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An electron spin resonance (ESR) study on the mechanism of ascorbyl radical production by metal-binding proteins

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The mechanism of ascorbate oxidation by metal-binding proteins (ceruloplasmin, albumin and transferrin) was investigated *in vitro* and in isolated plasma by the measurement of the ascorbyl free radicals (AFR) by electron spin resonance (ESR). In plasma of 13 healthy volunteers, a spontaneous and variable production of AFR was detected, which was increased by a 10^{-4} M ascorbate overloading; however, this increase was not correlated to the intensity of the spontaneous AFR signal. The addition of Cu^{2+} and ceruloplasmin to plasma increased the ESR signal, while the addition of transferrin decreased the signal intensity in a dose-dependent manner. *In vitro*, we demonstrated that ascorbate was oxidized by human serum albumin and by ceruloplasmin, and that this oxidase-like activity was lost by trypsin or heat treatment of these proteins. These two proteins positively interacted in the oxidation of ascorbate, since addition of crude albumin to a solution of ascorbate and ceruloplasmin increased the intensity of ESR signal in a dose-dependent manner. The treatment of albumin by a metal chelator (DDTC) abolished these positive interactions. The respective roles of copper and iron in ascorbate oxidation were studied and showed a dose-dependent effect of these ions on ascorbate oxidation. The role of iron was confirmed by the inhibiting effect of metal-free transferrin on iron-dependent ascorbate oxidation. Concerted actions between iron carrying albumin and copper carrying ceruloplasmin appear responsible for the production of AFR *in vitro* and *in vivo*.

Keywords: ascorbate, albumin, ceruloplasmin, copper, electron spin resonance, iron, transferrin

Introduction

Ascorbic acid (vitamin C) can be oxidized by one-electron steps into dehydroascorbic acid, by the way of two ascorbyl radicals ($A^{\cdot-}$ or $AH^{\cdot}$) (Bielski *et al.* 1981). Ascorbate free radicals (AFR) can be generated from ascorbic acid, either by reaction with free radicals, such as superoxide anion and hydroxyl (Bielski *et al.* 1971, 1981), or by oxidation in the presence of transition metals, such as iron and more efficiently, copper (Martell 1982). Moreover, it has recently been reported that peroxynitrite could induce the formation of free radicals, which can react with ascorbate to generate ascorbyl radicals detected by electron spin resonance (ESR) measurement (Vásquez-Vivar *et al.* 1996).

The first observation of AFR in blood was realized by Dodd and Giron-Conland (1975). Lohmann

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et al. (1981) observed a long-lived ESR signal of AFR in the blood of normal humans after an intravenous injection of ascorbate. Sasaki *et al.* (1982) reported that AFR can be spontaneously observed in the plasma and the serum of certain healthy humans, and established a gaussian curve repartition of AFR in 244 volunteers. Lohmann (1981) hypothesized that the blood might possess an ascorbate oxidase-like activity, and Sasaki *et al.* (1985) gave the evidence that both albumin and ceruloplasmin (Cp) were involved in this phenomenon. They also suggested that blood AFR could arise *in vivo* from the reaction of ascorbate with free radicals generated elsewhere in the organism. Later, Pietri *et al.* (1990, 1994) proposed the detection of AFR as a monitor of free radical generation during ischemia-reperfusion of human heart. Their observations were supported by Minakata *et al.* (1993), who observed a rise of AFR detected by ESR signal in human intoxicated with paraquat. The detection of AFR was also used as an index of free radical formation in tissues by several other teams (Buettner & Jurkiewicz 1993, Roginsky & Stegmann 1994, Sharma *et al.* 1994, Matsuo *et al.* 1995).

However, Minetti *et al.* (1992) demonstrated that AFR signal cannot be evidenced by ESR in the whole blood, but only in the plasma; moreover, these authors demonstrated that when the plasma was overloaded with iron, the ESR signal of AFR was significantly enhanced. In a recent paper, Lovstad (1995), using non-ESR techniques, demonstrated the interactions between albumin, Cp and iron in the formation of AFR in ascorbate solutions, and observed that citrate and apo-transferrin, acting as iron chelators, inhibited the oxidation of ascorbate.

