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INACTIVATION OF LYSOZYME BY ALKYLPEROXYL RADICALS

E.A. LISSI and N. CLAVERO

Departamento de Química, Facultad de Ciencias, Universidad de Santiago, Casilla 5659, Correo 2, Santiago, Chile

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Thermolysis of 2,2′-azo-bis-(2-amidinopropane) under air in the presence of lysozyme leads to extensive inactivation of the enzyme. The number of inactivated enzyme molecules per radical produced increases with the enzyme concentration up to values considerably larger than one. Enzyme inacitivation is accompanied by extensive tryptophan modification. Over the enzyme concentration range considered (1.7 to 130 μM) nearly 4 tryptophan groups are modified per enzyme molecule inactivated. Both the inactivation and tryptophan modification are prevented by micromolar concentrations of propyl gallate. The results are interpreted in terms of an efficient inactivation of the enzyme by the alkylperoxyl radicals generated by thermolysis of the azocompound.

KEY WORDS: Lysozyme inactivation, alkylperoxyl radicals, tryptophan modification, 2,2′-azo-bis-(2-amidinopropane)

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INTRODUCTION

Free-radical attack on biological membranes can lead to the oxidative destruction of the polyunsaturated fatty acids of the membrane in the well-documented process termed lipid peroxidation.[1] Also, radicals can damage proteins by fragmentation, cross-linking and aminoacid modification.[2-5] The relevance of this process is emphasized by data obtained on red blood cells exposed to oxidative stress. These show that protein modification occurs faster than lipid peroxidation and is not significantly prevented by lipid peroxidation inhibitors.[6]

Extensive studies have been performed on the interaction of hydroxyl radicals and different proteins.[2-8] However, in recent years the importance of hydroxyl radicals as the ultimate toxin in oxygen-related injury has been questioned, and the possibility has been raised that other radicals, such as the superoxide anion and the hydroperoxyl radical, were involved.[7] In particular, peroxyl radicals of general formula ROO·, present as lipid peroxidation chain carriers and formed each time a reactive radical (such as hydroxyl) attacks a lipid molecule, have seldom been considered. Willson has discussed extensively the role of these radicals as ultimate agents in oxygen toxicity[7] and has presented comprehensive data regarding the interaction of trichloromethyl peroxyl radicals with proteins. However, this radical is highly reactive and its reactions can be considerably different from those expected for non-chlorinated peroxyl radicals. Data on the capacity of such radicals to interact with proteins are scarce and generally indirect. For example, Dean and Cheeseman have suggested that membrane-bound proteins are damaged during lipid peroxidation due to their interaction with lipid radicals,[9] and Griffiths *et al.*[10] from the effect of added phenylalanine, considered that peroxyl radical attack may be an *in-vivo* mechanism for the

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denaturation of IgG. On the other hand, from the absence of lysozyme inactivation when the enzyme is irradiated in the presence of tert-butanol, Willson has concluded that peroxyl radicals do not inactivate the enzyme.[7]

2,2′-Azo-bis-(2-amidinopropane) (ABAP) is a water-soluble thermal source of alkyl radicals that, in the presence of oxygen, generates alkylperoxyl radicals.[11] In the present work, we report data bearing on the reactivity of these radicals towards the water-soluble enzyme lysozyme.

MATERIALS AND METHODS

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 enzyme activity. In the experimental conditions employed, the measured activity was proportional to the lysozyme concentration.

Tryptophan destruction, either as free aminoacid or when bound to the enzyme, was monitored by following the decrease of its fluorescence intensity (excitation at 295 nm, emission at 345 nm).

Solutions of the enzyme and/or tryptophan were incubated at 45°C in PBS buffer (0.07 M phosphate, 0.017 M NaCl, pH = 6.5) with 10 mM ABAP. Aliquots were withdrawn at different times and analyzed to obtain the tryptophan concentration and/or the remaining enzymatic activity.

