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Glabridin Protects Paraoxonase 1 from Linoleic Acid Hydroperoxide Inhibition via Specific Interaction: A Fluorescence-Quenching Study

Dana Atrahimovich,[†,‡] Jacob Vaya,[†,‡] Hagai Tavori,[†] and Soliman Khatib*[,†,‡]

[†]Oxidative Stress Research Laboratory, MIGAL—Galilee Technology Center, P.O. Box 831, Kiryat Shmona 11016, Israel

[‡]Tel-Hai College, Upper Galilee 12210, Israel

ABSTRACT: The enzyme paraoxonase 1 (PON1) binds to high-density lipoprotein (HDL) and is responsible for many of HDL's antiatherogenic properties. We previously showed that recombinant PON1 is inhibited by linoleic acid hydroperoxide (LA-OOH) present in the lipid fraction of the human carotid plaque (LLE) via oxidation of the enzyme's Cys284 thiol. Here we explore the effect of glabridin, an isoflavan isolated from licorice root, on preventing LA-OOH's inhibitory effect on rePON1 using the tryptophan-fluorescence-quenching technique and modeling calculations. Glabridin significantly prevented rePON1 inhibition by LLE or oxidized linoleic acid (by 22% and 15%, respectively), whereas ascorbic acid and Trolox, strong antioxidants, had no effect. Glabridin quenched the intrinsic fluorescence of rePON1 in a concentration-dependent manner. Binding parameters and modeling calculations demonstrated a major role for hydrophobic forces in the rePON1−glabridin interaction, indicating that it is not the antioxidant capacity of glabridin that protects rePON1 from LA-OOH inhibition, but rather its specific interaction with the enzyme.

KEYWORDS: *antioxidant, flavonoid, fluorescence, glabridin, HDL, paraoxonase 1*

INTRODUCTION

Paraoxonase 1 (PON1; EC 3.1.8.1) is a calcium-dependent enzyme that binds to high-density lipoprotein (HDL). It is synthesized in the liver and secreted into the bloodstream.[1] Many of the antiatherogenic functions of HDL are attributed to its associated PON1.[2,3] PON1-deficient mice have been found susceptible to the development of atherosclerosis,[4] while overexpression of human PON1 in mice inhibits its development.

Flavonoids are important dietary polyphenols found in fruits, vegetables, nuts, and tea.[5] The benefits of consuming flavonoids are assumed to stem from their antioxidant activity, which can prevent diseases such as cancer, cardiovascular disease, and neurodegenerative disorders.[6,7] However, antioxidant activity cannot be the sole explanation for flavonoids' cellular effects in vivo:[8−10] They are poorly absorbed through the gut into the blood and extensively metabolized in the small intestine, liver, and colon;[11,12] thus, their concentration in the blood scarcely reaches effective levels. In fact, no human clinical trial has yet been able to prove disease prevention by a particular pure flavonoid due to its antioxidant activity.[5,13,14] Recent studies have suggested that the cellular effects of flavonoids may be mediated by their interaction with specific proteins which are central to the intracellular signaling cascade and cell-signaling pathways.[8,15−17] The ability of flavonoids to interact with serum albumin and other serum proteins has also been investigated.[18,19] Several methods have been utilized to characterize flavonoid−protein interactions, such as fluorescence, absorption, circular dichroism, and lifetime measurements. The fluorescence assay provides many advantages, such as high sensitivity, high selectivity, easy operation, and low cost, and it has been widely applied to the study of protein−drug interactions.[19,20] We have previously shown that rePON1 activity is inhibited when incubated with human carotid lesion lipid extract (LLE), and the chemical structure of the inhibitor within the LLE was identified as linoleic acid hydroperoxide (LA-OOH).[21] The mechanism of the inhibition was established as a direct reaction of LA-OOH with rePON1's free thiol on Cys284, which is probably oxidized to sulfenic, sulfinic, and then sulfonic acids. Unlike the classical antioxidants such as vitamins C and E and Trolox, which were ineffective in preventing rePON1 inhibition, the isoflavan glabridin, extracted from licorice roots,[22] exhibited dose-responsive protection of rePON1. Thus, the aim of the present study was to explore the mechanism by which glabridin prevents LLE, or specifically LA-OOH, inhibition of rePON1 using the fluorescence technique and molecular-modeling calculations.

