needs further in vivo experiments or the use of other oxidative systems to investigate.

In addition, the monomers of the cysteine mutants were not isolated from purified recombinant proteins in this study. Therefore, we could not exclude the interference of the dimers in the assays, although they were present only in very small amounts.

In summary, although the cysteine mutations were located at a similar position in each helix, the mutants displayed different lipid binding and cholesterol efflux features. Unlike the natural cysteine mutants, the monomeric mutants displayed no significant cardioprotection. Finally, whether A-I_M possesses antioxidation activity still remains to be determined.

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