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Antioxidant reactivity toward nitroxide probes anchored into human serum albumin. A new model for studying antioxidant repairing capacity of protein radicals

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ABSTRACT

A new strategy to evaluate accessibility of antioxidants to radical proteins has been developed using nitroxide prefluorescent probes anchored into human serum albumin (HSA). Binding association constants for the nitroxide probes $C_{343}T$ and QT with HSA were 5×10^4 and 9×10^4 M^{-1}, respectively. Rate constants for the nitroxide reduction by antioxidants in HSA were determined finding k_{HSA}/k_{buffer} ratio of 0.8, 1.9, and 0.075 for ascorbic acid, Trolox, and caffeic acid, respectively, for the nitroxide $C_{343}T$ reduction.

Free radical damage to proteins can be extensive and more difficult to prevent than lipoperoxidation processes.[1] Protein oxidation involves complex mechanisms, such as propagation of the initial radical attacking by chain reactions and radical migration between amino acid residues within the protein structure.[2] Furthermore, free radicals species generated by decomposition of peroxides/hydroperoxides, derived from tryptophan and tyrosine residues oxidation, may extend the initial damage to more remote sites.[3] It has also been proposed that the accumulation of hydroperoxides could contribute to cellular dysfunction and alter redox signaling.[4] In this context, the ability of antioxidants to reduce peroxide/hydroperoxide products in proteins, or even prevent their formation, is of main importance in biological processes. In spite of the numerous studies on the evaluation of antioxidant capability in homogeneous media, there is little knowledge on the ability of antioxidants to access, repair and/or trap secondary free radical in proteins.[5] Most of the studies have been focused on the quantification of peroxides/hydroperoxides reduction by antioxidants.[6] However, at our best knowledge; there is no kinetic information regarding the accessibility of antioxidants to a hidden locus in the protein where secondary damage could have migrated.

Herein, we report a new strategy based on antioxidant mediated reduction of nitroxide prefluorescent probes anchored into human serum albumin (HSA) in order to mimic the antioxidant reaction with protein radicals. This approach takes advantage on the ability of nitroxide prefluorescent probes to monitor nitroxide reduction by following the increase in the chromophore fluorescence intensity due to the suppression of the intramolecular quenching of the nitroxide moiety.[7]

We explored the possibility of anchoring nitroxide probes by binding in HSA, a well known fatty acid transport protein present in blood plasma. HSA has the property of binding an extraordinary variety of drugs in two different binding sites, denominated as site I and II.[8] Thus, specific binding of the probes in those sites would permit easily to localize a nitroxide free radical in hydrophobic environments in HSA. This strategy would mimic a free radical located in the protein structure. In order to explore this possibility we used different methodologies to determine the nitroxide probes association in HSA.

The nitroxide probes QT and $C_{343}T$ (Fig. 1) were selected due to the high sensitivity of the photophysic properties of their chromophores to pH and polarity of the medium, respectively.[9] Thus, the association of probes to HSA can be monitored by the change on the absorbance and fluorescence spectra with HSA concentration (Fig. 2). In particular, the absorbance spectrum of QT in

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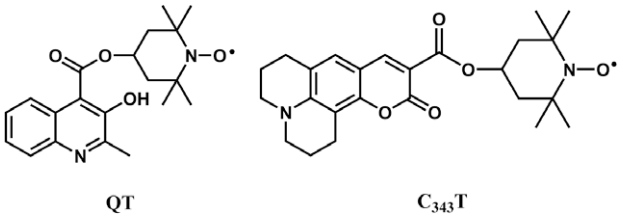

Figure 1. Nitroxide prefluorescent probes QT and $C_{343}T$.

