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Alpha lipoic acid (ALA) protects proteins against the hydroxyl free radical-induced alterations: rationale for its geriatric topical application

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Abstract

The well known $OH^{\bullet}$ free radical scavenging properties of α-lipoic acid (ALA) cannot be easily utilized for biological experiments, because the compound is practically insoluble in water. We elaborated a simple method of preparing its Na-salt (Na-ALA) which proved to be water soluble. It has been demonstrated by ESR spin trapping experiments with DMPO, using the Fenton reaction as the source of $OH^{\bullet}$ free radicals that Na-ALA maintains its $OH^{\bullet}$ free radical scavenging ability: it reacts nearly an order of magnitude faster with these radicals than the spin trap itself. It was tested in two different systems to determine whether Na-ALA was able to protect bovine serum albumin (BSA) against the $OH^{\bullet}$ free radical-induced polymerization and protein oxidation. (i) $OH^{\bullet}$ free radicals were generated by Fenton reaction in the presence of BSA. This protein is polymerized by these radicals shown by the loss of its water solubility; Na-ALA exerted a considerable protective effect against this type of protein damage. (ii) BSA oxidation was induced by Co-gamma irradiation of 80 krad, resulting in a strong increase in the protein carbonyl content. Na-ALA inhibited this carbonyl formation very efficiently. The data suggest that the interaction of the OH radical with Na-ALA takes place on the disulfide group, yielding thiosulfinate or thiosulfonate. The results indicate that the geriatric topical application of Na-ALA may have an established rationale.

Keywords: α-Lipoic acid; $OH^{\bullet}$ free radicals; Fenton reaction; Protein polymerization; ESR spin trapping; Co-gamma irradiation; Carbonyl formation

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1. Introduction

The compound α-lipoic acid (ALA) has been used in both animal experiments and as a therapy for various diseases in humans (Suzuki et al. 1992; Kahler et al., 1993; Guillausseau, 1994; Jacob et al., 1995; Ou et al., 1995), and further data reviewed by Packer et al. (1995). From a gerontological point of view, it is particularly important to note that ALA displayed a protective effect in various models against age-dependent cognitive deficits (Choi, 1988; Altenkirch et al., 1993; Stoll et al., 1993; Greenamyre et al., 1994). In addition, ALA proved to be useful also in topical applications against aging signs of the skin (Perricone, 1997, 1999).

The investigation of ALA began during the 1930s, originally identified as a bacterial growth factor which was designated 'acetate-replacing factor' or 'pyruvate oxidation factor' (Reed, 1957). The name ALA was given by Reed et al. (1951) who isolated a crystalline organic acid which proved identical to the acetate-replacing and pyruvate oxidation factors. In the same year, Patterson et al. (1951) isolated a derivative of ALA, and called it β-lipoic acid (BLA). The chemical structure of ALA was identified: it is 1,2-dithiolane-3-valeric acid, or 1,2-dithiolane-3-pentanoic acid, or dl-thioctic acid, or 6,8-dithiooctanoic acid, whereas BLA is an oxidized form of ALA, being a thiosulfinate version of it (Fig. 1) (Reed, 1957; Biewenga and Bast, 1995).

