Free Rad. Res. Comms., Vol. 14, Nos. 5–6, pp. 373–384
Reprints available directly from the publisher
Photocopying permitted by license only

© 1991 Harwood Academic Publishers GmbH
Printed in the United Kingdom

EFFECT OF ADDITIVES ON THE INACTIVATION OF LYSOZYME MEDIATED BY FREE RADICALS PRODUCED IN THE THERMOLYSIS OF 2,2′-AZO-BIS-(2-AMIDINOPROPANE)

E.A. LISSI, M. FAURE and N. CLAVERO

Departamento de Quimica, Facultad de Ciencias, Universidad de Santiago de Chile, Santiago, Chile

(Received December 23, 1990; in revised form February 11, 1991)

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

The inactivation of lysozyme caused by the radicals produced by thermolysis of 2,2′-azo-bis-2-amidino-propane can be prevented by the addition of different compounds that can react with the damaging free radicals. Compounds of high reactivity (propyl gallate, Trolox, cysteine, albumin, ascorbate, and NADH) afford almost total protection until their consumption, resulting in well-defined induction times. The number of radicals trapped by each additive molecule consumed ranges from 3 (propyl gallate) to 0.12 (cysteine). This last value is indicative of chain oxidation of the inhibitor. Uric acid is able to trap nearly 2.2 radicals per added molecule, but even at large (200 μM) concentrations, a residual inactivation of the enzyme is observed, which may be caused by urate-derived radicals.

Compounds of lower reactivity (tryptophan, Tempol, hydroquinone, desferrioxamine, diethylhydroxyl-amine, methionine, histidine, NAD^+ and tyrosine) only partially decrease the lysozyme inactivation rates. For these compounds, we calculated the concentration necessary to reduce the enzyme inactivation rate to one half of that observed in the absence of additives. These concentrations range from 9 μM (tryptophan and Tempol) to 5 mM (NAD^+).

KEY WORDS: lysozyme, inactivation; free radical, lysozyme inactivation; radical scavengers, lyzozyme inactivation; DFO; NADH; albumin.

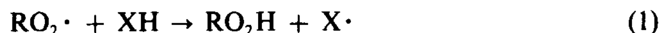
INTRODUCTION

The relevance of protein damage as one of the early events associated with oxidative stress is now widely recognized.[1] Several papers have pointed out that protein modification frequently precedes,[2–4] and is more difficult to inhibit,[4–6] than lipid peroxidation, the classical process associated with free radical toxicity in biological systems.[7] In spite of the possibility that proteins could interact with hydroxyl derived and/or chain carrying lipid peroxyl radicals, there are very few studies on the capacity of peroxyl radicals to inactivate enzymes,[4,5,8–15] and how the damage can be prevented and/or repaired.[5,10–15] In previous papers, we have shown that peroxyl radicals produced by the thermolysis of 2,2′-azo-bis-2-amidino-propane (ABAP) efficiently inactivate lysozyme,[11] horseradish peroxidase,[13] and glucose oxidase,[13] and that the inactivation can be decreased or totally supressed by addition of tryptophan or propyl gallate.[11]

†Correspondence to: Dr. Eduardo Lissi, Departamento de Química, Facultad de Ciencias, Universidad de Santiago de Chile, Casilla 307, Correo 2, Santiago, Chile. FAX: 56-2-6812108.

RIGHTSLINK

In order to be effective in the protection of an enzyme (EnzH), an additive (XH) must either react with the peroxyl radicals

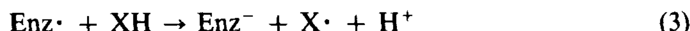


$$RO_2\cdot + XH \rightarrow RO_2H + X\cdot \qquad (1)$$

generating one or more radicals (X·) unable to react with critical sites of the enzyme,[14] and/or be able to repair the damage produced by the peroxyl radicals through a hydrogen atom

$$Enz\cdot + XH \rightarrow EnzH + X\cdot \qquad (2)$$

or electron



$$Enz\cdot + XH \rightarrow Enz^- + X\cdot + H^+ \qquad (3)$$

transfer followed by protonation of the enzyme anion.

In the present paper we present results regarding the efficiency of several molecules of biological relevance as inhibitors of lysozyme inactivation mediated by the radicals produced by thermolysis of ABAP in air saturated aqueous solutions. This enzyme is particularly sensitive to peroxyl radicals[15] and, related to its lack of cysteine groups, the damage is related to extensive tryptophan modification.[11]

MATERIALS AND METHODS

NAD^+, propyl gallate, uric acid, GSH, β-NADH, albumin (bovine), L-cysteine, L-tyrosine and Micrococcus lysodeikticus, lyophilized (Sigma, St. Louis, MO); Trolox, Tempol, hydroquinone and DL-methionine (Aldrich Chemical Co.); ascorbic acid, DL-tryptophan and L-histidine (Fluka); 2,2′-azo-bis-2-amidinopropane (Polysciences, Warrington, PA) and desferrioxamine (Ciba-Geigy) were used without further purification. Diethylhydroxylamine (Aldrich Chemical Co.) was vacuum-distilled prior to its use.

