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Biochemical Pharmacology

Biochemical Pharmacology 61 (2001) 1237–1242

Short communication

Structural damage to proteins caused by free radicals: asessment, protection by antioxidants, and influence of protein binding

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Received 11 September 2000; accepted 16 January 2001

Abstract

Oxidative damage to proteins results in biological dysfunctions such as perturbed activity in enzymes, transport proteins, and receptors. Here, we investigated structural damage to proteins induced by free radicals. Structural alterations to lysozyme, human serum albumin (HSA) and β-lactoglobulin A were monitored by capillary zone electrophoresis. Four well-known antioxidants (quercetin, melatonin, Trolox, and chlorogenic acid) were examined for their ability to inhibit protein damage and to bind to these proteins. Melatonin and chlorogenic acid, which did not bind to any of the three proteins under study, showed scavenging and protective activities well correlated with the amount of free radicals generated. Trolox, which bound only to HSA, was a better protector of HSA than of the two other proteins, indicating that its antioxidant capacity is increased by a shielding effect. Finally, quercetin was a good antioxidant in protecting lysozyme and β-lactoglobulin A, but its binding to HSA resulted in a pro-oxidant effect that accelerated HSA fragmentation. These results demonstrate that binding of an antioxidant to a protein may potentiate protection or damage depending on the properties of the antioxidant.

Keywords: Free radicals; Human serum albumin; Protein oxidation; Capillary electrophoresis; Antioxidants; Protein binding

1. Introduction

Oxidative damage to biomolecules is involved in a number of pathologies including the two major causes of death, namely cancer and atherosclerosis. Much interest has focused on lipid and DNA oxidation and their protection by antioxidants, while comparatively little is known about protein oxidative damage and its modification by antioxidants. Oxidative damage to proteins may be of particular importance *in vivo*, since the loss of protein function may affect the activity of enzymes [1], receptors, and membrane transporters, among others [2]. Moreover, oxidatively modified proteins may contain very reactive chemical groups that could contribute to secondary damage to other biomolecules [3,4].

As a result of free radical exposure, many changes can occur in proteins, including amino acid modification, fragmentation, aggregation, changes in absorption and fluorescence spectra [2], decrease or loss of biological function [1], or increase in proteolytic susceptibility [5]. All these modifications can be used as markers of protein damage by free radicals. In this study, structural damage to proteins was assessed by the fragmentation of three proteins (i.e. lysozyme, HSA, and β-lactoglobulin A) monitored by capillary zone electrophoresis [6].

We examined the ability of four reference antioxidants (quercetin, melatonin, Trolox, and chlorogenic acid) to protect these proteins against fragmentation. Equilibrium dialysis was used to assess the binding of the antioxidants to the investigated proteins. The results reveal that protein binding may potentiate protection by a shielding mechanism, or on the contrary potentiate oxidative damage by a pro-oxidant effect.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of analytical grade. Phosphate buffers, KCl, uric acid, melatonin, and chlorogenic acid were

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Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) 2 HCl; and HSA, human serum albumin.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00607-4

purchased from Fluka. Methanol of HPLC grade was obtained from Romil Chemicals. AAPH was from Wako Chemicals. Essentially fatty acid-free HSA (quality A-1887, lot 118F9311), β-lactoglobulin A from bovine milk, lysozyme from chicken egg white, and quercetin were obtained from Sigma Chemical Co. α-Chymotrypsinogen A from bovine pancreas was purchased from Serva Feinbiochemica. Trolox (6-hydroxy-2,5,7,8-tetraethylchroman-2-carboxylic acid) was purchased from Aldrich-Chemie. Mannitol was purchased from Siegfried.

2.2. Instruments

Capillary electrophoresis was performed in a P/ACE system 5510 controlled by the P/ACE™ Station software (Beckman Instruments). A P/ACE UV Absorbance detector was connected at the cathodic end of the capillary. The capillary cartridges were supplied by Beckman and fitted with a 50-μm i.d. fused silica column (eCAP™ Capillary Tubing, Beckman). The aperture window was 100 × 800 μM for UV/VIS detection.

Kinetic studies of AAPH decomposition were performed with a UV/VIS spectrophotometer UVIKON 941 (Kontron Instruments). The cells were maintained at 40 ± 0.2° or 45 ± 0.2° with a Haake D1 water-bath circulator (Digitana).

A UV/VIS scanning spectrophotometer (PU 8730 UV/VIS, Philips Analytical) was used in the binding experiments.

