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# Rapid Letter

# Dihydrolipoic Acid as an Effective Cofactor for Peptide Methionine Sulfoxide Reductase in Enzymatic Repair of Oxidative Damage to Both Lipid-Free and Lipid-Bound Apolipoprotein A-I

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## ABSTRACT

**The aim of this study was to examine the possible use of dihydrolipoic acid (DHLA), the reduced form of lipoic acid, in the reduction of oxidized apolipoprotein A-I mediated by peptide methionine sulfoxide reductase (PMSR), and to test the accessibility of methionine sulfoxides in the lipid-bound oxidized apolipoprotein A-I to this reaction. We show that DHLA acts as an effective cofactor for PMSR, mediating restoration of the native secondary structure, tertiary structure, stability, and lipid-binding properties of the native unoxidized protein. Reconstituted high-density lipoproteins were used to demonstrate effective enzymatic reduction of the lipid-bound oxidized protein in the presence of DHLA. These findings suggest that the enzymatic repair of oxidative damage to intact lipoproteins could provide a model of a possible repair mechanism active *in vivo* during oxidative stress and that the restoration of high-density lipoprotein function could be one of the therapeutic benefits of lipoic acid.** *Antioxid. Redox Signal.* 4, 553–557.

## INTRODUCTION

APOLIPOPROTEIN (apo) A-I, the major protein constituent of plasma high-density lipoproteins (HDL), plays a key role in the reverse cholesterol transport pathway. Both lipid-free and lipid-bound apo A-I have been shown to promote cholesterol efflux from cells (9), and HDL levels in plasma inversely correlate with the risk of coronary heart disease (7).

Oxidative modification of HDL can impair their protective actions *in vivo* (5). We have previously shown that oxidation of Met-112 and Met-148 in apo A-I occurs *in vivo*, and is accompanied by functionally important alterations in structure and stability of both lipid-free and lipid-bound apo A-I forms (11, 12). We have also demonstrated that cellular cholesterol efflux mediated by HDL depends upon the ratio of unoxidized/oxidized apo A-I, and that the difference in the extent of efflux can reach 30% (14). Finally, it was also shown that lipid-free and detergent-associated oxidized apo A-I molecules containing methionine sulfoxides at positions 112 and 148 can be readily repaired by peptide methionine sulfoxide reductase (PMSR) using dithiothreitol (DTT) as the source of reducing equivalents (11). However, it still remains unclear whether any naturally occurring sources of reducing equivalents can be used in enzymatic repair of oxidative damage to apo A-I. The reduction of oxidized apo A-I in actual HDL particles, as would be found *in vivo*, also has not been reported.

Dihydrolipoic acid (DHLA) is a metabolic product formed *in vivo* from lipoic acid (1), which is widely used as a therapeutic agent in a variety of diseases. Several lines of evidence suggest that the antioxidant properties of lipoic acid and its reduced form, DHLA, are at least in part responsible for the therapeutic effect. In this study, we show that DHLA acts as an effective cofactor for PMSR in the reduction of methionine sulfoxides in oxidized apo A-I molecules, and mediates restoration of the native secondary and tertiary structure and

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the thermodynamic stability and lipid-binding properties of the native unoxidized protein. We prepared well characterized reconstituted HDL particles (rHDL) with a defined molar ratio of unoxidized to oxidized apo A-I proteins, with or without apo A-II as found in some kinds of HDL, and determined that the rate and yield of the enzymatic reduction of methionine sulfoxides to methionines are identical for lipid-free apo A-I and apo A-I incorporated in different rHDL particles.

## MATERIALS AND METHODS

### *Chemicals, enzymes, and lipids*

DHLA and other chemicals were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). A homogeneous preparation of bovine PMSR (EC 1.8.4.6), overexpressed in *Escherichia coli* and provided as a 0.6 m*M* solution in 25 m*M* Tris-HCl, pH 7.4, was a gift from Prof. Nathan Brot (Cornell University Medical College, New York, NY, U.S.A.). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.).