MATERIALS AND METHODS

Materials. Ascorbic acid, Trolox, linoleic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), paraoxon, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich. Glabridin was isolated from licorice root extract.[22] 5-(Thiobutyl)butyrolactone (TBBL) was synthesized in our laboratory using a previously described method.[23]

LLE and OX-LA Preparation. The lipid fraction of the human carotid plaque (LLE) was extracted from carotid plaque in our laboratory as described elsewhere.[21,23] Oxidized linoleic acid (OX-LA) was prepared by incubating linoleic acid (20 mM stock solution in DMSO diluted to 4 mM in phosphate-buffered saline, pH 7.4) with $CuSO_4$ (200 μM stock solution in double-distilled water diluted to 5 μM) for 20 h at 37 °C. OX-LA was extracted from the aqueous solution by an organic solvent (ethyl acetate). The organic layer was evaporated under nitrogen and analyzed by LC−MS to give 20% 13-

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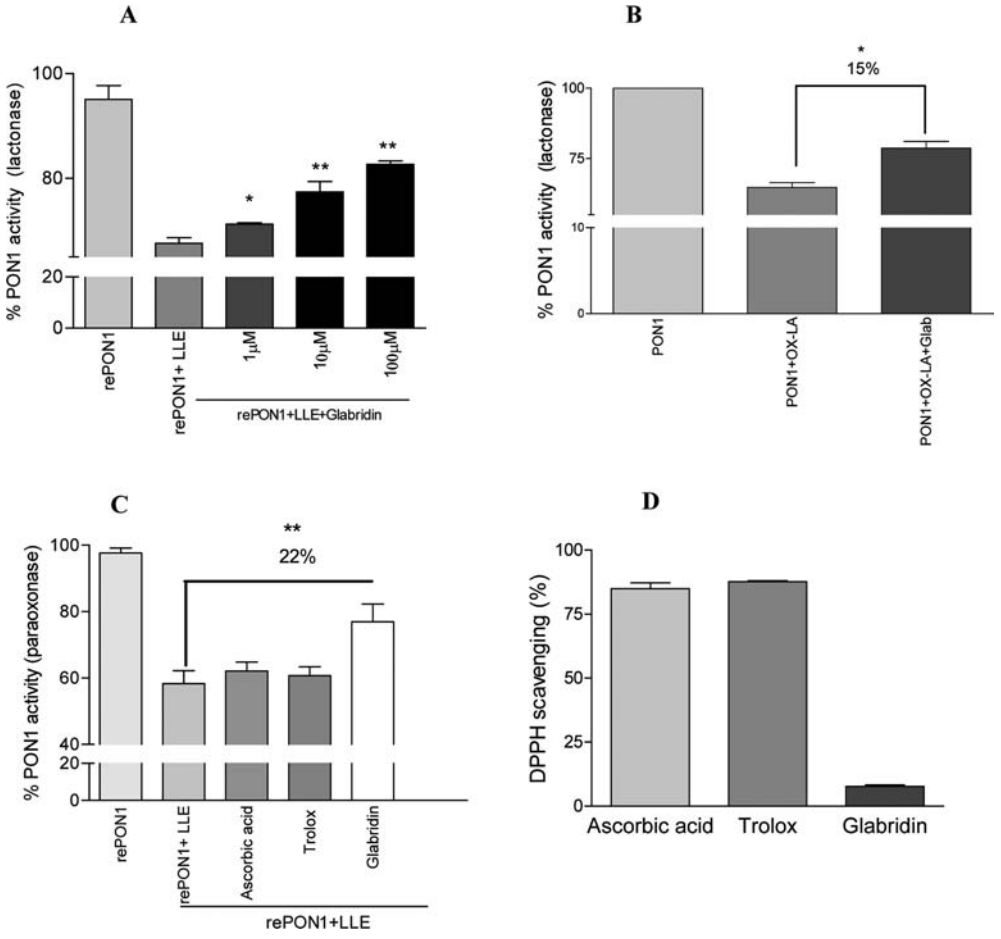

Figure 1. Effects of antioxidants on rePON1 inhibition by LLE or OX-LA: (A) dose-responsive effect of glabridin (1−100 μM) on lactonase activity of rePON1 (5 μg/mL) incubated with LLE (0.7 mg/mL) for 5 h, (B) protective effect of glabridin (10 μM) on lactonase activity of rePON1 (5 μg/mL) incubated with OX-LA (30 μM) for 2 h, (C) paraoxonase activity of rePON1 (10 μg/mL) with 0.7 mg/mL LLE in the presence of ascorbic acid, Trolox, or glabridin (100 μM), (D) scavenging capacity of ascorbic acid, Trolox, or glabridin (40 μM) in a methanolic solution of DPPH at a final concentration of 100 μM. Results are presented as the mean ± SD or as the percentage of activity, with significance determined at $p < 0.05$ (*) and $p < 0.001$ (**).