2.3. Measurement of protein oxidation by capillary electrophoresis

Oxidation of proteins induced by AAPH and the assessment of structural damage by capillary electrophoresis were performed as described with full details elsewhere [6]. Briefly, proteins (1 mg mL^{-1}) with antioxidants in various concentrations were incubated for 15 min, at a given temperature, in phosphate buffer (10 mM, pH 7.4 ± 0.1). Protein oxidations were performed in the presence of 10 mM AAPH with or without antioxidants at 40 ± 0.1° for 60 min for lysozyme, at 45 ± 0.1° for 45 min for HSA, and at 45 ± 0.1° for 60 min for β-lactoglobulin A, with continuous shaking under air. It was necessary to use a different set of conditions for each protein, since no single set of conditions existed that would allow degradation of the three proteins at reasonable rates [6]. However, a meaningful comparison between the three assays remains possible when based on the amount of free radicals generated (see Fig. 2 below).

Electrophoresis experiments were performed in a fused silica capillary (50 μm × 47 cm) filled with phosphate buffer 50 mM (pH 3.0 for lysozyme and β-lactoglobulin A, and pH 2.5 for HSA). The temperature of the capillary was kept constant at 30°, and a constant voltage of 15 kV for lysozyme and 20 kV for HSA and β-lactoglobulin A was applied. Detection was carried out by UV/VIS spectrophotometry at 200 nm. α-Chymotrypsinogen A was used as internal standard and percentages of inhibition were calculated by the ratio (peak height of the sample protein/peak height of the internal standard protein) relative to controls, i.e. samples with maximal oxidation (no antioxidant) and with no oxidation (no free radical generator). Each sample analysis was done in triplicate.

2.4. Measurement of protein binding by equilibrium dialysis

Solutions of the antioxidants (20 and 200 μM) and proteins (1 mg mL^{-1}) were prepared in the phosphate buffer used in the oxidation experiments. The antioxidants were diluted from stock solutions in methanol, such that cosolvent concentration after dilution was 1%. All dialysis experiments were performed using an equilibrium dialyser (Diachema) under constant stirring (12 rev min^{-1}) at temperatures equal to those used in the oxidation experiments. The two chambers (half-cells) were separated by a neutral cellulose semi-permeable membrane (Diachema, type 10.14, 5000 MW cut-off). The time required to reach transport equilibrium between the two half-cells was determined for each antioxidant (200 μM) by dialysis against buffer. Samples were analyzed by UV/VIS spectrophotometry. It must be remembered that after dialysis, the equilibrium concentration of the antioxidants was decreased by half, i.e. to 10 or 100 μM.

After dialysis, the unbound concentrations of the antioxidants in the buffer compartment were measured by UV/VIS spectrophotometry and compared to standards, i.e. dialyses where protein solutions had been replaced by buffer. The binding of antioxidants to proteins was calculated as a percentage of the free concentration in the absence of proteins.

3. Results

3.1. Amounts of free radicals produced by AAPH

In order to assess protein oxidation and its protection by antioxidants, it was indispensable to know the amount of free radicals present in each experiment and thus to generate radicals at a constant and quantifiable rate. The kinetics of decomposition of AAPH was determined at 40° and 45° by monitoring the decrease in absorbance at 205 nm. The degradation slopes at 40° and 45° were 6.13×10^{-5} and 1.12×10^{-4}, respectively. In other words, a 5° increase in temperature doubled the degradation slope and thus the rate of radical production, as also observed by Terao and Niki [7]. According to *Niki et al.* [8], the total amount of radical formed in an aqueous phase at pH 7.4 and 37° equals 1.36×10^{-6} [AAPH] × t, where t is time in seconds and [AAPH] is in mol L^{-1}. Since the rate of production doubles for every 5°, the amounts of free radical formed at 40° and 45° in the different assays can be estimated, i.e. the amount of free

Table 1
Antioxidant activities toward lysozyme, HSA, and β-lactoglobulin A fragmentation

Compound	IC_{50} [μM] against structural damage (measured by capillary electrophoresis)[a]		
	Lysozyme[b]	HSA[b]	β-Lactoglobulin A[b]
1 Quercetin	9 ± 3	Pro-oxidant	15 ± 3
2 Melatonin	80 ± 19	90 ± 36	130 ± 42
3 Trolox	31 ± 9	23 ± 7	60 ± 13
4 Chlorogenic acid	10 ± 2	14 ± 5	22 ± 10

[a] IC_{50} represents the concentration inhibiting oxidation by 50%. Values are the average of two experiments.

[b] The total amount of free radicals generated was calculated to be 76 μM (lysozyme assay), 111 μM (HSA assay), and 148 μM (β-lactoglobulin A assay).