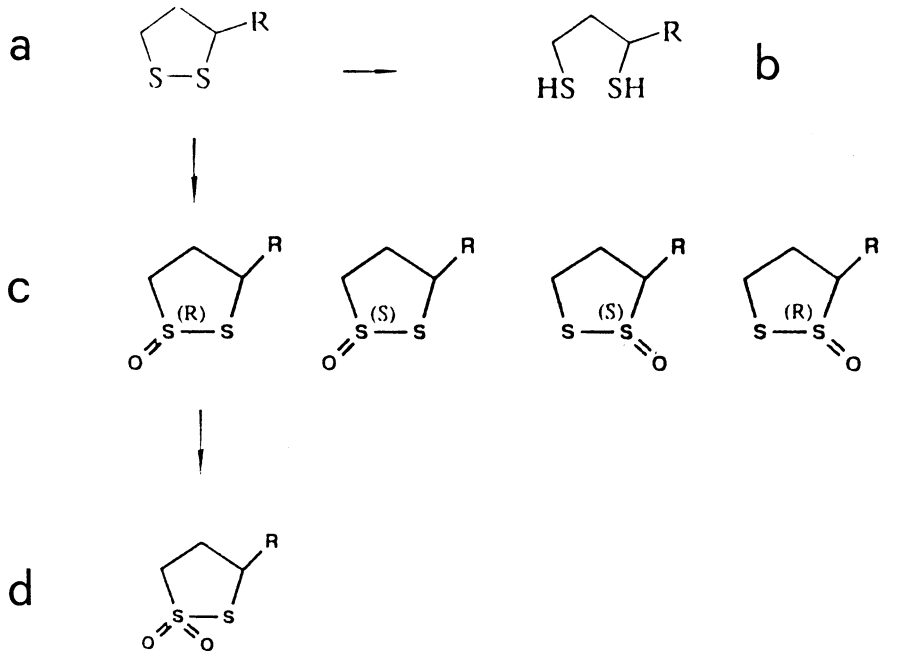

Fig. 1. The chemical structure of ALA and related compounds. a, ALA; b, DHLA; c, the four possible enantiomers of BLA, (R) and (S) mean the right and left rotating versions, respectively; d, the thiosulfonate version. R symbolizes the pentanoic acid.

ALA is an ubiquitous natural compound: it forms the prostethic group of the Coenzyme-A (CoA) in the mitochondrial (e.g. pyruvateor- or α-ketoglutarate-) dehydrogenase systems (Reed, 1957; Lehninger, 1970). ALA is a relatively small molecule (mwt: 206). Its heterocyclic 1,2-dithiolane ring contains an -S-S- group, and the C-atom of α-position to either of the S atoms is asymmetric, giving rise to two possible d- and two possible l-enantiomers of the molecule. In CoA only the d-AI A may be incorporated. ALA can be synthesized relatively easily in racemic form.

ALA is a partially oxidized variant of the molecule (Fig. 1a). This can easily be reduced to a compound called dihydrolipoic acid (DHLA) shown by Fig. 1b. As a matter of fact, this reduced form performs the temporary binding of the acetyl group, in the CoA, and it is reoxidized again at the end of the process, i.e. becomes ready for repeating the transacetylating function (Lehninger, 1970). It has been observed rather early that ALA prevented the consequences of vitamin C deficiency in guinea pigs, and of vitamin E deficiency in rats (Rosenberg and Culik, 1959), i.e. it acted as an antioxidant.

More recent studies have revealed that ALA and DHLA are potent quenchers of various reactive oxygen species, as reviewed by Packer et al. (1995). A number of studies in various model systems have demonstrated these molecules effectively neutralizing free radicals such as the $OH^{\bullet}$ free radical, hypochlorous acid, singlet oxygen, but not hydrogen peroxide (Suzuki et al., 1991; Scott et al., 1994; Passwater, 1996). Based on their properties, both the ALA and DHLA have been characterized as 'ideal antioxidants' (Packer et al., 1995), although DHLA has been shown to have both antioxidant and prooxidant effects (Suzuki et al., 1991; Scott et al., 1994).

The mechanism of the free radical scavenging processes performed by these compounds remains somewhat controversial. Most authors assume that DHLA is chiefly responsible for the antioxidant effects (Armstrong and Webb, 1966; Peinado et al., 1989; Muller and Menzel, 1990; Handelman et al., 1994) etc. This assumption is supported by the fact that when ALA is used in vitro or in vivo systems, a reduction to DHLA results. However, other experiments show that ALA is directly able to scavenge hypochlorous acid (HOCl) (Haenen and Bast, 1991; Biewenga and Bast, 1995) as well as the $OH^{\bullet}$ free radical (Scott et al., 1994; Passwater, 1996). This apparent contradiction is explained by the formation of BLA (Fig. 1c). One of the S-atoms in the dithiolane ring of BLA becomes tetravalent and the same S-atom may take up further oxygen, and thus becomes a thiosulfonate (Fig. 1d) in which the S-atom is hexavalent (Biewenga and Bast, 1995). These valence changes fully explain the ability of ALA to directly scavenge the $OH^{\bullet}$ free radical without reduction to DHLA.