Lysozyme Grade I (Sigma) was employed as received. Its activity was measured by following the loss of turbidity when added to suspensions of lyophilized Micrococcus lysodeikticus. Measurements were carried out in a Shimadzu UV-160 spectrophotometer at 436 nm, and the initial values of $-dA/dt$ (A = absorbance) were taken as a measure of the enzyme activity. In the experimental conditions employed, the activity measured was proportional to the lysozyme concentration.

Air saturated solutions of the enzyme (3.4 μM) were incubated at 45°C in PBS buffer (0.07 M sodium phosphate, 0.017 M NaCl, pH = 6.5) with 10 mM ABAP. Aliquots were withdrawn at different times and the remaining enzymatic activity measured.

RESULTS

Thermolysis of ABAP has been employed as a controlled source of alkylperoxyl radicals both in lipid peroxidation[16-18] and enzyme inactivation studies.[11,13]

Inactivation of lysozyme by the free radicals produced in the thermolysis of ABAP under aerobic conditions can be decreased by addition of different types of compounds. However, the changes in lysozyme activity with incubation time show different profiles for different additives. In principle, the data obtained in the present

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

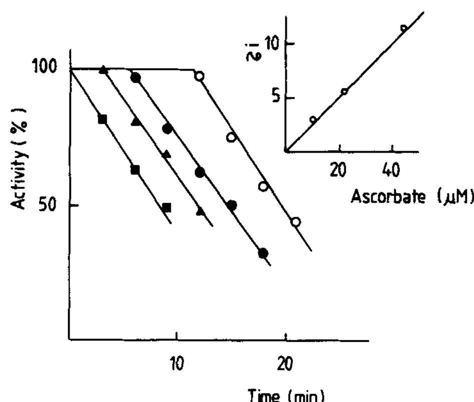


FIGURE 1 Lysozyme activity remaining after incubation in the presence of ABAP (10 mM) with (▲) 11 μM, (●) 22 μM or (○) 44 μM added ascorbic acid. (■) No ascorbic acid added. Insert shows the induction times as a function of the initial ascorbic acid concentration.

work conform to four types of such profiles, and they are shown in Figures 1 and 4 for ascorbate, desferrioxamine (DFO), uric acid and NADH.

The first type of behaviour (Figure 1) is that expected for "ideal" inhibitors, i.e., compounds that either react much faster with the radicals than the enzyme substrate, or that, at low concentrations, are able to quantitatively repair the induced damage. For these compounds, the only parameter that can be obtained from the present data is the number of radicals scavenged (n) by each added inhibitor molecule. The observed induction times (τ_i) are related to n through

$$\tau_i = n[\mathrm{XH}]/\mathrm{R}_r \qquad (4)$$

where [XH] is the initial inhibitor concentration, and R_r is the rate of radical input. Plots of τ_i against [XH] (insert, Figure 1) allow the evaluation of n/R_r. R_r can be evaluated by employing a reference inhibitor whose n value is known. By employing Trolox as reference with $n_{\mathrm{Trolox}} = 2$ (reference 19), a value of R_r of $4.1 \times 10^{-8}\ \mathrm{M\,l^{-1}\,s^{-1}}$ is obtained, in agreement with data from other laboratories.[19] the values of n obtained by this procedure are given in Table I.

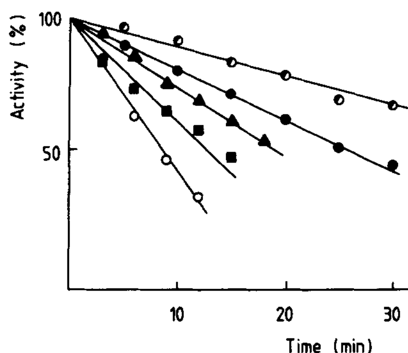


FIGURE 2 Lysozyme activity remaining after incubation in the presence of ABAP (10 mM) with (■) 19 μM, (▲) 38 μM, (●) 76 μM or (◐) 152 μM added DFO. (○) No DFO added.