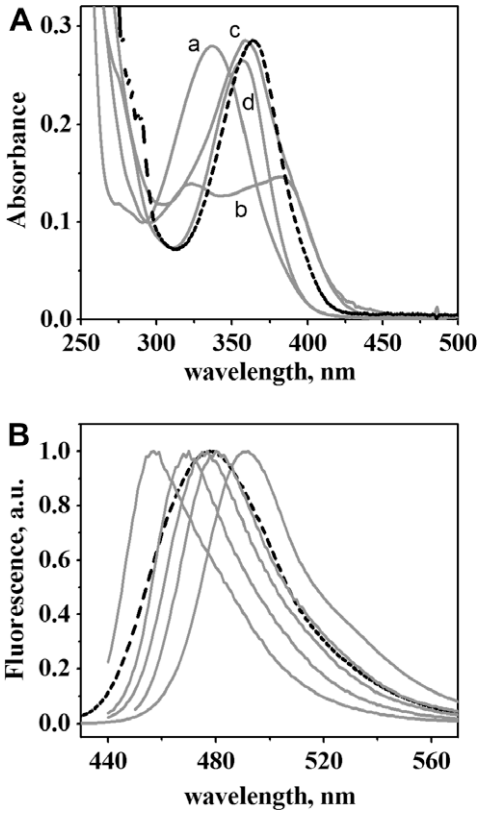

Figure 2. (A) Absorption spectra of 30 μM QT in aqueous solution at different pH: 1.5 (a); 5.6 (b); 7.0 (c) and 9.8 (d); (B) normalized fluorescence of 2.5 μM $C_{343}T$ in benzene, dichloromethane, acetonitrile, methanol, and phosphate buffer pH 7 (from left to right). The broken lines correspond to samples in 70 μM HSA in Pi buffer (50 mM, pH 7.0).

presence of HSA showed a decrease on the neutral and zwitterionic species present at pH 7.0 due to its association to HSA (Fig. 2A). On the other hand, the addition of HSA to $C_{343}T$ in buffer induced a hypsochromic shift of the absorbance and fluorescence spectra indicating a relative hydrophobic environment for $C_{343}T$ in the HSA.

An association constant for $C_{343}T$ of $4.8 \times 10^4\ M^{-1}$ was determined by employing the change on the absorbance of the probes with the concentration of HSA, according to the Benesi–Hildebrand method.[10] An accurate binding constant for QT was not possible to obtain using this methodology due to the little difference on the QT absorbance in HSA and buffer at pH 7.0. Alternatively, we evaluated association constants based on the effective tryptophan (W214) residue fluorescence quenching of HSA by the nitroxide probes (Fig. 3).[11] This method allowed to determine association constant values of $5.0 \times 10^4\ M^{-1}$ and $9.0 \times 10^4\ M^{-1}$ for $C_{343}T$ and QT, respectively. Interestingly, Stern–Volmer plots show a very efficient fluorescence quenching for both nitroxide probes in a concentration range where Tempol did not show any appreciable

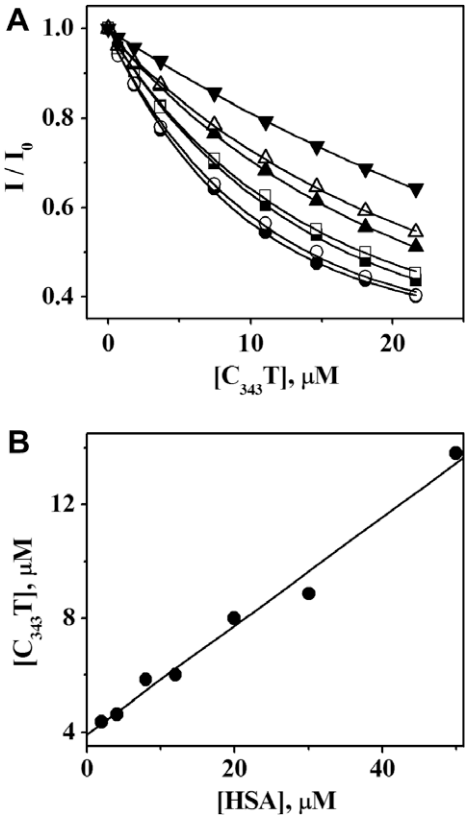

Figure 3. (A) Quenching of the HSA-tryptophan fluorescence elicited by $C_{343}T$ at different HSA concentrations: (●) 2, (○) 4, (■) 8, (□) 12, (▲) 20, (Δ) 30, (▼) 50 μM. (B) Plot of the $C_{343}T$ concentration required to produce $I/I^o = 0.75$ as a function of the HSA concentration. λ_{exc} 290 nm; λ_{em} 340 nm.

quenching. This result suggests a location of both nitroxide probes close to W214 residue located in site I in HSA.[8]