LA-OOH. OX-LA and LLE were dissolved in DMSO and stored at −20 °C.

Recombinant PON1 (rePON1). rePON1 was generated in *Escherichia coli* by directed evolution as described previously.[24] rePON1 storage buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM $CaCl_2$, and 0.1% (v/v) tergitol) was supplemented with 0.02% (w/v) sodium azide, and storage was at 4 °C. rePON1 was purchased from the Weizmann Institute of Science (Rehovot, Israel).

rePON1 Activity. The antioxidants ascorbic acid, Trolox, and glabridin (100 μM each) were incubated with rePON1 (5 μg/mL for lactonase activity or 10 μg/mL for paraoxonase activity) for 15 min. Then atherosclerotic LLE (0.7 mg/mL) or OX-LA (30 μM) was added and the resulting mixture incubated for another 5 or 2 h, respectively, in Tris−HCl buffer (50 mM, pH 8) containing 1 mM $CaCl_2$.

Lactonase Activity. A 145 μL aliquot of DTNB (0.5 mM in Tris−HCl buffer) was placed in a visible (vis) enzyme-linked immunosorbent assay (ELISA) microplate well. Then 5 μL of the rePON1 sample described above and 95 μL of TBBL (4 mM in Tris−HCl buffer) were added. The catalytic activity of rePON1 was measured spectrophotometrically at 405 nm for 10 min every 0.5 min. One unit of rePON1 lactonase activity was equal to 1 μmol of TBBL hydrolyzed per milliliter per minute as reported previously.[23]

Paraxonase Activity. A 95 μL aliquot of glycine buffer (50 mM, pH 10.5) containing 1 mM $CaCl_2$ was placed in a vis ELISA microplate well. Then a 10 μL rePON1 sample and 95 μL of paraoxon (4 mM in glycine buffer) were added. The catalytic activity of rePON1 was measured spectrophotometrically at 405 nm for 40 min every 2 min. One unit of rePON1 paraoxonase activity was equal to 1 nmol of paraoxon hydrolyzed per milliliter per minute.[25]

DPPH Assay. DPPH (100 μM in 1 mL of methanol) was incubated with ascorbic acid, Trolox, or glabridin (40 μM) for 10 min in a cuvette. The free-radical-scavenging capacity of the antioxidants was determined by diode-array spectrophotometry at 517 nm.[26] The antioxidant capacity was calculated by comparing the percentage of DPPH absorbance in the presence of the antioxidant to the control (methanolic DPPH solution without antioxidants).

Molecular Docking. The crystal structure of rePON1 was retrieved from the Protein Data Bank (PDB).[24] The enzyme was prepared for docking by the ADT (AutoDock Tools) program, an accessory program that allows the user to interact with AutoDock 4.2 from a graphic user interface. Water molecules were removed from the protein PDB file. Polar hydrogen atoms were added and Kollman united atom charges assigned. The solvation parameters were added by the Addsol program (part of the ADT program), and the grid points were set to 74, 74, and 74, the spacing value was equivalent to 0.375, and the grid center was set to 0.59, 38.29, and 13.84. Glabridin's structure was taken from the ZINC database. Ligand docking was carried out with the AutoDock 4.2 Lamarckian genetic algorithm (GA-LS).[27,28]

LC−MS Analysis. Glabridin (100 μM) was coincubated with either rePON1 (10 μg/mL) or bovine serum albumin (BSA) (10 μg/mL), or without protein, for 5 h at 37 °C. Then the unbound glabridin was removed using a 10 000 nominal molecular weight limit (NMWL)