RIGHTSLINK

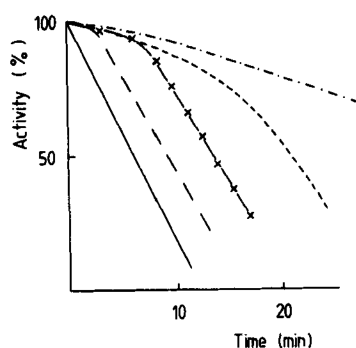


FIGURE 3 Lysozyme activity remaining after incubation in the presence of ABAP (10 mM) with (– – –) 3 μM, (-x-x-) 10 μM, (----) 20 μM or (-·-·-) 40 μM added uric acid. (——) No uric acid added.

TABLE I
Number of Trapped Radicals per Molecule of Additive

Additive	n
Propyl gallate	3.0 ± 0.3
Urate	2.2 ± 0.4
Trolox	2.0 (a)
GSH	0.70 ± 0.05
NADH	0.6 ± 0.2
Ascorbate	0.55 ± 0.05
Albumin	0.27 ± 0.02; 0.6 ± 0.02 (b)
Cysteine	0.12 ± 0.03

Errors given as standard deviations ($n \geq 3$).
(a) Taken as a reference value.
(b) n values decrease when the albumin concentration increases. The values given correspond to albumin concentrations of 50 and 5 μM, respectively.

The second type of behaviour (Figure 2) corresponds to additives that are less reactive towards the radicals than the enzyme and/or are poor repairers of the initial damage. In these systems, the efficiency of an additive in protecting the enzyme can be characterized by its $Q_{1/2}$ value, defined as the additive concentration needed to decrease the inactivation rate to one half the values observed without additives (Table II). These values can be employed only to estimate relative protection capacities since their absolute values, even at a given temperature, are expected to increase when the ABAP and lysozyme concentrations increase. These considerations are supported by results obtained employing tryptophan as additive that show, at 10 mM ABAP, $Q_{1/2}$ values of 23 μM, 12 μM and 7 μM for lysozyme concentrations of 10.2 μM, 6.8 μM and 1 μM, respectively.

If a simple mechanism in which the alkylperoxyl radicals react with themselves, the enzyme or the added substrate is considered, the inactivation rate in the absence of additive (Ro) and that observed in its presence (R) are related to the additive concentration [SH] by

$$Ro/(Ro - R) = \{1/(1 - f)\} + k/[XH] \quad (5)$$

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

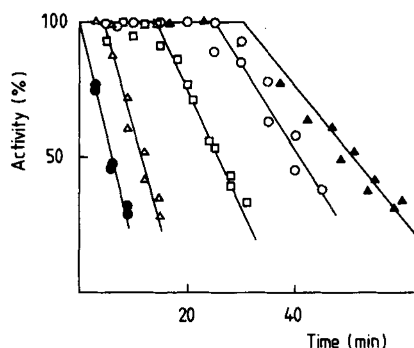


FIGURE 4 Lysozyme activity remaining after incubation in the presence of ABAP (10 mM) with (△) 14.1 μM, (□) 63.5 μM, (○) 141 μM or (▲) 200 μM added NADH. (●) No NADH added.

TABLE II
$Q_{1/2}$ values for different additives[a]

Additive	$Q_{1/2}$ (μM)	f
Tryptophan	9 ± 1	< 0.1
Tempol	9 ± 4	< 0.1
Albumin	10 ± 3(b)	≈ 0.1
Hydroquinone	10 ± 1	< 0.1
DFO	30 ± 5	< 0.1
Methionine	35 ± 6	< 0.1
Diethylhydroxylamine	45 ± 10	< 0.1
NADH	70 ± 6 (b)	–
Tyrosine	100 (c)	0.1
Histidine	1000 (c)	0.15
NAD^+	5000 (c)	0.23

(a) Obtained from eqn. (5) and taking $Q_{1/2} = k$. Errors given are standard deviations ($n \geqslant 3$).
(b) Measured after the induction time.
(c) Value obtained by plotting (Ro − R)/Ro against the additive concentration and interpolating to (Ro − R)/Ro equal to 0.5. Estimated error: ±20%.

where f is the ratio between the inactivation efficiency of the X· radicals and the $RO_2\cdot$ radicals. If a repair mechanism were operative, a similar equation applies with the factor f being the fraction of damage that cannot be repaired by the additive. If the radical X· produced is unable to attack the enzyme ($f = 0$), a reciprocal plot of the relative inactivation rate must extrapolate to one. This type of plot is shown in Figure 5. Plots of the left hand side of eqn. (5) against the reciprocal of additive concentration are nearly linear (Figure 5) and allow the evaluation of f. The values obtained by this procedure have been included in Table II.