RESULTS

Incubation of the enzyme at 45°C in the presence of oxygen and ABAP (10 mM) leads to its inactivation (Figure 1). The inactivation rate is considerably faster than that observed in absence of ABAP or when oxygen is excluded (nitrogen-purged solu-

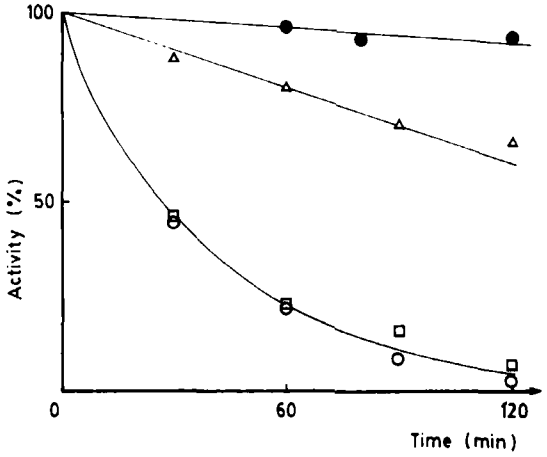

FIGURE 1 Loss of lysozyme activity as a function of the incubation time. Lysozyme concentration: 6.8 × 10^{-5} M. (●) Control experiment, under air, without added ABAP. (△) ABAP = 10 mM, under nitrogen. (○) ABAP = 10 mM, air-saturated. (□) ABAP = 10 mM, oxygen-saturated.

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tions). Futhermore, identical inactivation rates are measured in air or oxygen-saturated solutions. Controls performed by measuring the enzymatic activity in the presence of ABAP but without incubation at 45°C show that this compound does not modify the enzymatic activity of lysozyme.

Lysozyme incubation with ABAP also leads to a significant modification of its tryptophan moieties. Changes in the relative tryptophan group fluorescence intensities with incubation time are given in Figure 2. Similiar results are obtained when the free aminoacid is incubated with ABAP (Figure 2). Extensive oxidation of the enzyme promoted by ABAP leads to formation of kynurenine (excitation at 365 nm, emission in the 420–500 nm range) and formylkynurenine (excitation at 325, emission in the 360–480 nm range), as expected for the free-radical mediated oxidation of the tryptophan moieties.[12]

Both enzyme inactivation and tryptophan modification can be prevented by propyl gallate, a widely employed water-soluble antioxidant. Typical results are given in Figure 3. These data can be interpreted in terms of a very efficient trapping of the radicals by propyl gallate, leading to induction times proportional to the inhibitor concentration (Figure 3B). The rate of lysozyme inactivation can also be decreased by tryptophan addition. However, when present at concentrations comparable to those of the enzyme, this compound provides only a moderate protection (Table 1).

The dependence of the enzyme inactivation rate on the enzyme concentration was measured over the range from 1.7×10^{-7} M to 3.4×10^{-4} M. The results obtained, plotted as log(d[lysozyme]/dt) vs log[lysozyme], where d[lysozyme]/dt is the initial rate of lysozyme activity loss and [lysozyme] is the initial lysozyme concentration, are given in Figure 4. Figure 5 shows the loss of tryptophan groups (as evaluted from the fluorescence decrease) in the enzyme or the free aminoacid. In this figure, log(d-[tryptophan]/dt) vs log[tryptophan] has been plotted, where d[tryptophan]/dt is the initial rate of tryptophan group loss and [tryptophan] is the initial tryptophan group concentration.

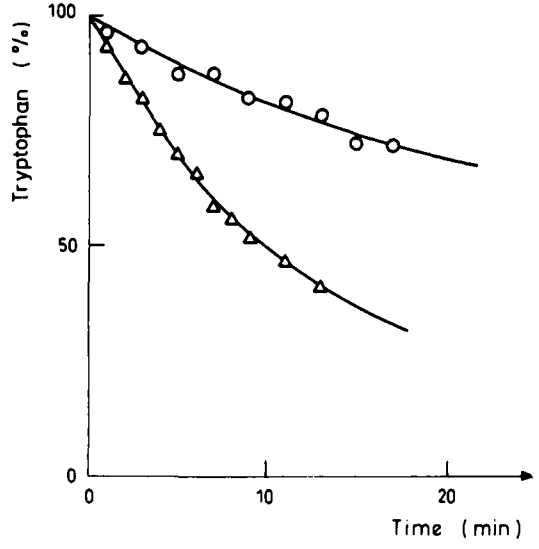

FIGURE 2 Loss of tryptophan groups as a function of the time of incubation with ABAP. (○) Free aminoacid (2.2 μM); (△) Lysozyme (3.4 μM).