Although the popularity of ALA as a possible therapeutic agent is growing, some basic problems still persist. Namely, because ALA is almost completely insoluble in water, the possibilities of experimental explorations of its effect in aqueous systems are limited. On the other hand, it seemed to be important to reveal whether the $OH^{\bullet}$ free radical scavenger activity of this compound may really be exploited as a protective effect on proteins against the $OH^{\bullet}$ free radical induced oxidations, conformational alterations or cross linking.

2. Materials and methods

The ALA used in these experiments was obtained from Maypro Industries Inc. (550 Mamaroneck Avenue, Harrison, NY 10528, USA), manufactured on 27 August, 1997, with an expiration date of 27 February, 2000. Purity was 99.8% as assayed by Maypro.

2.1. The problem of water-insolubility of ALA

ALA is soluble in a variety of organic solvents, but practically insoluble in water. ALA can first be dissolved in some organic solvents then mixed with water. However, none of the tested solvents (ethanol, methanol, DMSO, acetone, ethyl-methyl-ketone, etc.) yielded a clear solution; that is, some turbidity was also present. These organic solvents also strongly react with the $OH^{\bullet}$ free radical (as shown bv spin-trapping methods using DMPO) (Zs.-Nagy and Floyd, 1984a,b, 1990). It is, therefore, unreliable to measure the activity of ALA toward the $OH^{\bullet}$ free radical in the presence of these organic solvents. In order for these experiments to be relevant in biological systems, the radical scavenging experiments must be performed in aqueous systems.

In order to overcome the problem of solubility, we first tested the amide-salt of ALA (ALA-amide). Its water solubility was also unsatisfactory for our purposes, as it formed an opalescent, inhomogeneous system in water, making it impractical for the experiments. A Na-salt of ALA was then prepared by adding to its aqueous suspension 1 M NaOH drop by drop until a clear solution was achieved, then neutralized with 1 M HCl. This method was successful, with the Na-ALA salt remaining completely water-soluble even after neutralization. All experiments were performed with this Na-ALA salt, in the three test systems described below.

2.2. ESR spin trapping with DMPO (5,5-dimethyl-L-pyrroline-N-oxide)

Although similar experiments have already been performed with ALA, using iron-induced heterolysis of hydrogen peroxide for the generation of $OH^{\bullet}$ free radicals (Fenton reaction) (Suzuki et al., 1991; Scott et al., 1994) , the repetition was justified for the following reasons:

(i) The previous experiments were carried out using either unchelated divalent iron (Suzuki et al., 1991), or EDTA-Fe^{3+} complex + ascorbate (Scott et al., 1994), whereas in our system ADP-Fe^{2+} complex was utilized.

(ii) It was important to demonstrate that the Na-ALA salt prepared as described above really maintained its $OH^{\bullet}$ free radical scavenging ability.

The preparation and maintenance of the stock solutions of the reagents used for these experiments were essentially the same as described by Floyd and Lewis (1983), although the composition as well as the sequence of addition of reagents were slightly modified according to requirements of the actual experiments as described by Zs.-Nagy and Floyd (1984a,b). In the standard experimental model a total volume of 0.1 ml was used, consisting of the components added in the following order:

1. ADP (20 mM) 0.01 ml;
2. $Fe(NH_4)_2(SO_4)_2$ (1 mM in 0.0012 N HCl) 0.01 ml;
3. the Na-ALA solution (10, 25, 50 or 100 mM) (or water for control purposes) in 0.01 ml;
4. buffer (100 mM NaCl + 25 mM $NaHCO_3$, pH 7.1) 0.05 ml;
5. spin trap (DMPO, Aldrich, 200 mM in water) 0.01 ml;
6. H_2O_2 (0.3% in water) 0.01 ml.