The data obtained employing uric acid (Figure 3) and NADH (Figure 4) do not conform to the simple patterns previously described. The data given in Figure 3 show that, at long incubation times, the enzyme is inactivated with a rate similar to that obtained in the absence of additives, suggesting total uric acid consumption. However, the inactivation rates at low incubation times are substantially smaller than those of the control, and nearly independent of the uric acid concentration. This

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

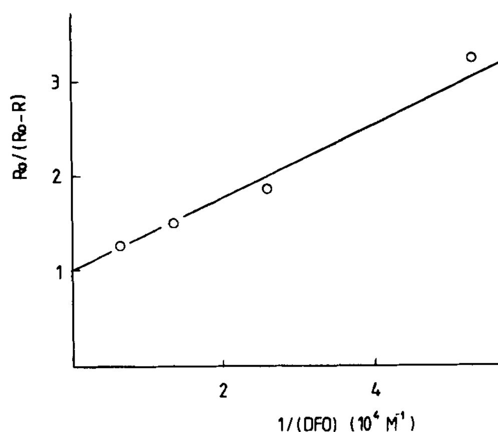


FIGURE 5 Plot of Ro/(Ro − R), where Ro and R are the initial lysozyme inactivation rates without and with added DFO, as a function of the reciprocal of DFO concentration.

behaviour could be explained in terms of residual damage due to urate-derived radicals. After total urate consumption, the displacement of the curves corresponds to the times required to consume the additive. Plotting these time lags against the initial urate concentrations gives a straight line, whose slope can be equated to n/R_r. From this procedure a value of $n = 2.2$ for uric acid (Table I) was obtained.

The data obtained employing NADH (Figure 4) show clear induction times, suggesting a high reactivity leading to the almost total consumption of the additive prior to significant enzyme inactivation rates. However, after the induction time, the inactivation rate is substantially slower than that observed in the absence of additives. This behaviour is the expected one if some of the products formed in the NADH consumption are able to act as mild protective agents. The ratio Ro/R′, where R′ is the rate observed after the induction times, are plotted, as a function of the initial NADH concentration, in Figure 6.

The effect of albumin on the lysozyme inactivation rate is similar to that of NADH. In this case also an induction time is observed after which the inactivation

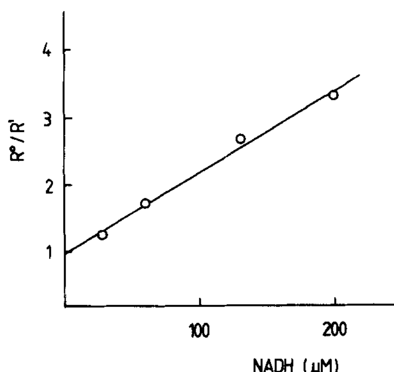


FIGURE 6 Plot of Ro/R′, where Ro is the initial rate of lysozyme inactivation in the absence of additives and R′ is the inactivation rate, after the induction time, as a function of NADH concentration.

RIGHTSLINK

rate depends upon the initial albumin concentration. However, for the protein the value of n was not constant, showing a noticeable decrease when the protein concentration increases.

DISCUSSION

None of the compounds considered in the present work affect modify lysozyme activity in the absence of added ABAP. However, all of them are able to protect lysozyme from inactivation mediated by the alkylperoxyl radicals produced by thermolysis of ABAP. This effect can be due either to a direct interaction of the alkylperoxyl radicals with the additives (Reaction 1) and/or to the occurrence of repair processes (Reaction 2 and 3). A clear-cut differentiation between these two mechanisms is difficult without direct monitoring of the enzyme- (Enz·) or additive-derived (X·) radicals. However, the dependence observed in $Q_{1/2}$ with the enzyme and ABAP concentration would favour, at least for tryptophan, a protective mechanism mainly due to competitive free radical trapping.

ADDITIVES OF HIGH REACTIVITY

All the compounds belonging to this class almost totally prevent the enzyme inactivation when employed at concentrations similar to that of lysozyme. Total protection of the enzyme requires a nearly quantitative formation of additive-derived radicals. The lack of measurable enzyme inactivation under these conditions implies that less than 5×10^{-3} enzyme molecules are inactivated by each X· radical generated in the system. The values of n obtained range from 3.0 for propyl gallate to 0.12 for cysteine. The present value for propyl gallate is higher than that previously reported,[11] the difference being due to the R_r value employed in its estimation. We consider that the present procedure, based on the use of Trolox as reference inhibitor under the same experimental conditions, is more accurate, and we thus favour the higher n value. This large value can be understood in terms of the presence of 3 reactive phenolic groups, and indicates that the compound retains its activity even after the reaction of one of these groups. On the other hand, values smaller than one imply some chain consumption of the inhibitor. This process is more significant for cysteine, where nearly 9 molecules are consumed by each radical introduced into the system, implying the occurrence of a chain oxidation of the type[20]

$$RS\cdot + O_2 \rightarrow RSOO\cdot \quad (6)$$

$$ROO\cdot + RSH \rightarrow \text{oxidized products} + RS\cdot \quad (7)$$

Reaction (6) is a reversible process that competes with[21]

$$RS\cdot + RS\cdot \rightarrow RSSR \quad (8)$$

and

$$RS\cdot + RS^- \rightarrow (RSSR)^- \quad (8)$$

making the efficiency of cysteine pH dependent.[22]