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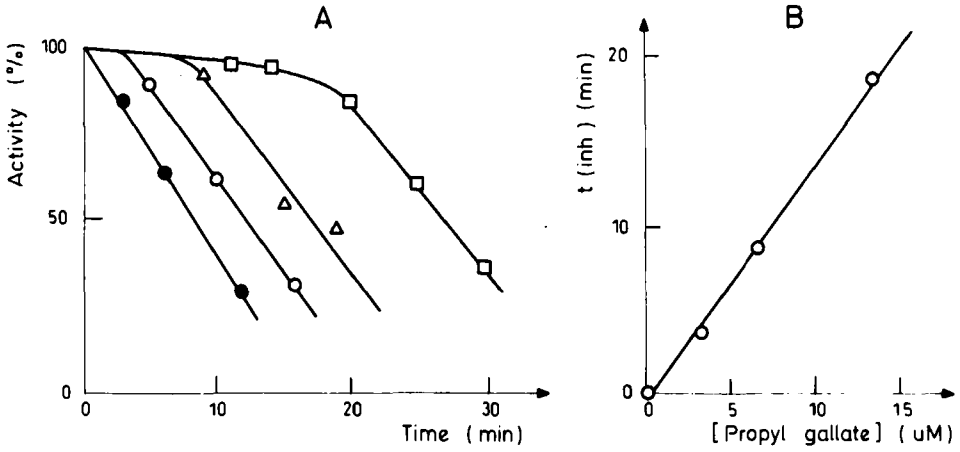

FIGURE 3 (A) Effect of Propyl gallate on the lysozyme inactivation rate. (●) no propyl gallate added; (○) 3.4 μM, (△) 6.8 μM, and (□) 13.6 μM propyl gallate. Lysozyme concentration = 3.4 μM. (B) Dependence of the inhibition time on the propyl gallate concentration; data from (A).

TABLE I
Effect of Tryptophan Concentration on Lysozyme Inactivation Rate by ABAP

[Tryptophan] (μM)	Relative Inactivation Rate
0	1.0
3.4	0.73
6.8	0.55
13.7	0.43
20.0	0.28

Lysozyme concentration: 3.4 μM.

DISCUSSION

The increase in lysozyme inactivation rate observed in the presence of oxygen indicates that the inactivation is due to the peroxyl radicals produced by the sequence of steps:

$$\text{ABAP} \rightarrow 2\,\text{R}^{\cdot} + \text{N}_2 \quad (1)$$

$$\text{R}^{\cdot} + \text{O}_2 \rightarrow \text{ROO}^{\cdot} \quad (2)$$

where R· stands for the tertiary carbon-centered radical. The formation of the alkylperoxyl radicals leads efficiently to lysozyme inactivation, with a concomitant destruction of tryptophan moieties. Propyl gallate is able to prevent the enzyme inactivation and tryptophan modifications, acting as an almost ideal inhibitor. In this situation the inhibition time (t_{inh}) can be related to the inhibitor concentration by

$$t_{inh} = a\,[\text{Inh.}]/(2\,k_{ABAP}[\text{ABAP}]) \quad (3)$$

where a is the number of free radicals scavenged by each propyl gallate molecule. The value of the ABAP cleavage rate constant (k_{ABAP}) at the employed temperature can be estimated as $5.4 \times 10^{-7}\,s^{-1}$.[11] If the slope obtained from the plot shown in Figure