In particular, the association for both nitroxide probes and therefore their location in HSA would be mainly conducted by the structural properties of the chromophore moiety. In fact, similar absorbance and fluorescence spectra were recorded for the isolated chromophores (quinoline methyl ester, Q, and the coumarin methyl ester, C_{314}) in presence of HSA. This was confirmed by the analysis of the fluorescence lifetime distribution by using the isolated chromophore of $C_{343}T$. A new distribution centered at 0.48 ns was observed in presence of HSA in addition to the distribution corresponding to the chromophore in buffer at 3.7 ns (see Supplementary data). This result would indicate that the association of this probe occurs only in one site of the protein. In order to evaluate binding to site I or II, displacement experiments employing dansyl amide and dansyl sarcosine were carried out. An effective displacement of $C_{343}T$ was observed only for dansyl amide suggesting a location of this probe in site I (Supplementary data). Similar experiments were not successful for QT due to the similar absorbance and fluorescence range to the dansyl probes. However, docking calculations showed a similar location of both nitroxide probes, QT and $C_{343}T$, with the nitroxide moiety interacting with the tryptophan residue in the hydrophobic site I (Fig. 4).[12]

Large values of the association constant of nitroxide probes in one specific site of HSA would guarantee an adequate location of nitroxides in a hidden locus in the protein. Furthermore, the properties of the nitroxide radical make it a good model of peroxyl radicals, thus, mimicking a radical protein.[7]

Apparent rate constant values for the nitroxide reduction by antioxidants (Eq. 1) in presence and absence of HSA could permit

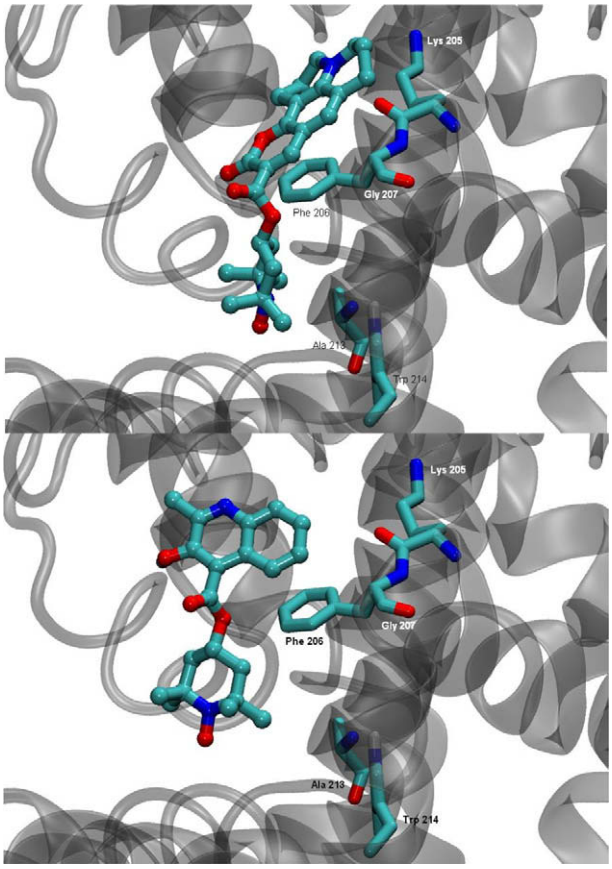

Figure 4. Docking model of the $C_{343}T$ (top) and anionic form of QT (bottom) in HSA. Interacting amino acid residues with the probes are labeled.

to evaluate the accessibility of antioxidants toward nitroxide radicals bound to HSA.

$$RNO^{\cdot} + PhOH \rightarrow RNOH + PhO^{\cdot} \quad (1)$$

It is important to note, that reduction of nitroxide by the antioxidant can take place from hydrogen or electron transfer reaction depending on the antioxidant.[7] However, independent on the reaction mechanism involved, reduction of the nitroxide moiety of the prefluorescent probes increase the fluorescence intensity allowing to determine the rate reaction constant of the process in conditions of pseudo first order employing an excess of the antioxidants (Fig. 5).[7,13]

The differences observed between rate constant values in phosphate buffer and the apparent rate constant in HSA can be interpreted in terms of the partition of nitroxide probes and/or accessibility of the antioxidants to the nitroxide bound to HSA (Table 1). It is noteworthy that the addition of antioxidants does not modify the absorbance or fluorescence of the probes, suggesting that the antioxidants do not alter the nitroxide association to HSA.