### *Apolipoproteins and lipoproteins*

Apo A-I and apo A-II were isolated and purified according to our previously published procedure (13). Unoxidized and oxidized apo A-I, as well as unoxidized apo A-II, were isolated from the initial preparations by preparative HPLC and characterized as previously described (11, 12). We use the terms "unoxidized" to describe naturally occurring apo A-I as found in the unoxidized form (apo A-$I_{unox}$), "oxidized" to describe both naturally occurring or chemically oxidized apo A-I containing sulfoxides at methionines 112 and 148 (apo A-$I_{ox}$) and "reduced" to describe apo A-I obtained by reduction of the oxidized protein using PMSR (apo A-$I_{red}$). Mass spectra of unoxidized and oxidized apo A-I were measured using a Voyager Elite STR mass spectrometer from PerSeptive Biosystems (Cambridge, MA, U.S.A.). Chemically oxidized apo A-I was obtained from the unoxidized protein using hydrogen peroxide, and then was purified by preparative HPLC, as previously described (11, 12).

The rHDL complexes were prepared and characterized as previously described in detail (12).

### *DMPC binding kinetics*

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) in chloroform solution was dried under argon and then solubilized in TBS (Tris-buffered saline containing 0.01 *M* Tris-HCl, 0.14 *M* NaCl, 0.25 m*M* EDTA-$Na_2$, 0.15 *M* $NaN_3$), pH 8.0 (0.5 mg/ml final) above its transition temperature (>24°C). DMPC/protein molar ratios of 50:1 were used, and the reaction was followed at 24°C in a thermostated cell compartment of a Hitachi U-3110 spectrophotometer by monitoring the decrease in absorbance at 325 nm. The data were analyzed according to pseudo–first-order kinetics, and $t_{1/2}$ was determined as the time required for a 50% decrease in turbidity.

### *Circular dichroism*

Circular dichroism (CD) spectra of lipid-free apo A-I (3.6 μ*M* or 0.1 mg/ml) in TBS, pH 7.4, were recorded with an AVIV 62A DS spectropolarimeter (AVIV, Lakewood, NJ, U.S.A.). An apparent fractional percent α-helix content was calculated from the molar ellipticities at 222 nm ($[\phi]_{222} = -30{,}300\ f_H - 2{,}340$, where $f_H$ is the fraction of α-helical structure) (4).

In experiments on temperature-induced unfolding, CD spectra were measured as a function of temperature (25–95°C) using solutions of 3.6 μ*M* (0.1 mg/ml) lipid-free apo A-I in TBS, pH 7.4.

### *Isothermal denaturation studies*

The effect of guanidine hydrochloride (GdnHCl) concentration on the structure of lipid-free apo A-I was monitored by fluorescence emission of Trp using a FluoroMax-2 spectrofluorimeter (SPEX Industries, Inc., Edison, NY, U.S.A.). Denaturation curves were analyzed as previously described in detail (12). Stability of lipid-free apo A-I was also determined by plotting the 353 nm/333 nm fluorescence emission ratio versus the molar GdnHCl concentration, and expressed as the concentration of the denaturant that reduced this ratio by 50% ($D_{1/2}$).

### *Enzymatic reduction of methionine sulfoxides*

Reduction of lipid-free apo A-$I_{ox}$ (65–130 μg) and apo A-$I_{ox}$ in rHDL particles (15 μg) was carried out essentially as described (11) at 37°C in 33 m*M* Tris-HCl, 13 m*M* $MgCl_2$, 13 m*M* DHLA (or DTT), pH 7.5, containing 4–8 μg of PMSR in a total volume of 30–90 μl. For HPLC analysis, solid GdnHCl was added to the reaction mixtures containing rHDL complexes to a final concentration of 6 *M*.

## RESULTS

Incubation of apo A-$I_{ox}$ with PMSR in the presence of DHLA resulted in the appearance of a new peak in the analytical reverse-phase HPLC chromatogram with the same retention time as for apo A-$I_{unox}$ (data not shown). Molecular mass determination indicated that the new species (apo A-$I_{red}$) was 32 atomic mass units lighter than the starting material, confirming repair of the two methionine sulfoxides and regeneration of apo A-$I_{red}$. The reaction yielded 50–60% apo A-$I_{red}$ after 5 min of incubation. Longer incubation time (up to 2.5 h), increased PMSR amount (4–8 μg), or increased apo A-I or DHLA concentration (12.5–125 μ*M* or 13–39 m*M*, respectively) did not significantly increase the final yield of reduced protein, as observed previously for the reaction using DTT (11). Incomplete reduction with PMSR may be a result of the high enzymatic diastereoselectivity (10).