Final concentrations of each reagent were ten times lower. In order to achieve uniformity of the experiments, 30 s elapsed after the addition of each component following the ferrous-ammonium sulfate. All the experiments were carried out at 20°C. After the last component was added, 0.030 ml of the sample was transferred into a sealed glass micro capillary of 0.1 ml, then placed into the standard cavity of the ESR instrument. The spectrum was recorded after 2 min subsequent to the addition of H_2O_2.

The ESR instrument was a JEOL JESRE1X type X-band spectrometer operating in the first derivative mode. Typical instrumental parameters during the DMPO experiments were as follows: scan range 100 Gauss (= 10 mT), field set 3373 Gauss, time constant 0.1 s, scanning time 4 min, modulation amplitude 2.0 Gauss, modulation frequency 100 kHz, receiver gain 5×10^2, microwave power 4 mW, microwave frequency 9.451 GHz, temperature 20°C.

All spin-trapping measurements were repeated at least five times, and curve fitting procedures were performed with the averages of these repeated experiments at each Na-ALA concentration.

2.3. The protective effect of Na-ALA against the protein cross linking

Bovine serum albumin (BSA) is a completely water-soluble protein. When $OH^{\bullet}$ free radicals are generated by Fenton reaction (Walling, 1975), the protein becomes polymerized and loses its water-solubility in a concentration dependent manner (Zs.-Nagy and Nagy, 1980). If $OH^{\bullet}$ free radical scavengers are added to such a system, it slows down the polymerization and thus decreases the loss of water solubility of BSA, yielding a simple way to study this system.

The reaction mixture of 0.5 ml final volume was composed of the following (the sequence of additions is important):

1. 0.1 ml aqueous solution of BSA (0.4%);
2. water to reach 0.5 ml total volume (see items d and e);
3. 0.21 ml of NaCl (0.1 M)-$NaHCO_3$ (0.025 M) buffer, pH 7.1;
4. 0.1 ml Na-ALA solution of various dilutions, resulting in final concentrations of 1, 2.5, 5 and 10 mM (in controls only water is added);
5. 0.005–0.030 ml of ADP-Fe^{2+} solution (Floyd, 1983; Floyd and Lewis, 1983), in a 20:1 molar ratio, in order to protect Fe^{2+} against the autoxidation at nearly neutral pH), resulting in final concentrations of Fe^{2+} of 1–6 mM;
6. 0.05 ml of 2% H_2O_2.

When mixing these components, various degrees of BSA precipitation can be observed due to the polymerizing effect of the $OH^{\bullet}$ free radicals. One can then

centrifuge the precipitated fraction and measure the remaining water-soluble fraction of BSA in the supernatant, as shown in Section 3.

2.4. The protective effect of Na-ALA against radiation-induced protein oxidation

This model differed from the previous one, in that the source of $OH^{\bullet}$ free radicals was not the Fenton reaction, but the result of gamma rays of ^{60}Co as described by Nagy et al. (1994). A BSA solution (0.33 mg/ml) was exposed to gamma radiation of 80 krad total dose, at room temperature, under oxygen atmosphere. This level of radiation causes a measurable increase in the carbonyl content of the protein (Davies, 1987), as a result of the radiation-induced $OH^{\bullet}$ free radical production.

The protein carbonyl content was determined spectrophotometrically by means of the 2,4-dinitrophenylhydrazine (DNPH) method of Levine et al. (1990). The final results were calculated from an extinction coefficient of 21.0 mM^{-1} cm^{-1} for the aliphatic hydrazones (Levine et al., 1994), and expressed as nmole carbonyl/mg BSA.

The protective effects of Na-ALA against this type of protein oxidation were measured in 0.5, 1.0, 2.5, 5.0 and 10.0 mM final concentrations. Each experiment was repeated at least three times, and curve fitting calculations were performed by using the means of these measurements.