Until now, no experiment using plasma overloaded with ascorbate has been done, to better evidence the 'ascorbate oxidase-like' activity (AOA) of plasma. The question also remains debated whether the ascorbyl radical level of plasma can be taken as a specific monitor of radicalar reactions occuring in the organism? Copper exerts a strong oxidative action on ascorbate (Martell 1982), and because the copper and Cp levels of plasma are under the control of sexual hormones and cytokines (Linder 1991a), other factors than radicalar reactions can be involved in the generation of AFR signals in blood.

The aim of this work was to contribute to the better understanding of the mechanism of ascorbate oxidation in plasma using ESR technique. We especially focused on the role of Cp in ascorbate oxidation and its interactions with copper, iron and albumin.

Material and methods

Reagents

L(–)+ ascorbic acid (vitamin C, 99%), dehydroascorbic acid, diethyldithiocarbamate (DDTC) were from Aldrich (Bornem, Belgium). Human albumin (Cohn's fraction V), human ceruloplasmin, human holotransferrin and heparin were from Sigma (Sigma-Aldrich, Bornem, Belgium). Bovine trypsin was from Boehringer Biochemicals (Mannheim, Germany). All the other reagents, iron(II) and copper(II) sulfates, tri-(hydroxymethyl)-aminomethane (Tris), phosphate salts, sodium chloride, sodium citrate and ethylenediamine tetraacetic acid (EDTA) were analytical grade from Merck (Darmstadt, Germany). Chelex100 Resin (200–400 mesh, sodium form) was from Bio-Rad Laboratories, Nazareth, Belgium). Dialysis tubes were from Medicell International LTD (Vel, Leuven, Belgium). Polyacrylamide electrophoresis gels (Excel Gel SDS, gradient 8–18) and Coomassie Brillant Blue were from Pharmacia Biotech (The Netherlands).

Blood sampling

Blood samples were taken on heparin (12.5 IU mL^{-1}) from 13 healthy volunteers (6 men, 7 women; mean age : 38 ± 7 years) by venipuncture of radial vein. None of them had taken supplementary vitamin C for 10 days before the blood sampling. In one volunteer (woman, 52 years), blood samples were also drawn on 0.15 M citrate (1 ml for 9 ml of blood), on EDTA (1 mg ml^{-1}) and without anticoagulant. After drawing, blood was immediately centrifuged (2000 *g*; 5 min) and plasma was used directly after centrifugation. Serum was isolated by centrifugation after blood clotting for 30 min at 37°C in glass tube. The spontaneous ascorbyl ESR signal was measured in serum or plasma; immediately after, 1×10^{-4} M ascorbate (chelexed solution, pH 7.4) was added and the ESR signal was monitored in the same conditions.

Reagent solutions

All the solutions for ESR studies were prepared in milli-Q water. Stock solutions of copper, iron and ascorbate were prepared daily and dilutions were made extemporaneously.

Protein solutions

Albumin solutions. The solution of human serum albumin (HSA) was prepared daily in phosphate buffer (pH 7.4, NaCl 0.15 M) and used immediately at the final concentration of 60 mg ml^{-1}, a value near the normal concentration of plasma. For some experiments HSA was treated either by DDTC or by trypsin.

(i) *DDTC treatment.* This treatment was performed to remove by complexation the metal ions which are carried by this protein. DDTC was added at the final concentra-

tion of 0.01 M to 1.2 g of HSA dissolved in 20 ml 0.2 M Na acetate 0.15 M NaCl at pH 6.0. This mixture was dialysed against 0.001 M DDTC in 0.02 M Na acetate at + 4°C for 48 h with 10 changes of the external milieu. After dialysis, HSA was concentrated by lyophilization. This HSA was used in comparison with untreated (crude) HSA.

(ii) *HSA denaturation.* HSA was denatured either by heat treatment (70°C, 15 min) or by proteolytic digestion with increasing concentrations of bovine trypsin (from 0.2 to 1%) for 1 h at 37°C in 0.2 M Tris–HC1 buffer added with 20 mM $CaCl_2$ at pH 7.4. Trypsic digestion was monitored by polyacrylamide gel electrophoresis (followed by Coomassie Brillant Blue staining). The degradation of the protein was calculated after scanning of the electrophoresis bands and computerized measurement of the density. With 1% trypsin, albumin was degraded at 87%.