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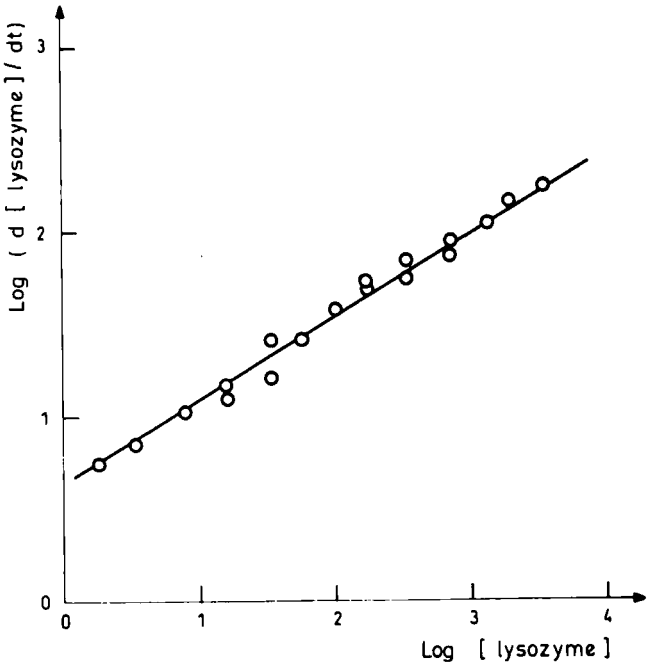

FIGURE 4 Dependence of lysozyme inactivation rate on the enzyme concentration. Inactivation rates given in $10^{-2}\,\mu$M/min. Lysozyme concentrations given in units of 10^{-7}M.

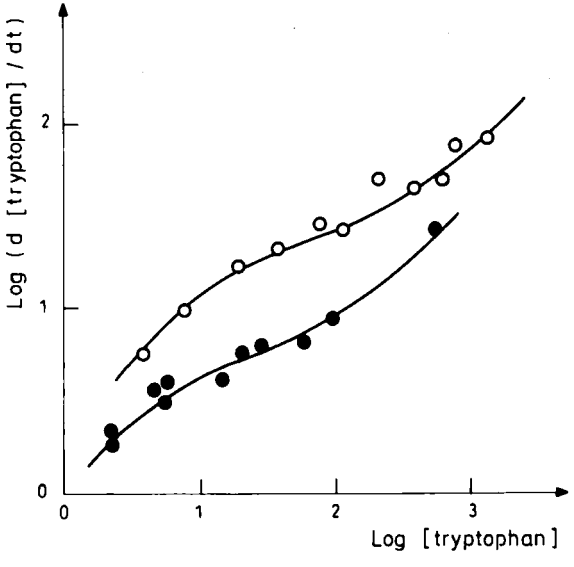

FIGURE 5 Dependence of tryptophan groups consumption rates with the tryptophan concentration for the free aminoacid (●) and in the enzyme (○). Rates and concentrations are given in 0.1 μM/min. and μM units, respectively.

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3(B) is multiplied by $2k_{ABAP}[ABAP]$, a value of 0.96 is obtained, showing that under these conditions each propyl gallate molecule is able to trap nearly one free radical.

The minimum set of reactions needed to interpret the present results must comprise, besides reactions (1) and (2), self-reactions of the peroxyl radicals and their reactions with the enzyme and/or the additives:

$$ROO^{\cdot} + \text{lysozyme} \rightarrow \text{modified lysozyme} \quad (4)$$

$$2\,ROO^{\cdot} \rightarrow \text{termination} \quad (5)$$

$$ROO^{\cdot} + XH \rightarrow ROOH + X^{\cdot} \quad (6)$$

where XH is the additive (either propyl gallate or tryptophan).

At low enzyme concentration (i.e. the rate of process (4) is much lower than that of process (5)), and in the absence of added XH

$$-(d[\text{lysozyme}]/dt) = (2k_1[ABAP]/k_6)^{0.5} k_4 [\text{lysozyme}] \quad (7)$$

At higher enzyme concentrations, termination by the radicals derived from the enzyme must be considered, leading to a rather complex dependence of the inactivation rate on the enzyme concentration. However, when most of the $ROO^{\cdot}$ radicals react with the enzyme, this simple reaction scheme predicts that

$$-(d[\text{lysozyme}]/dt) = 2k_1[ABAP] \quad (8)$$

and the inactivation rate must become independent of the enzyme concentration. Plots like those shown in Figures, 4 and 5 should have slopes ranging from 1 (at low substrate concentrations), to 0 at high substrate concentrations. The data show an intermediate behaviour. For enzyme inactivation, a slope of nearly 0.5 is obtained over the concentration range considered, while for tryptophan destruction an S-shaped behaviour is obtained. In both cases, it is remarkable that the limit of slope 0 is not obtained even at the higher concentrations considered. This cannot be related to an incomplete trapping of the radicals, since under these conditions more than one enzyme molecule is inactivated and more than one tryptophan group is modified per radical produced (see Table 2). These data could be explained in terms of short reaction chains such as