3. Results and discussion

3.1. Some general considerations

The main characteristics of the $OH^{\bullet}$ free radical generating system based on the Fenton reaction (Walling, 1975) used in our first two experimental models have been treated in detail in our previous works (Zs.-Nagy and Floyd, 1984a; Gutteridge et al., 1990). An essential requirement of such chemical systems is that Fe^{2+} should remain ferrous iron even at a nearly neutral pH, until the H_2O_2 is added to the mixture. This is assured by using an ADP-Fe^{2+} complex in which the iron is not autoxidized (Floyd and Lewis, 1983). On the other hand, one also has to assure that the antioxidant compound tested should not have any direct oxidative effect on the iron complexed by ADP. This statement requires an experimental control of the situation in the reaction mixtures before H_2O_2 is added to the system. Such a control can be performed by checking the state of iron by means of the ferrozine (Stookey, 1970) reaction. This compound reacts only with Fe^{2+} resulting in an intense pink-colored complex absorbing light of 562 nm, whereas it remained colorless if reacting with Fe^{3+}. The ferrozine reaction, if performed in the presence of the tested substance, before and after addition of hydrogen peroxide may reveal whether the tested compound interferes with the valence state of iron. We have performed experiments according to Carter (1971), and established that even the highest concentration of Na-ALA (10.0 mM) did not influence the Fe^{2+}-ferrozine color reaction before the hydrogen peroxide was added. On the other hand, this

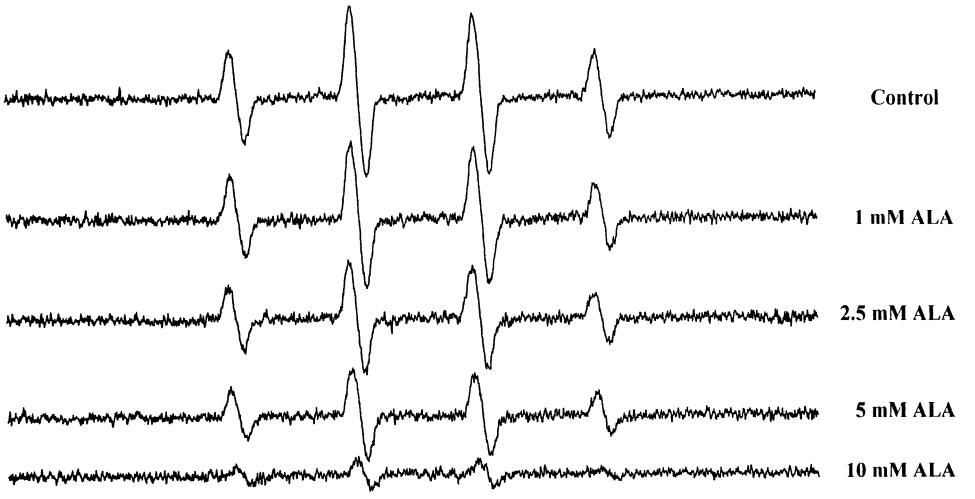

Fig. 2. ESR spectra of the DMPO-OH spin adduct under the influence of various concentrations of Na-ALA. The peak heights decrease proportionally as the Na-ALA concentration increases.

color reaction completely disappeared after hydrogen peroxide was added. These results indicate that Fe^{2+} remained fully accessible for the Fenton reaction in the presence of Na-ALA.

It is known (Floyd and Lewis, 1983) that DMPO reacts with $OH^{\bullet}$ free radicals at a rate constant of 3.4×10^9 $M^{-1}s^{-1}$. This results in the formation of the DMPO-OH spin adduct displaying a characteristic ESR signal. It is important to note that the range of reaction rates for any organic substance with the $OH^{\bullet}$ free radicals is estimated (Walling, 1975) to be 10^7–10^{10} $M^{-1}s^{-1}$. This implies that the $OH^{\bullet}$ free radicals formed in the Fenton reaction (or by the gamma-irradiation) can be captured in a competitive way by the tested compounds present in the reaction mixture. Obviously, the molar ratios of the scavenging components involved, as well as their rate constant ratios will determine the actual amount of various oxidized products. This statement is valid for all three systems used in the present experiments.