The apo A-$I_{red}$ prepared using DHLA was purified and characterized in detail. Electrophoretic mobility, protease susceptibility, CD spectra, and isothermal and thermal denaturation profiles were all identical to those observed for apo A-$I_{unox}$, as well as for apo A-$I_{red}$ prepared using DTT (data not shown, see also 11, 12).

The ability of apo A-I to promote cholesterol efflux is partially determined by its ability to bind with lipids. The kinetics of this binding was assessed by measuring the rate of DMPC liposome turbidity clearance (Fig. 1). The $t_{1/2}$ values

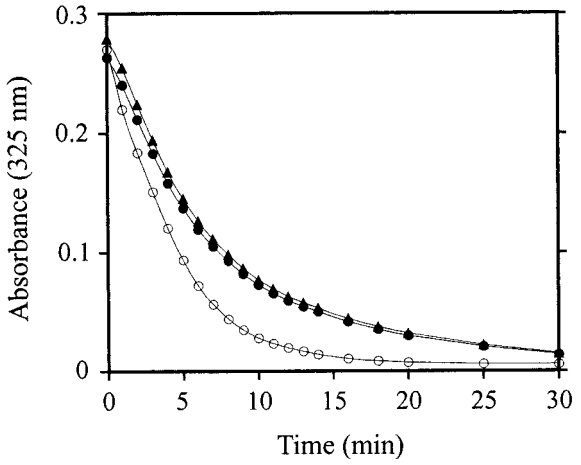


**FIG. 1. DMPC kinetic binding with unoxidized, oxidized, and reduced apo A-I proteins.** DMPC solubilized above its transition temperature (>24°C) in TBS, pH 8.0, was diluted by the same buffer to 0.5 mg/ml and preincubated for 10 min at 24°C. Then unoxidized (●), oxidized (○), and reduced (▲) apo A-I proteins dissolved in the same buffer were added (final DMPC/protein molar ratio, 50:1), and the reaction was followed at 24°C for 30 min at 325 nm.

were estimated to be 5.0 and 3.5 min, for apo A-$I_{unox}$ and apo A-$I_{ox}$, respectively. Thus, apo A-$I_{ox}$ binds DMPC more rapidly than apo A-$I_{unox}$, although the final binding levels are equal. Similar results were obtained for the 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine/cholesterol mixture. The observed faster rate of apo A-$I_{ox}$ association may reflect a partial unfolding induced by methionine oxidation (see below and 11, 12) that is consistent with significantly decreased conformational stability and reduced fractional helix content of the lipid-free oxidized protein (Table 1; 11, 12). Our findings are also in line with the hypothesis that lipid association by apo A-I requires some unfolding to expose hydrophobic domains for lipid binding (8).

The reduction of apo A-$I_{ox}$ by PMSR in the presence of DHLA completely restores DMPC binding kinetics (Fig. 1), protein secondary and tertiary structural features, and thermodynamic parameters (Table 1) characteristic of the native apo A-$I_{unox}$. Thus, enzymatic reduction of apo A-$I_{ox}$ using PMSR with DHLA as its cofactor can reverse the dramatic damage caused by the oxidation of two of three methionine residues (Met-112 and Met-148).