Ceruloplasmin solutions. Cp was dissolved in phosphate buffer (pH 7.4, NaCl 0.15 M) and used immediately at the final concentration of 11 U $ml.^{-1}$ For some assays, Cp was denatured by heating or by proteolytic digestion with bovine trypsin in the same manner as for HSA. Denaturation of Cp was monitored by polyacrylamide gel electrophoresis (followed by Coomassie Brillant Blue staining); for 1% trypsin, Cp was degraded at 72%.

Transferrin solutions. Human holo-transferrin (10 mg ml^{-1}) was dissolved in phosphate buffer (pH 7.4, NaCl 0.15 M) and treated with chelex (column : 10 cm length, 1 cm dia.) at room temperature in order to remove metal ions. The chelexed protein was used immediately.

In vitro *studies of ascorbate oxidase-like activity*

The AOA of HSA, Cp and transferrin was measured by monitoring the height of ESR signal of ascorbyl radicals produced by the oxidation of ascorbate. The addition of metal ions was always performed before the addition of ascorbate to the reaction milieu. The final volume was 1 ml and the final concentration of ascorbate was 1×10^{-4} M. the ESR spectrum of the reaction milieu before the addition of ascorbate was taken as control value (no signal of ascorbyl radical).

Electron spin resonance (ESR) spectroscopy

For ESR studies, the ascorbate solution was added to the reaction milieu just before ESR signal measurement. The sample was then immediately transferred into the flat quartz cell of the spectrometer. ESR spectra were recorded at room temperature on a Bruker ESP 300 E spectrometer operating at X-band (9.78 GHz) with 100 kHz frequency modulation. The instrumental settings were : microwave field 3480 G, amplitude modulation 1.0 G, receiver gain 2×10^4, time constant 81.92 ms, time conversion 164 ms, sweep width 50 G, sweep time 82 s $scan^{-1}$ and total scan time of 7 min. Hyperfine coupling constants were measured directly from the experimental spectra and compared with literature values (Yamasaki & Piette 1961, Pietri *et al.* 1990, 1994). Signal heights were determined from measurements of line intensities on spectra recorded with identical instrumental settings, and expressed in arbitrary units.

Results

Studies on isolated plasma

Presence of a spontaneous ascorbyl radical and effects of overloading with ascorbate. The ESR signal of AFR could be detected in the isolated heparinized plasma of the 13 healthy volunteers, but the intensity of this ESR signal was highly variable as it appeared on Figure 1 (spectra B) for 2 chosen volunteers and in Figure 2 (black histograms) for the 13 volunteers. The overloading of the plasma sample with 1×10^{-4} M ascorbate always resulted in an increase of the ESR signal indicating an 'ascorbate oxidase' activity of the plasma. But, this increase was variable and not correlated to the intensity of the signal measured before overloading (Figure 1,

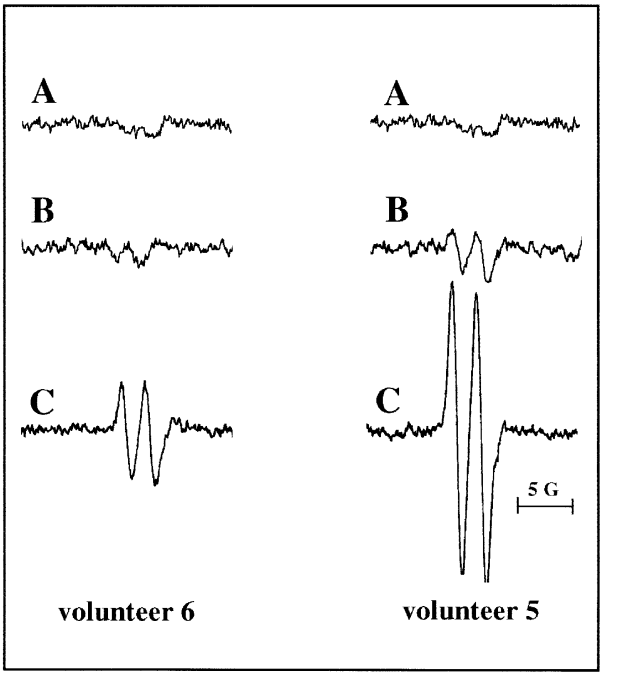

Figure 1. ESR spectra of ascorbyl radicals in the heparinized plasma of two healthy volunteers (subjects 5 and 6) before (spectra B) and after overloading with 1×10^{-4} M ascorbate (spectra C). Spectra A are the ESR controls obtained for the chelexed ascorbate solution (1×10^{-4} M at pH 7.4) before its addition to the plasma.