$$ROO^{\cdot} + \text{lysozyme} \rightarrow \text{lysozyme}^{\cdot} + ROOH \quad (9)$$

$$\text{lysozyme}^{\cdot} + O_2 \rightarrow \text{lysozymeOO}^{\cdot} \quad (10)$$

$$\text{lysozymeOO}^{\cdot} + \text{lysozyme} \rightarrow \text{modified lysozyme} + \text{lysozyme}^{\cdot} \quad (11)$$

TABLE 2
Dependence of Lysozyme Inactivation and Tryptophan Modification on Substrate Concentration

Substrate	Concentration μM	Enzyme*	Tryptophan #
lysozyme	1.7	0.37	1.6
	70	0.92	4.0
	130	3.4	12
tryptophan	2.3		0.3
	43		1.54

(*) Number of inactivated enzyme molecules per radical generated.
(#) Number of modified tryptophan groups per radical.

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that can be particularly important at high enzyme concentrations, leading to enzyme consumption rates larger than the free radical production and dependent on the enzyme concentration.

A further complication that precludes a complete kinetic analysis of the system is that peroxyl radicals, either the primary $ROO^{\cdot}$ or those derived from the enzyme, can self-react to generate more reactive alkoxyl radicals[13]

$$2\,ROO^{\cdot} \rightarrow 2\,RO^{\cdot} + O_2 \qquad (12)$$

a reaction particularly important for tertiary peroxyl radicals. The alkoxyl radicals generated in processes such as reaction (12) could contribute further to enzyme inactivation and/or tryptophan modification. Nevertheless, flash photolysis experiments in which ABAP was irradiated in the presence of tryptophan (0.1 mM) showed the typical absorption of the tryptophan radical at 510 nm,[14] with a rise time shorter than 0.2 ms (data not shown). Given the low concentration of radicals that could be present in the system after irradiation (less than 0.1 mM) and the rather low reaction rate expected for a process such as reaction (12) involving tertiary peroxyl radicals (less than $10^5\ M^{-1}\ s^{-1}$),[15] the rise time observed is incompatible with the previous occurrence of reaction (12) and must be attributed to the direct interaction of the primary peroxyl radicals and tryptophan in a process whose rate constant must be higher than $10^7\ M^{-1}\ s^{-1}$. Furthermore, the data obtained (Table 2) would imply that the free aminoacid and the tryptophan moieties bound to the enzyme react at similiar rates.

Extensive tryptophan modification has been observed in other systems in which lysozyme was exposed to free radicals or to excited carbonyl triplets.[7,8,16,17] The data given in Table 2, although only semi-quantitative since the extent of tryptophan modification in the enzyme was derived from fluorescence intensity measurements, show that more than one tryptophan is modified in each inactivated enzyme molecule, pointing to a rather indiscriminate attack on essential and non-essential tryptophan groups.

The larger inactivation rates observed in the presence of air or oxygen (see Figure 1) indicate that alkylperoxyl radicals are much more efficient than the carbon-centered radicals in inactivating the enzyme. This difference can be due to a smaller reactivity of the alkyl radical, a lower selectivity of these radicals,[7,8] and/or a smaller steady state radical concentration as a consequence of a faster recombination rate. This last interpretation seems particularly attractive due to the large difference in recombination rates of alkyl and peroxyl radicals.[15] In the present system, the interaction between peroxyl radicals and lysozyme can be particularly favoured due to two factors. First, the low recombination rate expected for the tertiary peroxyl radicals, resulting in a high steady state concentration. Second, the low rate of radical generation favouring processes that are first-order in radical concentration.

In conclusion, our data show conclusively that alkyl peroxyl radicals can lead to lysozyme inactivation with high efficiency, and that the inactivation is accompanied by extensive tryptophan modification.

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