Well defined rHDL particles with varying proportions of apo A-$I_{ox}$, apo A-$I_{unox}$, and apo A-II were reacted each with PMSR in the presence of DHLA. Conversion of lipid-bound apo A-$I_{ox}$ to apo A-$I_{red}$ proceeded to ~50–60% after 5 min of incubation independent of rHDL protein composition (Fig. 2). Rate and yield of the reaction were similar to those observed for lipid-free apo A-I. No changes in size distributions or shapes in rHDL particles were observed after PMSR reaction as analyzed by nondenaturing gradient gel electrophoresis and electron microscopy (data not shown). These data indicate that both of the apo A-I methionine sulfoxide residues (Met-112 and Met-148) are as accessible to enzymatic reduction in rHDL particles as they are in lipid-free apo A-$I_{ox}$. Furthermore, the reaction does not depend on the molar ratio of apo A-$I_{unox}$/apo A-$I_{ox}$ or the presence of apo A-II. Because these experiments were performed with discoidal rHDL particles including those that contain apo A-II, these findings are especially relevant to native nascent HDL, but they also should apply to mature HDL (3), including those containing only apo A-I or both apo A-I and apo A-II.

Thus, oxidatively damaged apo A-I molecules in rHDL particles can be readily repaired using PMSR with DHLA as its cofactor.

## DISCUSSION

Only one published study described the use of DHLA as a cofactor for PMSR (2), in which oxidative damage to α-1-proteinase inhibitor was reversed by PMSR with full restoration of the protein biological activity. However, in that study DHLA alone was effective in reducing methionine sulfoxides in the absence of PMSR, with ~94% conversion in the presence of 40 m*M* DHLA. In contrast, we did not observe production of reduced apo A-I protein using DHLA (or DTT) alone in the absence of PMSR, even at concentrations up to 65 m*M*, indicating a requirement for enzymatic catalysis.

The current study shows that the rate and yield of the enzymatic reduction of methionine sulfoxides to methionines are

TABLE 1. SECONDARY STRUCTURE AND THERMODYNAMIC STABILITY OF LIPID-FREE APO A-I

| *Complex* | α-*Helix*[a] *(%)* | $D_{1/2}$[b] *(M GdnHCl)* | $\Delta G_D^{\circ}$[c] *(kcal/mol of apo)* | $\Delta n$[d] *(mol of GdnHCl/mol of apo)* | $T_m$[e] *(°C)* |
|---|---|---|---|---|---|
| apo A-$I_{unox}$ | 62 ± 4 | 1.0 ± 0.1 | 4.7 ± 0.3 | 29 ± 4 | 64 ± 3 |
| apo A-$I_{ox}$ | 42 ± 4[f] | 0.4 ± 0.1[f] | 1.4 ± 0.1[f] | 15 ± 3[f] | — |
| apo A-$I_{red}$ | 64 ± 4 | 0.9 ± 0.1 | 4.4 ± 0.3 | 27 ± 4 | 62 ± 3 |

Results are given as means ± SD ($n$ = 3).
[a]Determined from molar ellipticities at 222 nm.
[b]Midpoints of GdnHCl denaturation.
[c]Free energy of denaturation at zero GdnHCl concentration.
[d]The number of the GdnHCl moles bound during denaturation.
[e]Midpoints of thermal denaturation.
[f]$p < 0.05$, comparison versus apo A-$I_{unox}$.