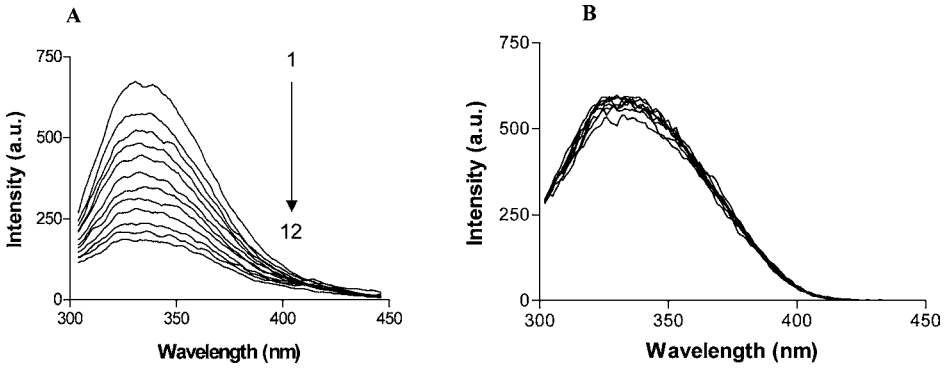

Figure 2. Fluorescence spectra of rePON1 (λ_{ex} = 290 nm, pH 7, T = 298 K) in the presence of (A) glabridin at concentrations of (1) 0, (2) 0.4, (3) 0.8, (4) 1.2, (5) 1.6, (6) 2, (7) 2.4, (8) 2.8, (9) 3.8, (10) 4.8, (11) 5.8, and (12) 6.8 μM and (B) Trolox at 2, 5, 10, 15, 20, and 25 μM. In all solutions, the total concentration of rePON1 was held constant at 1.16 μM. Each experiment was repeated separately at least three times.

cutoff membrane and centrifugal filtration at 4000 rpm and 4 °C. The remaining glabridin was re-extracted in methanol and injected for LC–MS analysis (this experiment was repeated three times).[29] The MS instrument (Micromass Quattro Ultima MS, United Kingdom) was operated in ES^- mode for multiple-reaction-monitoring (MRM) analysis. The MS parameters were as follows: source temperature 150 °C, cone gas flow 22 L/h, desolvation gas flow 600 L/h, capillary voltage 3–3.5 kV. Collision-induced dissociation MS was performed with a collision energy of 30–35 eV. A standard curve was generated for glabridin to evaluate glabridin concentrations in the samples.

Fluorescence-Quenching Measurements. The solution was prepared in a 0.7 mL quartz fluorescence cuvette. For each data point, 1 μL of glabridin (in ethanol) was added to 10 μL of rePON1 (2.5 mg/mL) diluted to 0.5 mL in Tris–HCl buffer (pH 7.0/8.0, 25 °C) to give a final glabridin concentration in the range of 0.4–6.8 μM and a final rePON1 concentration of 1.16 μM. Fluorescence-emission intensity was measured within 5 min of addition of glabridin or Trolox to the rePON1 solution. To measure the effects of temperature, each sample was placed in a 37 °C thermostat-controlled water bath for 5 min and then immediately transferred to the cuvette for measurement. pH measurement was carried out with a pH510 digital pH meter (Eutech Instruments, Malaysia). An electronic thermostat was used to control the temperature in the water bath.

Fluorescence measurements were performed in a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) equipped with a xenon lamp source and 1.0 cm quartz cell. The widths of both the excitation and emission slits were set at 5 nm. Emission spectra were recorded from 300 to 450 nm with an excitation wavelength of 290 nm. At each concentration of glabridin, the inner filter effect, which can decrease the fluorescence intensity, was corrected by using the following relationship:

$$F_{corr} = F_{obsd} e^{(A_{ex}+A_{em})/2} = F_{obsd} e^{(\varepsilon_{ex}CL+\varepsilon_{em}CL)/2} \quad (1)$$

where F_{corr} and F_{obsd} are the corrected and observed fluorescence intensities, respectively, ε_{ex} is 0.0165 μM^{-1} cm^{-1}, ε_{em} is 0.0034 μM^{-1} cm^{-1}, and L is the cuvette path length (0.2 cm for excitation and 1 cm for emission).[20,30]

Statistical Analysis. Statistical analysis was carried out using Graphpad Prism 5.03. Student's paired t test was used to compare the means of two groups. Analysis of variance (ANOVA) was used when more than two groups were being compared. Each experiment was repeated separately at least three times and was always performed in triplicate. Results are presented as the mean ± SD or as the percentage of activity, with significance determined at $p < 0.05$ or $p < 0.001$.