Ascorbic acid, the most reactive antioxidant toward nitroxide radical, showed a lower apparent rate constant (k_{app}) in HSA than rate constant in buffer (k_{buffer}). This result would indicate a lower accessibility of the antioxidant toward a nitroxide located in a hydrophobic environment. In contrast, Trolox showed a larger k_{app} than k_{buffer} suggesting also a role of the environment on the reaction in HSA (vide infra). In this context, it is important to mention that a pseudophase model cannot be applied for the antioxidant in HSA.

A simple kinetic analysis considering the partition of the nitroxide probe into the HSA and buffer is shown in Eq. 2.

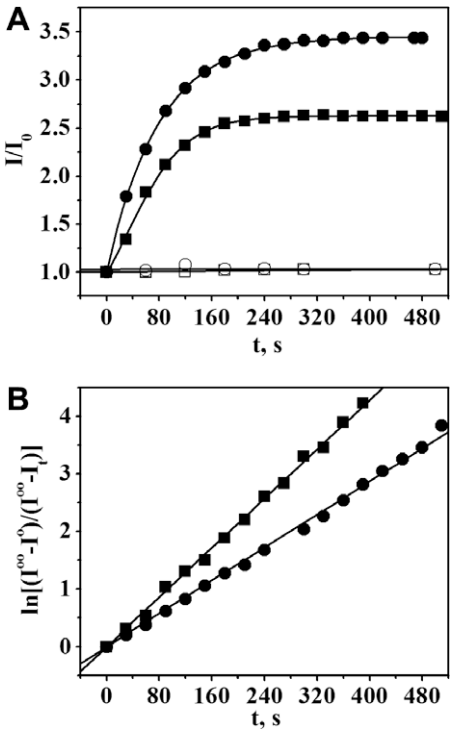

Figure 5. (A) $C_{343}T/C_{343}TH$ fluorescence profile evaluated at 490 nm in buffer (■) and in HSA (●) after ascorbic acid addition; and in the absence of ascorbic acid in buffer (○) and HSA (□) with time (λ_{exc} 450 nm). (B) Fluorescence kinetic adjusted. Experimental conditions: 2.5 μM $C_{343}T$, 1 mM ascorbic acid, 70 μM HSA, 50 mM phosphate buffer, pH 7.0.

Table 1
Rate constant values for the reaction of nitroxide prefluorescent probes with antioxidants in buffer and HSA

Nitroxide	Antioxidant	k_{Buffer}[a,b] (M^{-1} s^{-1})	$k_{HSA,\ app}$[a,b] (M^{-1} s^{-1})	k_{HSA} (M^{-1} s^{-1})
$C_{343}T$	Ascorbic acid	13.8 (0.6)	8.8 (0.6)	11.1
	Trolox	0.21 (0.02)	0.35 (0.04)	0.39
	Caffeic acid	0.04[c]	0.011	0.003
QT	Ascorbic acid	13.7 (0.9)	10.9 (0.7)	10.4
	Trolox	0.21 (0.02)	0.31 (0.01)	0.33
	Caffeic acid	0.04[c]	0.009	0.003

[a] Values are means of three experiments, standard deviation is given in parentheses.
[b] Experimental conditions: nitroxide concentrations were 10 μM QT and 2.5 μM $C_{343}T$; and 70 μM HSA. Antioxidant concentration range: 1–10 mM. All reactions were carried out in 50 mM phosphate buffer, pH 7.0.
[c] Ref. 7c.

$$k_{app} = (k_{Buffer} \cdot X_{H_2O} + k_{HSA} \cdot X_{HSA}) \quad (2)$$

where X is the molar fraction of the nitroxide probe in buffer and HSA. Thus, considering the association constant of QT and $C_{343}T$ and the rate constant in buffer is possible to estimate a rate constant value for the nitroxide probe in HSA, k_{HSA} (Table 1).