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

RIGHTSLINK

The nearly total protection afforded by cysteine before the induction time implies that RS· and RSOO· radicals are, under the present conditions, much less effective in lysozyme inactivation than the original peroxyl radicals. With regard to GSH, the data indicate that the chain process is considerably shorter but that the glutathione derived radicals are also ineffective in lysozyme inactivation. The present data show that, although sulfur derived radicals can inactivate some enzymes,[22,23] this is not a general phenomenon. In particular, Simone *et al.*[22] have reported that glutathione-derived peroxyl radicals are biologically active in vitro and that both lysozyme and trypsin are inactivated in the presence of glutathione and oxygen. However, since the experiments were carried out with different rates of radical input and, in the previous work, no inactivation quantum yields are reported, both sets of data cannot be considered as contradictory. The difference in conclusions emphasizes the relative nature of statements regarding the "activity" or "inactivity" of a given radical.[2]

The efficient protection afforded by ascorbate is in agreement with previous reports showing that ascorbic acid is an excellent scavenger of water-soluble peroxyl radicals.[15,24] The value of $n = 0.55$ found for this compound indicates that nearly two ascorbic acid molecules are consumed by each free radical introduced into the system. The mechanisms of antioxidant action of ascorbate have been extensively discussed[1,25] and essentially rely on the low reactivity of the semidehydroascorbate radical,[25] leading to its disproportionation to ascorbate and dehydroascorbate. However, the occurrence of these processes would lead to an n value of 1. The lower value found in the present work implies that secondary processes, perhaps involving peroxidized semidehydroascorbate and/or $HO_2\cdot$ ($O_2^{\bar{}}$) radicals produced by the reaction

$$O_2 + \text{semidehydroascorbate} \rightarrow HO_2\cdot + \text{dehydroascorbate} \quad (9)$$

may contribute to the ascorbate consumption. Wayner *et al.*[26] have reported, in the inhibition of the ABAP-initiated autoxidation of plasma lipids, n values for ascorbate that decrease when the additive concentration increases. The values reported in Table I correspond to the average value for ascorbate concentrations between 10 μM and 40 μM. The values reported by Wayner *et al.*[26] change from 0.8 to 0.5 in the same concentration range, in reasonable agreement with the average value found in the present system.

Uric acid is one of the main antioxidants present in biological fluids.[16,19] However, it has been shown that the radicals resulting from the attack of hydroxyl radicals upon uric acid can inactivate yeast alcohol dehydrogenase[9] and human α_1-antiproteinase,[10] leading, in some systems, to an increase in inactivation rates in the presence of uric acid. The data of Figure 3 show that in the present system uric acid addition protects lysozyme from inactivation induced by ABAP-derived radicals. However, even large (200 μM) excess concentrations do not completely suppress the inactivation. The residual inactivation, which might be due to uric acid-derived radicals, amounts to nearly 12% of that observed in absence of additives and implies that 0.015 enzymes are inactivated by each uric acid-derived radical produced.

The value of $n = 2.2$ obtained for uric acid can be explained in terms of a reaction of the uric acid derived radical with peroxyl radicals and/or by disproportionation of the uric acid-derived radicals to regenerate the parent compound. The near constancy of n over the concentration range considered would favour the second mechanism.

Wayner *et al.*[16] have reported n values of 1.3 for urate in plasma in the presence of natural antioxidants and unmodified proteins, but a higher value, about 1.65, was found in the absence of other plasma antioxidants and in the presence of proteins

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

RIGHTSLINK

modified by free radical attack. On the other hand, Niki *et al.*[19] have reported an n value of 2.0 for urate in liposomes and micellar systems undergoing oxidation induced by ABAP at 37°C, in good agreement with the value obtained in the present work.

ADDITIVES OF LOWER REACTIVITY

Compounds whose reactivity towards the radicals is smaller than that of the enzyme show only a protective action that remains almost constant in time due to their relatively low consumption in the time scale required to produce significant inactivation of the enzyme (Figure 2). Compounds showing this type of behaviour comprise radical traps such as Tempol and antioxidants such as diethylhydroxylamine.