RESULTS

Glabridin Protects rePON1 from Inhibition. We have recently shown that rePON1's lactonase activity is inhibited by LA-OOH present in LLE.[21] The effect of glabridin, a potent antioxidant, on the lactonase activity of rePON1 incubated with LLE or with OX-LA alone was examined. Glabridin protected rePON1 from inhibition by LLE in a dose-responsive manner (1–100 μM glabridin prevented the enzyme's inhibition by up to 22%) (Figure 1A). Similarly, upon incubation of rePON1 with OX-LA, glabridin (10 μM) prevented the enzyme's inhibition by 15% (from 64.6 ± 3 to 78.6 ± 4% rePON1 activity) (Figure 1B). Glabridin's protective effect of rePON1 paraoxonase activity was also compared to those of ascorbic acid and Trolox. Neither Trolox nor ascorbic acid (100 μM) had any effect on the inhibition of rePON1 paraoxonase activity, whereas glabridin significantly prevented this inhibition by 22% when LLE was the inhibitor (Figure 1C). rePON1 protection from LLE by ascorbic acid, Trolox, or glabridin was not correlated with these antioxidants' capacity to donate an electron—a major pathway for antioxidant activity—and scavenge the free radical DPPH. Ascorbic acid and Trolox demonstrated strong antioxidant activity by inducing rapid radical scavenging of DPPH, resulting in 85% and 88% reduced absorbance at 517 nm, respectively, whereas glabridin showed a radical-scavenging capacity of only 8% (Figure 1D). These results suggested that glabridin's protective effect is most probably not via its antioxidant activity.

rePON1-Glabridin Interactions Using Fluorescence-Quenching Method. We hypothesized that the protective effect of glabridin on rePON1 might occur via specific interaction of the isoflavan with the enzyme—an interaction that other, nonflavonoid antioxidants cannot form. The rePON1–glabridin interaction was investigated by the Trp-fluorescence-quenching method. Figure 2 shows the emission spectra of rePON1 in the presence of various concentrations of glabridin (Figure 2A) or Trolox (Figure 2B) in the 300–450 nm range with an excitation wavelength of 290 nm (pH 7). The same trends were observed at pH 8 (data not shown). In comparison to Trolox, which appeared to have no effect on the fluorescence intensity of the enzyme, glabridin quenched the fluorescence of rePON1 in a concentration-dependent manner.

Fluorescence quenching can occur via two different major mechanisms: static and dynamic. Both quenching pathways can be described by the Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + K_{sv}[Q] \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, K_{sv} is the Stern–Volmer quenching constant, and [Q] is the quencher concentration.

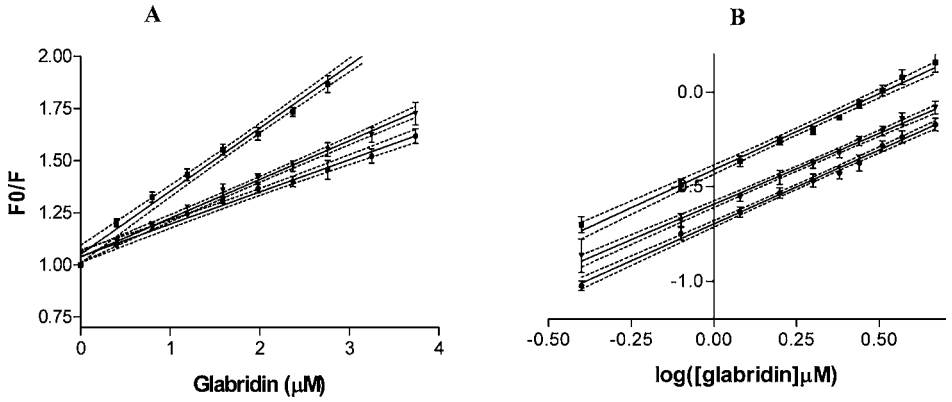

Figure 3. Glabridin's quenching of rePON1 fluorescence: (A) Stern–Volmer plot for the quenching of rePON1 by glabridin, (B) double-log plot for the quenching of rePON1 by glabridin. Key: ■, 310 K, pH 7; ▼, 298 K, pH 7; ●, 298 K, pH 8. Each experiment was repeated separately at least three times. Results are presented as the mean ± SD. $R^2 > 0.99$ and $p < 0.0001$ for all linear plots.