Rate constant values for the reaction in HSA (k_{HSA}) show a similar trend that buffer (k_{buffer}). Ascorbic acid was the most reactive antioxidant toward nitroxide both in buffer and HSA. However, the ratio between the rate constant in buffer and HSA clearly allows observing an impediment of ascorbic acid to react with nitroxide probes QT and $C_{343}T$ in protein (k_{HSA}/k_{buffer} of 0.8 and 0.76 for $C_{343}T$ and QT, respectively). Similar behavior was observed for caffeic acid with a ratio of 0.075 for both nitroxide probes. Interestingly, larger rate constant values were observed for Trolox in HSA with k_{HSA}/k_{buffer} of 1.9 and 1.6 for $C_{343}T$ and QT, respectively. The values for Trolox would suggest that also environmental factors,

such hydrophobicity, have to be considered for the reaction. In fact, the value k_{HSA} is very close to the reaction for α-tocopherol in apolar solvents as hexane ($0.33\ M^{-1}\ s^{-1}$) according to the increment on the rate reaction in non hydrogen bond solvents.[7]

In summary, a model employing nitroxide probes associated into HSA could be proposed as a new strategy to study antioxidant efficiency. Our results show that the antioxidant accessibility and environment parameters are factors that should be considered in the antioxidant repairing ability of protein radicals.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.09.070.

References and notes

1. (a) Richards, D. M.; Dean, R. T.; Jessup, W. *Biochim. Biophys. Acta* **1988**, *946*, 281; (b) Davies, M. J. *Biochim. Biophys. Acta* **2005**, *1703*, 93.
2. (a) Hawkins, C. L.; Davies, M. J. *Biochim. Biophys. Acta* **2001**, *1504*, 196; (b) Davies, M. J. *Biochim. Biophys. Acta* **2005**, *1703*, 93.
3. (a) Silvester, J. A.; Timmins, G. S.; Davies, M. J. *Arch. Biochem. Biophys.* **1998**, *350*, 249; (b) Wright, A.; Budd, W. A.; Hawkins, C. L.; Davies, M. J. *Photochem. Photobiol.* **2002**, *76*, 35.
4. (a) Gracanin, M.; Davies, M. J. *Free Radical Biol. Med.* **2007**, *42*, 1543; (b) Linton, S.; Davies, M. J.; Dean, R. T. *Exp. Gerontol.* **2001**, *36*, 1503.
5. Morgan, P. E.; Dean, R. T.; Davies, M. J. J. *Free Radical Biol. Med.* **2004**, *36*, 484.
6. (a) Filipe, P.; Morliére, P.; Patterson, L. K.; Hug, G. L.; Maziére, J. C.; Maziére, C.; Freitas, J. P.; Fernandes, A.; Santus, R. *Biochim. Biophys. Acta* **2002**, *1572*, 150; (b) Salvi, A.; Carrupt, P. A.; Tillement, J. P.; Testa, B. *Biochem. Pharmacol.* **2001**, *61*, 1237.
7. (a) Aliaga, C.; Aspée, A.; Scaiano, J. C. *Org. Lett.* **2003**, *5*, 4145; (b) Aspée, A.; Aliaga, C.; Scaiano, J. C. *Photochem. Photobiol.* **2007**, *83*, 481; (c) Aliaga, C.; Juárez-Ruíz, J.; Scaiano, J. C.; Aspée, A. *Org. Lett.* **2008**, *10*, 2147.
8. (a) Artarli, R.; Bombieri, G.; Calaba, L.; Del Pra, A. *Il Fármaco* **2005**, *60*, 485; (b) Sudlow, G.; Birkett, D. J.; Wade, D. N. *Mol. Pharmacol.* **1975**, *11*, 824.
9. Bueno, C.; Mikelsons, L.; Maretti, L.; Scaiano, J. C.; Aspée, A. *Photochem. Photobiol.* **2008**, *84*, 1535.
10. Abuin, E.; Aspée, A.; Lissi, E.; León, L. *J. Chil. Chem. Soc.* **2007**, *52*, 1088.
11. Abuin, E.; Calderón, C.; Lissi, E. *J. Photochem. Photobiol., A.* **2008**, *195*, 295.
12. Docking simulation in HSA structure was done in AutoDock4.3 'GaussView: Version 3.09' (see Supplementary data for details).
13. Observed rate constants were determinate in pseudo first order employing an excess of antioxidant, according to:

$$\ln\left(\frac{I^{\infty}-I^{o}}{I^{\infty}-I^{t}}\right)=k_{obs}t$$

where I^{∞}, I^{o}, and I^{t} represent the fluorescence intensities in the plateau region, initial and at time "t", respectively. Rate constants values were obtained from the slope of k_{obs} versus the initial concentration of the antioxidant. Values for the reaction in HSA are defined as apparent rate constants when is not consider the partition of the probe in HSA.