The relatively high $Q_{1/2}$ value (9 μM) obtained for Tempol is rather surprising given the high reactivity of nitroxides towards free radicals.[27] However, it must be taken into account that nitroxides react relatively slowly with peroxyl radicals.[28] For Tempol, a "repair" process cannot be envisaged and hence the protection must be due to trapping of the parent alkyl radical of the peroxyl radical produced by its reaction with molecular oxygen.[11] Experiments conducted in air or oxygen saturated solutions give similar $Q_{1/2}$ values (data not shown) and hence trapping of the peroxyl radicals is the most probable protection pathway.

Diethylhydroxylamine has proved to be a powerful antioxidant,[29] due to the lability of the hydroxyl hydrogen.[30] However, in biological systems its role is extremely complex and, at least in the brain homogenate system, it acts as a promoter of the lipid peroxidation process.[31] In the present system, diethylhydroxylamine behaves as a weak inhibitor.

DFO, a preventive antioxidant of very high efficiency for iron mediated processes,[31] has proved to be only of moderate efficiency in non-iron mediated processes.[33,34] This last action has been attributed to the presence of hydroxylamine groups. In agreement with this, the value of $Q_{1/2}$ for DFO is very similar to that obtained for diethylhydroxylamine. In both processes it can be considered that a nitroxyl radical is formed.[29,35] These nitroxyl radicals appear unable to inactivate lysozyme efficiently, as evidenced by the total protection afforded at high concentrations. These results are in agreement with the protective action of Tempol, but contrast with the reported effect of DFO in the hydroxyl radical-mediated inactivation of yeast alcohol dehydrogenase, where it caused a significant enhancement in inactivation rate.[36] This different behaviour may be due to the higher damaging capacity of the radicals involved in the present work, and emphasizes the fact that the behaviour of a given additive can be totally different, depending both on the substrate considered and the type of radicals involved in the initial damage.[1,37]

Hydroquinone, due to its high reactivity towards free radicals and rather low oxidation potential,[38] could be expected to be an efficient inhibitor of the enzyme inactivation. The data of Table II show that, in the present system, its activity is similar to that of tryptophan.

Willson[8,14] has reported that addition of tryptophan, methionine or cysteine (1 mM) protects lysozyme completely, and histidine to a lesser extent, from its inactivation mediated by trichloromethylperoxyl radicals. The results obtained in the present work are in fair agreement with those obtained employing the trichloromethylperoxyl radicals since they indicate that the relative protective action of the amino acids considered follows the order

cysteine > tryptophan > methionine > tyrosine > histidine

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

RIGHTSLINK

Cysteine, tryptophan and methionine afford almost complete protection when the data are extrapolated to infinite concentration. On the other hand, when tyrosine and histidine are employed, no total protection is obtained, irrespective of the additive concentration. This implies that the radicals derived from these compounds are capable of inactivating the enzyme or, if a repair mechanism is operative, that they are not able to repair all types of damage produced by the peroxyl radicals. The fact that the less reactive compounds are unable to afford complete protection even when employed at "infinite" concentrations is compatible with the smaller stability (and hence higher reactivity) expected for their radicals.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

ADDITIVES ACTING AS INHIBITORS AND RETARDERS

Albumin and NADH show a behaviour that can be interpreted in terms of a high protective efficiency which is followed, after their consumption, by a retardation of the inactivation process.

The results obtained in the present work show that albumin, in μM concentrations, is able to suppress the enzyme inactivation at short times and, afterwards, to decrease its rate. Albumin has been reported to contribute to the defences of human body fluids against free radical damage,[39] most probably due to the presence of –SH groups. The short induction times observed, indicative of a very low n value (Table I) are compatible with an inhibition due to the presence of the cysteine residues. The n values lower than one, as well as their diminution when the albumin concentration increases, may be due to a chain oxidation of the active residues. The decreased rate observed afterwards might be attributed to the presence of other residues, most probably tryptophan, that can partially trap the damaging radicals. The total protection afforded by albumin at the concentrations employed in the present work might be of interest due to the considerably higher concentrations present in human plasma.[39]

NADH, because of its capacity to donate a hydrogen atom or an electron, can be considered a compound with the potential both to trap free radicals and to repair the initial damage. Willson[8] has shown that NADH readily react with trichloromethyl-peroxyl and peroxyl radicals derived from alkanols. The data obtained in the present work show that it also react with the radicals derived from the thermolysis of ABAP, probably by the reaction

$$RO_2\cdot + NADH \rightarrow RO_2^- + NAD\cdot + H^+ \tag{10}$$

followed by

$$NAD\cdot + O_2 \rightarrow NAD^+ + O_2^{\cdot-} \tag{11}$$

giving complete protection. However, this mechanism predict an n value of one, while the experimentally determined value is considerably smaller. Hence, secondary reactions of the radicals must significantly contribute to the NADH consumption. In agreement with this, it is observed that the plot of induction time against the initial NADH concentration presents a downward curvature, suggesting that the value of n decreases with the NADH concentration from 0.6 at low NADH concentrations to 0.2 at 200 μM NADH, suggesting a chain oxidation of NADH. The small decrease in inactivation rate observed after the induction time must be due to the scavenging capacity of the products. In order to see if this effect was due to the NAD^+ produced,

RIGHTSLINK

we have evaluated the effect of this compound (Table II). Its small protective capacity is not enough to explain the effect observed after NADH consumption.