The Stern–Volmer curve (F_0/F versus [Q]) was linear at the tested concentrations of glabridin (with $R^2 > 0.99$) (Figure 3A), indicating that the quenching type is single static or dynamic.[31] For dynamic quenching, K_{sv} can be written as $K_q\tau_0$:

$$K_{sv} = K_q\tau_0 \tag{3}$$

where K_q is the quenching rate constant of the bimolecule and τ_0 is the lifetime of the fluorophore in the absence of quencher, approximately 10^{-8} s for a Trp residue.[32,33] The quenching constants (K_q) were calculated to be 3.02×10^{13} M^{-1} s^{-1} (310 K, pH 7), 1.86×10^{13} M^{-1} s^{-1} (298 K, pH 7) and 1.54×10^{13} M^{-1} s^{-1} (298 K, pH 8). The K_q value of rePON1 quenching initiated by glabridin was much greater than 2×10^{10} M^{-1} s^{-1}, the maximum diffusion collision quenching rate constant of various drugs with proteins (maximal dynamic quenching constant). This indicated that the quenching is not initiated by dynamic collision but via formation of a complex.[34,35]

Binding Constant and Binding Sites. For static quenching, the equilibrium between free and bound molecules can be described by

$$\log\left(\frac{F_0 - F}{F}\right) = \log K_a + n \log [Q] \tag{4}$$

where K_a is the binding constant, reflecting the degree of interaction between rePON1 and glabridin, and n is the number of binding sites specifying the number of glabridin molecules bound to a rePON1 macromolecule. Thus, a plot of $\log(F_0 - F)/F$ versus log [glabridin] can be used to determine K_a and n (Figure 3B). The highest K_a value for the association of glabridin with rePON1 was obtained for 310 K and pH 7 (3.88×10^5 M^{-1}) and the lowest for 298 K and pH 8 (2.01×10^5 M^{-1}). The values obtained for n were not affected by the pH or temperature and were equal to ~0.8 (Table 1).

Table 1. Binding Constants (K_a), Number of Binding Sites (n), and Thermodynamic Parameters for the rePON1–Glabridin Interaction

pH	T (K)	K_a (M^{-1})	n	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (J/mol)
7	310	3.88×10^5	0.8 ± 0.03	26.86	−33.17	193.2
7	298	2.51×10^5	0.75 ± 0.02	26.86	−30.80	193.2
8	298	2.01×10^5	0.78 ± 0.02	26.86	−30.26	193.2

Thermodynamic Parameters and Nature of the Binding Forces. The thermodynamic parameters enthalpy (ΔH), entropy (ΔS), and free energy (ΔG) were calculated to characterize the rePON1–glabridin type of interaction. ΔH can be estimated indirectly by examining the temperature dependence of K_a and using eq 5. ΔG can be estimated from eq 6 on the basis of the binding constants at different temperatures, and ΔS is estimated from their relationship (eq 7). K_{a1} and K_{a2} are the binding constants at temperatures T_1 and T_2, respectively, and R is the gas constant. Table 1 shows negative values for ΔG and positive values for ΔH and ΔS.

$$\ln \frac{K_{a2}}{K_{a1}} = \left(\frac{1}{T_1} - \frac{1}{T_2}\right)\frac{\Delta H}{R} \tag{5}$$

$$\Delta G = -RT \ln K_a \tag{6}$$

$$\Delta G = \Delta H - T\Delta S \tag{7}$$

Modeling Calculation of rePON1–Glabridin Interactions. Docking analysis of glabridin into rePON1's potential binding groove site was carried out to predict the location of the glabridin-binding site within the complete rePON1 variant crystal structure and to compare the modeling predictions with the Trp-quenching-fluorescence results. The approximate calculated binding energy (−9.5 kcal/mol) revealed strong binding of glabridin with rePON1. Figure 4A shows that glabridin is directed toward the hydrophobic (blue) site of the enzyme. The interactive forces between glabridin and rePON1 are mainly hydrophobic, in accordance with the Trp-fluorescence measurements. In addition, there are hydrogen bonds between the hydroxyl groups of glabridin and the amino group of Lys338 or the carbonyl oxygen of Val336 (Figure 4B).