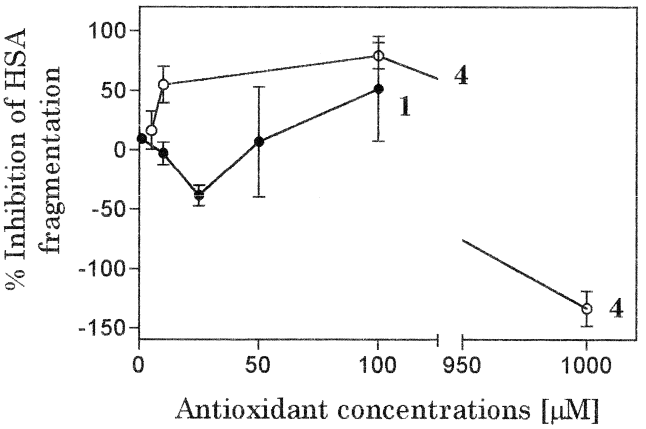

Fig. 1. Pro-oxidant effects induced by quercetin (**1**) and chlorogenic acid (**4**) on HSA fragmentation. Experiments were carried out as described in Materials and Methods. Values are the average of two experiments.

radical generated in lysozyme, β-lactoglobulin A, and HAS oxidation analyses were 76, 148, and 111 μM, respectively.

Complementary investigations indicated that the free radical generator AAPH did not bind to any of the proteins used (100% unbound in all experiments), and thus free radical generation occurred in solution only. Furthermore, should the antioxidants react with AAPH directly and thus affect the rate of radical generation, the AAPH/antioxidant ratios used (around 1000) would render this effect negligible (see, however, the last paragraph in the Discussion concerning the effects of very high concentrations of chlorogenic acid). All the evidence obtained here suggests that the radical species were generated at a constant and quantifiable rate, allowing comparison between the different assays.

3.2. Inhibition by antioxidants of functional damage to proteins and antioxidant binding to proteins

Four reference antioxidants (quercetin, melatonin, Trolox, and chlorogenic acid) were evaluated for their abilities to protect lysozyme, HSA, and β-lactoglobulin A against fragmentation as analyzed by capillary electrophoresis. Table I summarizes antioxidant concentrations inhibiting degradation by 50%. Chlorogenic acid was a very good antioxidant in all systems, except at very high concentrations (see below). Quercetin also showed very good activities, except for HSA fragmentation, where a pro-oxidant effect was detected. Trolox displayed good activities and melatonin was only moderately active. The pro-oxidant effects of quercetin (**1**) and chlorogenic acid (**4**) in HSA fragmentation are seen in Fig. 1 to be concentration-dependent in opposite directions. For quercetin, this pro-oxidant effect was maximal at 25 μM and had disappeared at 50 μM. Chlorogenic acid behaved differently, since it protected HSA up to concentrations of 100 μM and beyond, but was a strong pro-oxidant at very high concentrations (1 mM).

In order to determine whether binding to the target protein influenced the activity of antioxidants, their binding was measured under experimental conditions (pH, temperature, protein concentration) identical to those used in each oxidation assay. The results are expressed in Table 2 as percent of unbound antioxidant. None of the four compounds had any affinity for lysozyme and β-lactoglobulin A. In contrast, quercetin and Trolox showed marked binding to HSA.

4. Discussion

4.1. Nature of free radicals produced by AAPH

Peroxyl radicals are usually considered to be the species responsible for the oxidation of proteins induced by AAPH. Although $HO^{\cdot}$ and carbon-centered radicals may be generated during the breakdown of peroxyl radicals [9,10], preliminary results (data not shown) indicated that mannitol failed to protect HSA even at a very high concentration (100 mM), suggesting that $HO^{\cdot}$ was not significantly involved [11]. Moreover, uric acid was an effective antioxidant (equiactive with Trolox) against HSA fragmentation induced by AAPH. Since uric acid is known to be a poor scavenger of carbon-centered radical [12], it can be assumed that the free radicals responsible for protein damage in our assays were peroxyl radicals.