Acknowledgments

This work was supported by DICYT (University of Santiago). M.F. thanks the Fundación Andes for a fellowship.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

References

1. B. Halliwell (1990) How to characterize a biological antioxidant. *Free Radical Research Communications* **10**, 1-32.
2. D.M.C. Richards, R.T. Dean and W. Jessup (1988) Membrane proteins are critical targets in free radical mediated cytolysis, *Biochimica et Biophysica Acta* **946**, 281-288.
3. B. Deuticke, V. Henseleit, C.M.W. Haest, K.B. Heller and T.M.A.R. Dubbelman (1989) Enhancement of transbilayer mobility of a membrane lipid probe accompanies formation of membrane leaks during photodynamic treatment of erythrocytes, *Biochimica et Biophysica Acta* **982**, 55-61.
4. H. Yasuda, M. Miki, Y. Takenaka, H. Tamai and M. Mino (1989) Changes in membrane constituents and chemiluminescence of Vitamin E-deficient red cells induced by the xanthine oxidase reaction. *Archives of Biochemistry and Biophysics* **272**, 81-87.
5. M. Miki, H. Tamai, M. Mino, Y. Yamamoto and E. Niki (1987) Free-radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-tocopherol. *Archives of Biochemistry and Biophysics* **258**, 373-380.
6. K.J.A. Davies (1986) Oxidative stress causes protein degradation and lipid peroxidation by different mechanisms in red blood cells. In *Lipid Peroxidation in Biological Systems* (A. Sevanian, ed.) American Oil Chemists' Society, Illinois, pp. 100-116.
7. N.A. Porter (1984) Chemistry of lipid peroxidation. *Methods in Enzymology* **105**, 273-282.
8. R.L. Willson (1985) Organic peroxy free radicals as ultimate agents in oxygen toxicity. In *Oxidative Stress* (H. Sies, ed.), Academic Press, London, pp. 41-72.
9. K.J. Kittridge and R.L. Wilson (1984) Uric acid substantially enhances the free radical-induced inactivation of alcohol dehydrogenase *FEBS Letters* **170**, 162-164.
10. O.I. Aruoma and B. Halliwell (1989) Inactivation of α_1-antiproteinase by hydroxyl radicals. The effect of uric acid. *FEBS Letters* **244**, 76-80.
11. E.A. Lissi and N. Clavero (1990) Inactivation of lysozyme by alkylperoxyl radicals. *Free Radical Research Communications* **10**, 117-184.
12. K.O. Hiller, P.L. Hodd and R.L. Willson (1983) Anti-inflammatory drugs: Protection of a bacterial virus as an in vitro biological measure of free radical activity. *Chemico-Biological Interactions* **47**, 293-305.
13. E.A. Lissi, M. Salim-Hanna, M. Faure and L.A. Videla, 2,2′-Azo-bis-amidinopropane as a radical source for lipid peroxidation and enzyme inactivation studies. *Xenobiotica* (in press).
14. R.L. Willson (1982) Iron and hydroxyl free radicals in enzyme inactivation and cancer. In *Free radicals, Lipid Peroxidation and Cancer* (D.H. McBrien and T.F. Slater, eds) Academic Press, London, pp 275-303.
15. J. Van der Zee, J. Van Steveninck, J.K. Koster and T.M.A.R. Dubbelman (1989) Inhibition of enzymes and oxidative damage of red blood cells induced by *t*-butylhydroperoxide-derived radicals. *Biochimica et Biophysica Acta* **980**, 175-180.
16. D.D.M. Wayner, G.W. Burton, K.U. Ingold, L.R.C. Barclay and S.J. Locke (1987) The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. *Biochimica et Biophysica Acta* **924**, 408-419.
17. B. Frei, R. Stocker and B.N. Ames (1988) Antioxidant defenses and lipid peroxidation in human blood plasma. *Proceedings of the National Academy of Sciences, USA* **85**, 9748-9752.
18. E. Niki (1987) Antioxidants in relation to lipid peroxidation. *Chemistry and Physics of Lipids* **44**, 227-253.
19. E. Niki, M. Saito, Y. Yoshikawa, Y. Yamamoto and Y. Kamiya (1986) Oxidation of lipids. XII. Inhibition of oxidation of soybean phosphatidylcholine and methyl linoleate in aqueous dispersions by uric acid. *Bulletin of the Chemical Society of Japan* **59**, 471-477.