3.2. Spin trapping experiments with Na-ALA

In these experiments quantitative data can be obtained about the $OH^{\bullet}$ free radical scavenging ability of the tested substances. Fig. 2 demonstrates the typical ESR curves recorded without (control) or with various concentrations of Na-ALA. The amount of DMPO-OH formed in the system is proportional to the peak height (P) which is always measured at the second peak of the ESR curve on the actual recording. It can be compared in terms of percentile (P%, or ΔP%, the latter indicating the decrease in peak height) between various experiments, provided that the instrumental parameters remain identical. It is obvious from Fig. 2 that additions of increasing concentrations of Na-ALA reduce P considerably.

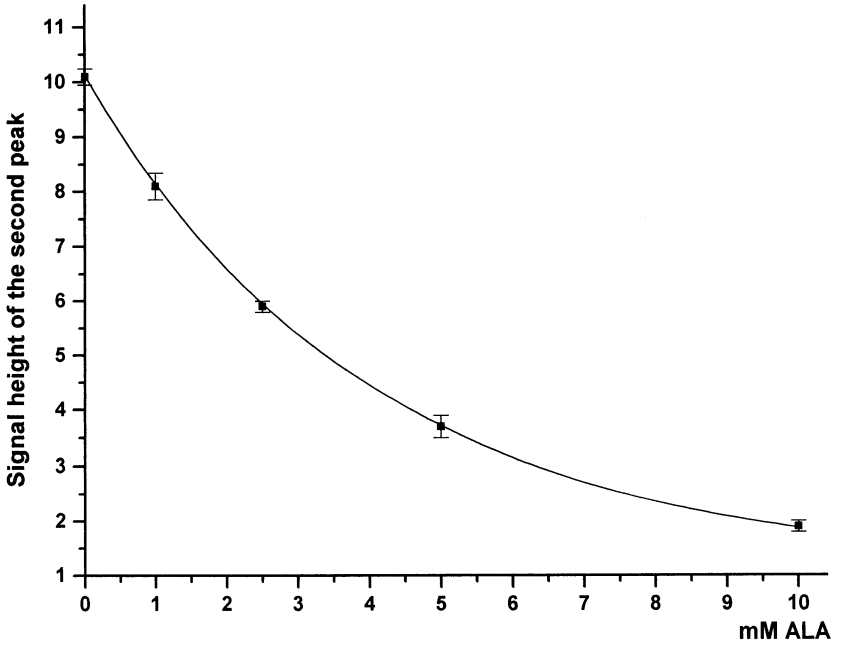

Fig. 3. Statistical evaluation of the peak height of the DMPO-OH spin adduct (measured always on the second peak, shown on Fig. 2) in cm (vertical scale). The horizontal scale indicates the Na-ALA concentrations added to the system.

Fig. 3 summarizes the changes in P caused by Na-ALA as average values with their statistical scatter. They are also compared in terms of percentile ($P\%$) in Table 1. Table 1 contains also $\Delta P\%$ values, meant as the loss of signal height from DMPO-OH. This parameter can be utilized to estimate the rate constant of the $OH^{\bullet}$ free radical reaction of Na-ALA (k_A) as follows:

$$k_A = k_D(\Delta P\%/P\%)(M_D/M_A) \quad (1)$$

where $k_D = 3.4 \times 10^9\ M^{-1}\ s^{-1}$ (Floyd and Lewis, 1983), $\Delta P\%$ and $P\%$ are

Table 1
Spin-trapping experiments with DMPO and Na-ALA[a]

Composition of the test system	$P\%$	$\Delta P\%$	$(\Delta P\%/P\%)$ (M_D/M_A)
DMPO+FR	100.00		
DMPO+1.0 mM Na-ALA+FR	80.43	19.57	4.80
DMPO+2.5 mM Na-ALA+FR	58.79	41.21	5.60
DMPO+5.0 mM Na-ALA+FR	36.66	63.34	6.92
DMPO+10.0 mM Na-ALA+FR	18.58	81.42	8.76

[a] FR, Fenton reactant; for other symbols see the text.