4.2. Inhibition by antioxidants of functional damage to proteins and antioxidant binding to proteins

To detect how protein binding might affect protein degradation, it was necessary to compare the three assays despite the different sets of conditions used. This was possible based on the amounts of free radicals generated. Fig. 2 indicates that, when no protein binding had occurred as was the case for lysozyme and β-lactoglobulin, the increase in IC_{50} values between the lysozyme and β-lactoglobulin as-

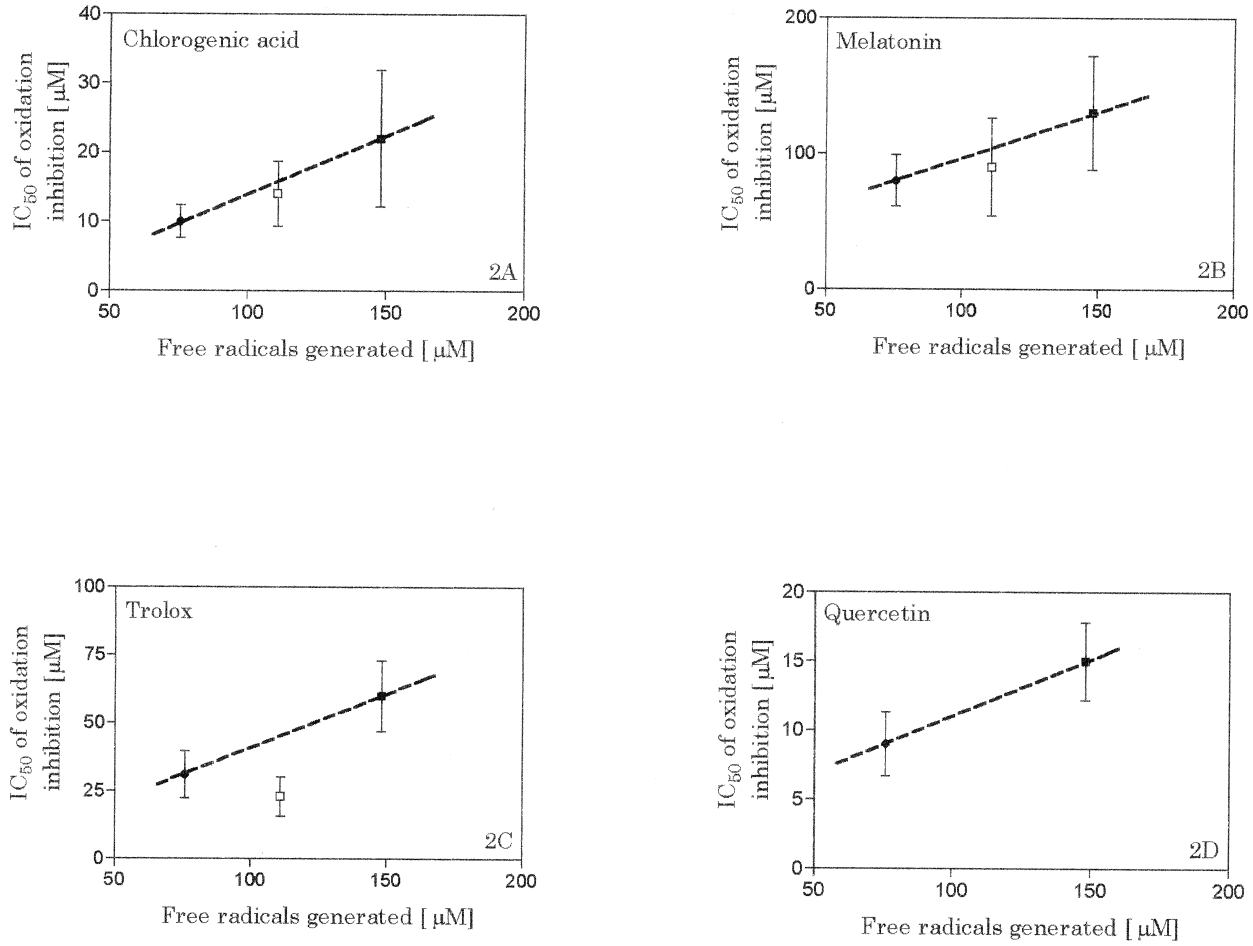

Fig. 2. IC_{50} as a function of free radicals present in the three fragmentation assays, namely 76 μM in the lysozyme assay (●), 111 μM in the HSA assay (□), and 148 μM in the β-lactoglobulin A assay (■). The data and error bars are from Table 1. The lines join the lysozyme and β-lactoglobulin A assays where protein binding never occurred.

says (where free radical production was 76 and 148 μM, respectively) was practically the same for the four antioxidants, namely a factor of 1.9 ± 0.3. As shown in Fig. 2, A and B, the antioxidant activities of chlorogenic acid and melatonin, which did not bind to any of the three proteins, were well correlated with the amounts of free radicals liberated in the three assays. These two antioxidants appear to act solely by a scavenging effect, the importance of which depends only on their structure and on the amounts of free radicals in solution.