RIGHTSLINK

20. T. Motoyama, M. Miki, M. Mino, M. Takahashi and E. Niki (1989) Synergistic inhibition of oxidation in dispersed phosphatidylcholine liposomes by a combination of Vitamin E and cysteine. *Archives of Biochemistry and Biophysics* **270**, 655–661.
21. K.D. Asmus (1990) Sulfur-centered free radicals. *Methods in Enzymology* **186**, 168–180.
22. G. Simone, J.C.M. Bremner, M. Tamba, B.R. Guerra and M. Quintiliani (1987). 4th International Congress in Oxygen Radicals, La Jolla. Contributed Abstracts pp 28–31.
23. O.I. Aruoma, B. Halliwell, J. Butler and B.M. Hoey (1989) Apparent inactivation of α_1-antiproteinase by sulphur-containing radicals derived from penicillamine. *Biochemical Pharmacology* **38**, 4353–4357.
24. A. Bendich, L.J. Machlin, O. Scandurra, G.W. Burton and D.D.M. Wayner (1986) The antioxidant role of vitamin C. *Advances in Free Radicals in Biology and Medicine* **2**, 419–444.
25. B.H.J. Bielski, H.W. Richter and P.C. Chan (1975) Some properties of the ascorbate free radical. *Annal of New York Academy of Sciences* **258**, 231–237.
26. D.D.M. Wayner, G.W. Burton and K.U. Ingold (1986). The antioxidant efficiency of vitamin C is concentration-dependent. *Biochimica et Biophysica Acta* **884**, 119–123.
27. K.U. Ingold (1973) Rate constants for the free radical reactions in solution. In *Free Radicals* (J.K. Kochi, ed) John Wiley, N.Y. Vol I, pp 37–112.
28. S.A. Maslov and G.E. Zaikov (1987) Nitroxy-radicals in the liquid-phase oxidation reactions of organic compounds. *Russian Chemical Reviews* **56**, 715–725.
29. E. Abuin, M.V. Encina, S. Diaz and E.A. Lissi (1978) On the reactivity of diethylhydroxylamine towards free radicals. *International Journal of Chemical Kinetics* **10**, 677–686.
30. T. Caceres, E.A. Lissi and E. Sanhueza (1978) Autoxidation of diethylhydroxylamine towards free radicals. *International Journal of Chemical Kinetics* **10**, 677–686.
31. E.A. Lissi, T. Caceres and L.A. Videla (1986) Visible chemiluminescence from rat brain homogenates undergoing autoxidation. I. Effect of additives and products accumulation. *Journal of Free Radicals in Biology and Medicine* **2**, 63–69.
32. L.A. Videla, M.I. Villena, C. Salgado, P. Canales and E.A. Lissi (1987) Antioxidant capacity of desferrioxamine in biological systems. *Biochemistry International* **15**, 205–214.
33. L.A. Videla, T. Caceres and E.A. Lissi (1988) Antioxidant capacity of desferrioxamine and ferrioxamine in the chemically-initiated lipid peroxidation of rat erythrocyte ghost membranes. *Biochemistry International* **16**, 799–807.
34. V.M. Darley-Usmar, A. Hersey and L.G. Garland (1989) A method for the comparative assessment of antioxidants as peroxyl radical scavengers. *Biochemical Pharmacology* **38**, 1465–1469.
35. O. Hinojoso and T.J. Jacks (1986) Interface by desferrioxamine of spin trapping oxy-radicals for ESR analysis. *Analytical Letters* **19**, 725–733.
36. M.J. Davies, R. Konkor, C.A. Dunster, C.A. Gee, S. Jonas and R.L. Willson (1987) Desferrioxamine (Desferal) and superoxide free radicals. Formation of an enzyme-damaging nitroxide. *Biochemical Journal* **246**, 725–729.
37. B. Halliwell and J.M.C. Gutteridge (1990) The antioxidants of human extracellular fluids. *Archives of Biochemistry and Biophysics* **280**, 1–8.
38. S. Steenken and P. Neta (1982) One-electron redox potentials of phenols. Hydroxy- and aminophenols and related compounds of biological interest. *Journal of Physical Chemistry* **86**, 3661–3667.
39. B. Halliwell (1988) Albumin — an important extracellular antioxidant? *Biochemical Pharmacology* **37**, 569–571.

Accepted by Professor B. Halliwell

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

RIGHTSLINK