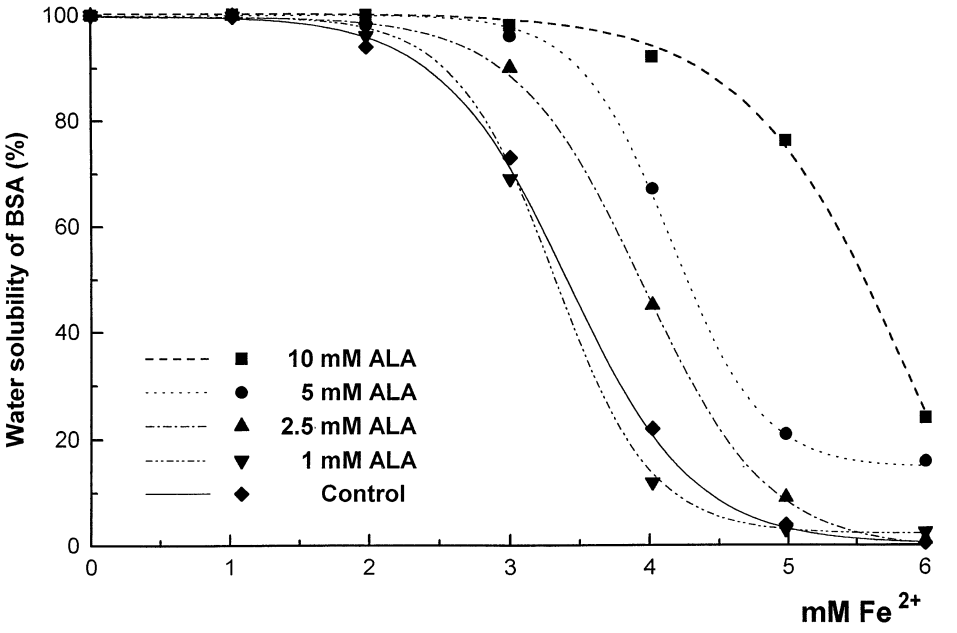

Fig. 4. The effect of Fenton reactants and various Na-ALA concentrations on the water solubility of BSA. Each symbol represents the average of three parallel measurements. Other details are described in the text.

explained above, M_D and M_A indicate the molar concentrations of DMPO and Na-ALA, respectively, present in the utilized system.

For Eq. (1) we calculated the factor $(\Delta P\%/P\%)(M_D/M_A)$ and reported in Table 1. The values of this factor are somewhat different from each other for various Na-ALA concentrations. This is most probably due to the fact that the $OH^{\bullet}$ free radical reactions are not usually increasing linearly with the concentration. One can assume that the most realistic value for this factor is the one belonging to 10 mM Na-ALA, as this is the ALA concentration closest to that of DMPO (always 20 mM). Taking this latter figure for Eq. (1), one obtains for $k_A = 3.0 \times 10^{10}\ M^{-1}\ s^{-1}$. This value is quite close to that calculated by (Scott et al., 1994) amounting to $4.7 \times 10^{10}\ M^{-1}\ s^{-1}$, and indicates that Na-ALA is also a very potent $OH^{\bullet}$ free radical scavenger, which may be competitive against almost all biological compounds whose rate constant (Walling, 1975) is in the range of 10^7–$10^{10}\ M^{-1}\ s^{-1}$.

3.3. Na-ALA and the protein cross linking

In the chemical system where BSA was exposed to the Fenton reaction, BSA loses its water solubility according to a sigmoid curve: the decay is slow up to about 2 mM Fe^{2+} concentration, and becomes much faster above it (Fig. 4). A loss of 50% solubility is recorded around 3.2 mM iron, and practically no water solubility remains at 6.0 mM. Control experiments have shown that omission of either

ADP-Fe^{2+} or the hydrogen peroxide from the reaction mixture result in a constant maintenance of the water solubility of BSA (data not shown).