■ DISCUSSION

PON1 has been shown to be present in atherosclerotic lesions at increasing levels with disease progression.[36] We have recently shown that this enzyme, when incubated with LLE, acts to reduce the LLE-oxidizing potential by affecting the LLE composition.[37] In parallel, incubation of rePON1 with LLE inhibits the enzyme's lactonase and paraoxonase activities. Interestingly, inhibition of rePON1 was not associated with the formation of ketoprotein, which suggests that it was not via the protein's oxidation.[21] The structure of the inhibitor present in the LLE was elucidated as LA-OOH, and the mechanism of inhibition was shown to occur via its specific interaction with

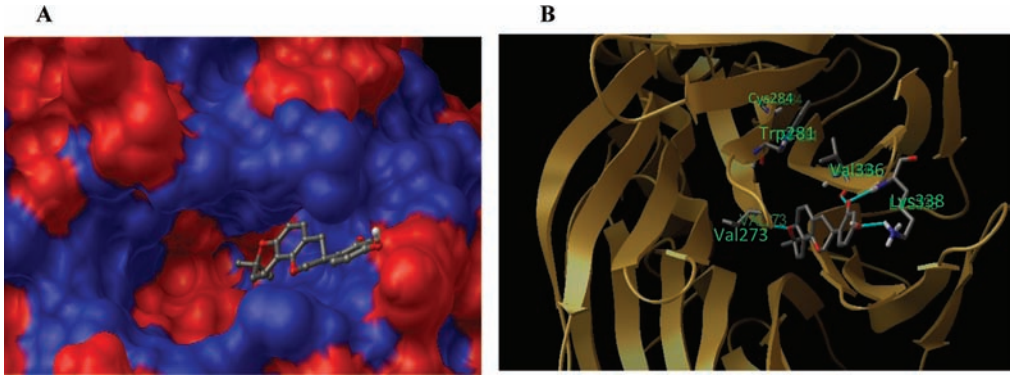

Figure 4. Orientation of glabridin within the potential rePON1 binding site. (A) Molecular surface of rePON1 showing hydrophilic (red) and hydrophobic (blue) zones docked with glabridin. The flavonoid is directed toward the hydrophobic part of the groove. (B) Interactions of glabridin with the enzyme's amino acids in the binding groove (red, oxygen; blue, nitrogen; gray, carbon). Hydrogen bonds are shown in light blue.

rePON1's free Cys at position 284.[21] The beneficial effects of antioxidants in general, and of vitamins C and E in particular, in protecting against coronary heart disease have been widely studied.[38] These antioxidants, with different redox potentials and solubilities, do not protect rePON1 from LLE inhibition.[21] The flavonoids glabridin and punicalagin have also been studied as natural compounds with antiatherogenic potential.[39,40] Unlike the classical, well-known antioxidants which were ineffective at protecting rePON1, glabridin exhibited dose-responsive protection of rePON1, despite its inability to donate electrons to DPPH (Figure 1D). On the basis of a study showing that flavonoids do not act as conventional hydrogen-donating antioxidants but may exert modulatory actions in cells through specific interactions,[10] and on the basis of the results presented here, we hypothesize that the mechanism governing glabridin's protection of rePON1 from LLE inhibition is not related to its antioxidant capacity but rather to its interaction with rePON1. This interaction may affect the enzyme's 3D structure and thus the susceptibility of Cys284 to oxidation by LA-OOH.

Protein−polyphenol associations are well-known,[20,35] and the effects of polyphenol−enzyme complexes formed by interaction of various enzymes with flavonoids on the enzyme's structure and activity have been widely explored.[41,42] In this study, we focused on characterizing the mechanism governing glabridin's protection of rePON1, suggesting that it proceeds via a physical interaction with the enzyme. LC−MS analysis revealed a possible interaction between rePON1 and glabridin, with 2-fold more glabridin binding to rePON1 than to BSA.[29]

Fluorescence spectroscopy is well-suited to determining interactions between small molecules and biomacromolecules, and several studies on Trp-fluorescence quenching of proteins induced by flavonoids or other polyphenols have been reported.[19,20] Changes in the emission spectra of Trp can be seen in response to protein ligand binding or denaturation. rePON1 has four Trp residues, three of which are located at the surface of the protein, while Trp281 is located at a hydrophobic site and is responsible for most of the Trp (and protein) fluorescence.[30,43] Therefore, fluorescence quenching can be used to measure its binding affinities. As demonstrated in Figure 2A, increasing glabridin concentration markedly decreased the intensity of rePON1 fluorescence with no shift in λ_{em}.