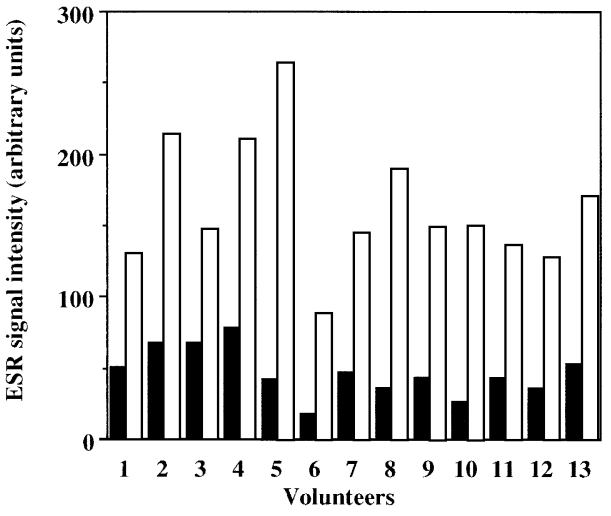

Figure 2. Height of ESR signal of ascorbyl radicals measured in the heparinized plasma of 13 healthy volunteers before (■) and after overloading with 1×10^{-4} M ascorbate (□).

spectra C and Figure 2, grey histograms). When plasma samples were overloaded with dehydroascorbate instead of ascorbate, no change of the ESR signal could be observed.

Role of the anticoagulant on the ascorbyl signal of isolated plasma. The height of ESR signal measured in serum was taken as control value (100%). Compared to this control value, the highest ESR signal was found in EDTA anticoagulated plasma (Table 1), while ESR values were similar for heparinized and citrated plasma and lower than in serum and EDTA. After overloading with ascorbate, an increase in ESR signal was observed in all the samples, but this increase was the highest in EDTA plasma and was different for each sample compared to the value measured before overloading.

Table 1. Effects of anticoagulant on the ESR signal intensity (arbitrary units) of ascorbyl radicals. The value measured in serum without overloading with ascorbate was taken as 100%

Anticoagulant	1×10^{-4} M ascorbate addition	
	No	Yes
None	72.5 (100%)	159.5 (220%)
Heparin	51.3 (71%)	130.2 (180%)
Citrate	50.6 (70%)	166.0 (229%)
EDTA	119.7 (165%)	294.7 (407%)

Effects of plasma overloading with ceruloplasmin and copper. The addition of increasing concentrations of Cp to ascorbate overloaded plasma led to a slight increase of ESR signal, which was not proportional to the concentration of added Cp (Table 2). Addition of Cu^{2+} strongly enhanced the ESR signal, but the supplementary addition of Cp did not further increase the height of the signal. For the lowest Cp concentration, there was even a decrease of the signal compared to that obtained with copper alone.

Effects of transferrin. Two control assays were performed : without ascorbate overloading (control 1) and with ascorbate addition (control 2). The ESR signal height of ascorbyl radical for these controls was taken as 0% inhibition in parts A and B of Table 3. The addition of transferrin to a fresh plasma sample induced an inhibitory effect of 45.86% on the ESR signal height (Table 3A). When increasing doses of transferrin were added to the ascorbate overloaded plasma sample, a dose-dependent inhibitory effect on ESR signal height was observed (Table 3B).

Table 2. Effects of ceruloplasmin and copper added to heparinized plasma overloaded with 1×10^{-4} M ascorbate, on the ESR signal intensity (arbitrary units) of ascorbyl radicals.