When various concentrations of Na-AlA were added to this system, a protective effect was observed against the cross linking of proteins.

Fig. 4 demonstrates the shifts of the control curve under the effects of 1.0–10.0 mM Na-ALA. concentrations. The lowest Na-ALA concentration (1 mM) was able to maintain a higher water solubility of BSA up to about 3 mM Fe^{2+} concentration, and was not effective above this concentration, whereas the higher Na-ALA concentrations displayed a considerable protective effect in the whole range of iron contents studied.

These results show unanimously that Na-ALA is an efficient competitor in capturing $OH^{\bullet}$ free radicals for BSA, and most probably any other proteins which have a similar reactivity with those radicals.

3.4. Protective effects of Na-ALA against radiation-induced carbonyl formation

When BSA (0.33 mg/ml) solution was exposed to 80 krad Co-gamma irradiation, a significant increase of the protein carbonyl content was detected, on the average of 86.6 nmol carbonyl/mg BSA. This value was taken as 100% in the following experiments. (The original carbonyl content of the non-irradiated BSA is included in the blank value, therefore, it was virtually considered to be zero).

When adding various concentrations of Na-ALA to the irradiated systems, a considerable decrease of carbonyl formation was observed. Table 2 summarizes the results obtained. It is evident from the data that 1 mM Na-ALA concentration causes an 88% inhibition of the radiation-induced protein oxidation, and the higher concentrations (2.5, 5.0 or 10.0 mM are able to increase this effect up to about 94–95%. Taking into account the stochastic nature of carbonyl formation, this inhibition level can be considered as the maximally aehievable one. These results were so highly reproducible that it was sufficient to repeat them only three times, and the observed scatters remained within a few relative percents.

The significance of these experiments, compared to those obtained in the previous two models is underlined by the fact that we used a completely different method of $OH^{\bullet}$-free radical generation. Yet the observations confirmed again that Na-ALA is

Table 2
Protective effect of Na-ALA against protein oxidation[a]

Composition of the test system	*C*%	Δ*C*%
BSA + IR	100.00	
BSA + 0.5 mM Na-ALA + IR	21.36	78.64
BSA + 1.0 mM Na-ALA + IR	11.78	88.22
BSA + 2.5 mM Na-ALA + IR	5.77	94.23
BSA + 5.0 mM Na-ALA + IR	5.54	94.46
BSA + 10.0 mM Na-ALA + IR	5.08	94.92

[a] IR, 80 krad Co-gamma irradiation; BSA was present in 0. 33 mg/ml; *C*%, the relative carbonyl contents (l00% = 86.6 nmole carbonyl/mg BSA; Δ*C*%, the perceptual inhibition of carbonyl formation.

a very potent protector of the proteins against $OH^{\bullet}$ free radical-induced alterations, independently from the origin, or the mechanism of generation of these free radicals.

3.5. The possible mechanism of the Na-ALA reactions

Na-ALA was not reduced in any of the above experiments. Therefore, the most probable reaction site involved in the $OH^{\bullet}$ free radical reactions seems to be the disulfide group of Na-ALA. Oxidation of this group may easily take place (Haenen and Bast, 1991; Scott et al., 1994; Biewenga and Bast, 1995; Passwater, 1996), giving thiosulfinate (BAL) or thiosulfonate. Although the present experiments did not elucidate further details of this type of chemistry, they call the attention to the importance of this aspect.

ALA is a natural compound with extremely low toxicity (Packer et al., 1995; Passwater, 1996). Thus it is worth pursuing further studies on the protein-protective effects of this agent not only in vitro, but also in cell cultures and eventually in vivo. Na-ALA may be considered a potential biologically useful free-radical scavenger for human therapy, explaining also the beneficial effects of its topical application against certain age-dependent skin alterations (Perricone, 1997, 1999).

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