As for Trolox (Fig. 2C), its protective activity against HSA fragmentation was higher (i.e. lower IC_{50}) than expected from its protective activity against lysozyme and β-lactoglobulin A fragmentation. Since this antioxidant bound to HSA but not to the other two proteins, its higher than expected efficacy suggests that binding (about 20% bound at the IC_{50}) potentiates the protection of HSA against fragmentation. This is postulated to involve a shielding effect in addition to radical scavenging in solution.

Quercetin is expected to be a very effective scavenger due to its catechol and phenol functions and its low redox potential [13]. Indeed, previous results have demonstrated its high scavenging activity toward peroxyl radicals [14]. Here, a good protection is seen against lysozyme and β-lactoglobulin A fragmentation (Fig. 2D). Quercetin also exhibited a high-binding affinity toward HSA (about 50% at the IC_{50}), and an excellent protection of HSA was therefore expected. However, a pro-oxidant effect was seen such that the fragmentation of HSA was potentiated. Our results again show the ambivalent behavior of phenols having a very low redox potential. Like other phenols, they act as radical scavengers due to their capacity to donate a H-atom to peroxyl radicals. The reaction transforms the phenol into a phenoxyl radical which, when resonance-stabilized, does not propagate the chain reaction. However, some phenoxyl radicals, especially those originating from phenols with a very low redox potential, can be very reactive toward biomolecules [15]. Indeed, the difference in radical stabilization could explain the difference observed between Trolox and quercetin. Another pro-oxidant mechanism involves generation of superoxide and hydrogen peroxide, followed by a Fenton reaction in the presence of transition metal ions

Table 2
Binding of antioxidants (at a final concentration of 10 or 100 μM) to lysozyme, HSA, and β-lactoglobulin A[a]

Compound	Lysozyme[b]		HSA[b]		β-Lactoglobulin[b]	
	100 μM	10 μM	100 μM	10 μM	100 μM	10 μM
1 Quercetin	ca. 100[c]	ca. 100[c]	71 ± 3	56 ± 4	ca. 100[c]	ca. 100[c]
2 Melatonin	100 ± 4	100 ± 11	97 ± 2	92 ± 6	99 ± 0.1	93 ± 2
3 Trolox	97 ± 7	98 ± 3	94 ± 2	78 ± 4	91 ± 0.4	96 ± 9
4 Chlorogenic acid	97 ± 0.4	97 ± 3	98 ± 0.7	95 ± 2	93 ± 1	98 ± 1

[a] Values are expressed as unbound fraction in % ± SD (N = 3).
[b] Protein concentration was 1 mg mL^{-1}.
[c] Difference from control too small to be quantifiable.

to produce hydroxyl radicals [16]. Quercetin in the presence of Cu^{2+} indeed behaves as a pro-oxidant [17,18]. This mechanism seems excluded here toward lysozyme and β-lactoglobulin A, since no pro-oxidant effect was detected. In contrast, HSA has the physiological ability to bind and transport copper ions, and it is likely that commercial albumin retains trace amounts of Cu^{2+}. The binding of quercetin to HSA and the generation of hydroxyl radicals in the close proximity of the protein thus appears as another likely mechanism to explain the pro-oxidant behavior toward HSA.

A finding that remains unexplained is the pro-oxidant effect of chlorogenic acid at very high concentrations (1 mM) toward HSA. This is a fortuitous observation of little or no relevance to the objective of this study, but it is an intriguing one. Chlorogenic acid does not bind to HSA (Table 2), but its anionic nature and high water solubility suggests that it may form ion-pairs with the protonated AAPH at high concentrations, perhaps accelerating the generation of radicals by AAPH. A specific study would be needed to examine whether the same pro-oxidant effect occurs with other proteins and to understand its mechanism.

4.3. Conclusion

It appears from this work that binding between an antioxidant and a target protein is an important factor to take into account when predicting antioxidant capacity from scavenging capacity and redox potential. As a general observation, the activity of antioxidants in the absence of protein binding is correlated to the number of free radicals generated, and appears to result only from a scavenging effect. The binding of an antioxidant to a target protein can either enhance its protection by a shielding effect, as seen for Trolox, or on the contrary potentiate pro-oxidant effects.

These opposite effects add a new dimension to the already complex interactions between antioxidants and proteins. Given the many deleterious consequences of oxidative damage to proteins, the *in vitro* and *in vivo* consequences of such interactions should be better understood.

Acknowledgments

The authors thank Drs. F. Brée and R. Zini of the Department of Pharmacology, University of Paris XII, for their helpful advice in protein oxidation techniques.

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