To interpret the data from the fluorescence-quenching studies, it is important to understand what type of interaction is occurring between the fluorophore (rePON1) and the quencher (glabridin). As already mentioned, the fluorescence-quenching mechanism usually involves either static or dynamic quenching.[34] In our study, static quenching was demonstrated by the fact that the Stern−Volmer plot did not show any deviation from linearity toward the y axis at the reported glabridin concentrations (Figure 3A) and by the quenching constant (K_q) values, indicating a static quenching process initiated by glabridin. These results suggest that a specific interaction is occurring between rePON1 and glabridin and that the binding parameters can be determined.

K_a, the binding constant, and n, the number of binding sites per rePON1, are associated indicator values. The obtained values for K_a increased with increasing temperature and decreased with increasing pH, with the best association between glabridin and rePON1 appearing to be at 310 K and pH 7. The values obtained for n were not affected by the pH or temperature and were found to be ∼1, indicating the existence of a single binding site for glabridin in rePON1 (Table 1). Thermodynamic parameters were calculated to elucidate the glabridin−PON1 interaction. A negative ΔG was observed, indicating that the interaction is spontaneous. Positive values for ΔH and ΔS indicated that the binding is mainly entropy-driven, and thus, hydrophobic forces play a major role.[20]

Docking calculations can predict the fit of the evaluated ligand within the protein, where the shape is complementary to the binding site. The binding site is considered to be rigid for the protein and flexible for the ligand.[28] AutoDock 4.2 was employed to dock glabridin at a specific groove site on rePON1's surface using the protein's 3D structure. The modeling calculations not only confirmed the demonstrated rePON1−glabridin interaction, but also predicted the specific location of glabridin in the groove site on rePON1. On the basis of the modeling results, it can be inferred that the glabridin-binding site in the enzyme is different from the predicted substrate-binding site.[1,24] Moreover, as can be seen in Figure 4B, most of the direct interactions of rePON1 with glabridin, in agreement with the spectrofluorometric results, are hydrophobic. It can be assumed that the fluorescence quenching of rePON1 originates primarily from a Trp281 residue that is presumably at the glabridin-binding site. This assumption is corroborated by the docking results, which showed that Trp281 lies in close proximity to glabridin with a distance of 5.3−7.1 Å (Figure 4B).

Glabridin protects rePON1's Cys284 thiol group from oxidation by LLE (Figure 1A) or from LA-OOH (Figure 1B) present in the LLE to only a limited degree (22% and 15%,

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respectively). This limited protective effect may be explained by the suggested mechanism of glabridin action. The hydrophobic interaction between glabridin and the enzyme shown here may affect the enzyme's structure and the ability of LA-OOH to get close enough to the thiol group of Cys284 and oxidize it: thus, this ability is decreased but not completely abolished. We assume that the glabridin−protein interaction makes thiol oxidation more difficult due to a steric effect. This assumption still needs to be confirmed. In addition, a PON1 mutant in which Cys284 is replaced by Ser284 is still active, although less so than the wild-type PON1.

In summary, our results suggest that the protective effect of glabridin on rePON1 is not due to its antioxidant activity, but rather to its specific binding with rePON1. Glabridin binds to an allosteric, hydrophobic site of the enzyme, which may change the enzyme's conformation at Cys284 and hamper its oxidation by LA-OOH. Glabridin binding to the enzyme was characterized by two methods: MS and the Trp-fluorescence-quenching technique. Results of both approaches were further evaluated using modeling calculations. PON1 is an antiatherogenic element, part of HDL: as such it is constantly circulating in the blood with the lipoprotein and could come into contact with carotid lesion constituents. To validate glabridin's protective mechanism, additional protein−flavonoid interactions must be evaluated. Such studies would enable correlation of the structure of flavonoids with their ability to interact with PON1 and protect it from the deleterious effects of LA-OOH present in human carotid lesions.

AUTHOR INFORMATION

Corresponding Author

*Phone: 972-4-6955009. Fax: 972-4-6944980. E-mail: solimankh@migal.org.il.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

DPPH, 1,1-diphenyl-2-picrylhydrazyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HDL, high-density lipoprotein; LLE, human carotid lesion lipid extract; LA-OOH, linoleic acid hydroperoxide; OX-LA, Oxidized linoleic acid; PON1, paraoxonase 1; rePON1, recombinant PON1; TBBL, 5-(thiobutyl)-butyrolactone

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