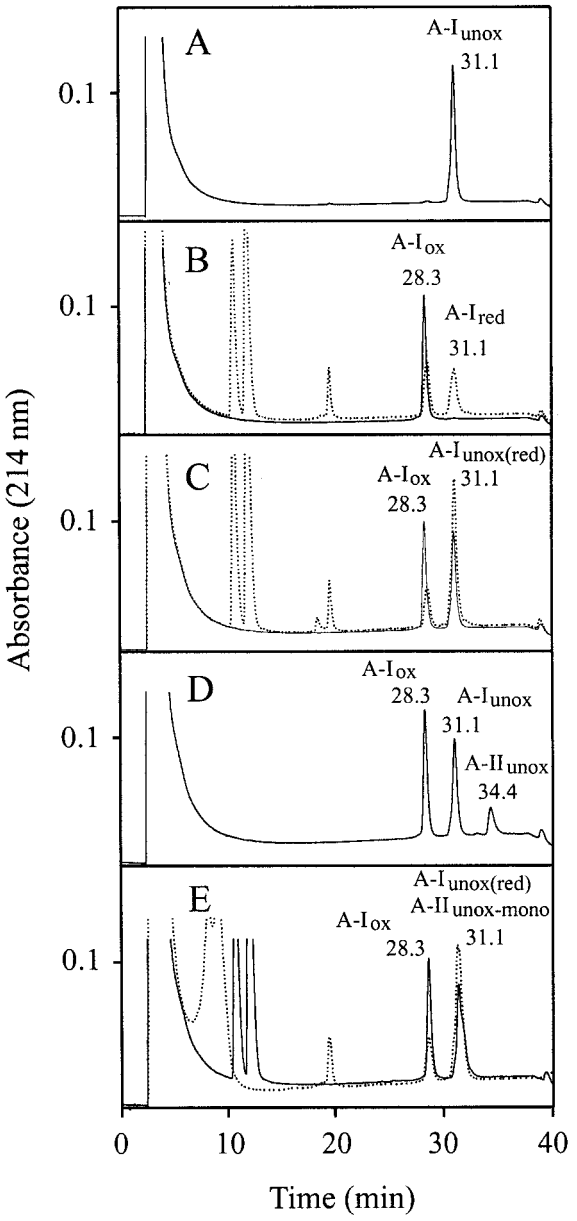


**FIG. 2. Analytical reversed-phase HPLC profiles of apo A-I in different rHDL particles.** (**A**) rHDL containing only apo A-$I_{unox}$. (**B**) rHDL containing only apo A-$I_{ox}$ before (solid line) and after (dotted line) the treatment by the PMSR enzyme in the presence of DHLA. (**C**) rHDL containing apo A-$I_{unox}$ and apo A-$I_{ox}$ with a molar ratio of 1:1 before (solid line) and after (dotted line) the treatment by the PMSR enzyme in the presence of DHLA. (**D**) rHDL containing apo A-$I_{unox}$, apo A-$I_{ox}$, and apo A-$II_{unox}$ with a molar ratio of 3:3:1 in TBS, pH 7.4. (**E**) rHDL containing apo A-$I_{unox}$, apo A-$I_{ox}$, and apo A-$II_{unox}$ with a molar ratio of 3:3:1 in the enzyme reaction buffer containing DHLA in the absence of the enzyme (solid line) and after PMSR treatment (dotted line). As DHLA reduces the disulfide bond in native apo A-II dimer, it dissociates into identical 77-residue monomers. The retention time of this apo A-II monomer is similar to that for apo A-$I_{unox}$. Retention times (in minutes) are shown above each peak.

identical for both lipid-free apo A-I and for apo A-I incorporated in different rHDL particles. As shown by a variety of criteria, the enzymatic treatment in the presence of DHLA restores the structure, conformational thermodynamic stability, and lipid-binding properties of the native unoxidized apolipoprotein. It could be expected that this enzymatic repair may also restore biological functions of HDL. Considering that enhancement of the specific pathway of cholesterol removal by HDL is now considered as a novel target in the therapy of atherosclerosis (6), the curative rather than preventive antioxidant action of DHLA toward damaged lipoproteins may be of clinical relevance. In addition, the enzymatic repair of oxidative damage to intact lipoproteins could provide a model of a possible repair mechanism active *in vivo* during oxidative stress (1).

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## ABBREVIATIONS

apo A-I, apolipoprotein A-I; apo A-$I_{unox}$, unoxidized apo A-I as found in serum; apo A-$I_{ox}$, oxidized apo A-I contained in serum apo A-I or obtained from unoxidized apo A-I using hydrogen peroxide; apo A-$I_{red}$, reduced apo A-I obtained by reduction of oxidized apo A-I using PMSR; apo A-II, apolipoprotein A-II; apo A-$II_{unox}$, unoxidized apo A-II contained in serum; CD, circular dichroism; DHLA, dihydrolipoic acid; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DTT, dithiothreitol; GdnHCl, guanidine hydrochloride; HDL, high-density lipoproteins; PMSR, peptide methionine sulfoxide reductase; rHDL, reconstituted HDL; TBS, Tris-buffered saline containing 0.01 *M* Tris-HCl, 0.14 *M* NaCl, 0.25 m*M* EDTA-$Na_2$, 0.15 m*M* $NaN_3$, pH 7.4 or 8.0.

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