Ceruloplasmin [IU ml^{-1}]	Height of ESR signal	
	no Cu^{2+}	Cu^{2+} (5×10^{-5} M)
0	130	447
8.8	132	397
17.6	147	457
36.0	157	473

Table 3. Effects of the addition of transferrin on ESR signal intensity of ascorbyl radicals in isolated plasma without or with overloading with 1×10^{-4} M ascorbate

		Transferrin [mg ml^{-1}]	% inhibition of ESR signal
A	Plasma	0	0
		3.37	45.86
B	Plasma + ascorbate	0	0
		0.48	12.70
		0.96	26.99
		3.67	50.80

In vitro *studies on the mechanisms of ascorbate oxidase-like activity*

Effect of human serum albumin. Crude HSA exerted an AOA on ascorbate, leading to the formation of AFR (Figure 3). The effects of increasing concentrations of ascorbate added to the HSA solution (60 mg ml^{-1}) on the intensity of this ESR signal were determined and a maximal intensity of the ESR signal with 6.25×10^{-4} M ascorbate was found; however, at 1×10^{-4} M ascorbate, the physiological normal value, an important ESR signal was already observed, and we choose this value for further studies (Figure 3).

When HSA was denatured by heat or proteolytic digestion, its AOA was diminished proportionally to the % of trypsin used for the digestion (Figure 4). After treatment with 1% trypsin, albumin only conserved 28.84% of its AOA. Electrophoresis performed on trypsin-treated HSA confirmed that the denaturation was increased with the increase of the trypsin concentration. In the same manner, HSA treated by heat partially lost its AOA.

Effect of ceruloplasmin. Cp (155 IU ml^{-1}) also presented an AOA, which generated an ESR signal. As for HSA, the denaturation of Cp by heat or with increasing concentrations of trypsin decreased its AOA (Figure 4), proportionally to the concentration of trypsin used for Cp digestion. After treatment with 1% trypsin, Cp only retained 49.3% of its AOA. The confirmation of this proteolytic digestion was obtained by electrophoresis (Figure 4).

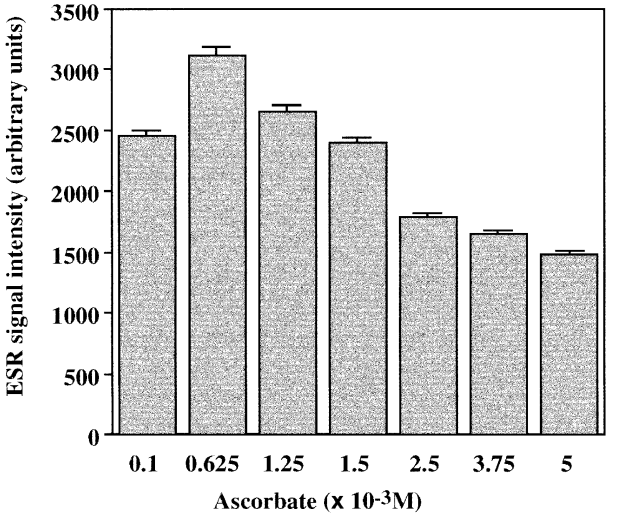

Figure 3. Role of the ascorbate concentration added to an albumin solution (60 mg ml^{-1}) on the intensity of the ESR signal of ascorbyl radicals (mean value; $n = 3$).

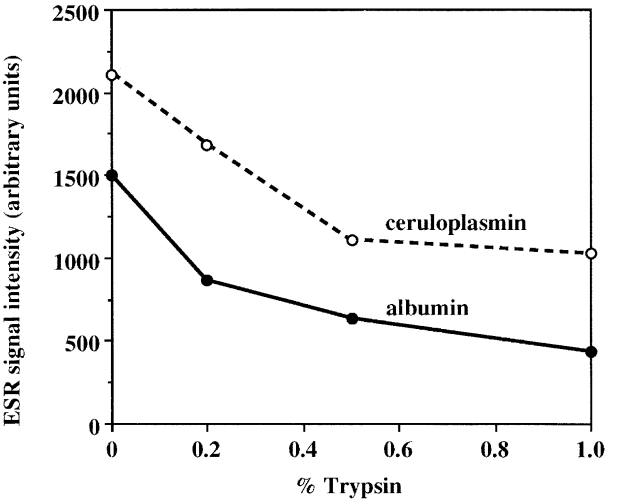

Figure 4. Effect of the denaturation of human serum albumin and human ceruloplasmin by increasing concentrations of trypsin on the ascorbate oxidase-like activity (measured by the height of ESR signal of ascorbyl radicals) of these two proteins on a 1×10^{-4} M ascorbate solution.

Interaction between HSA and ceruloplasmin. The addition of crude HSA enhanced the AOA of Cp in a dose-dependent manner (Figure 5). This enhancement activity of HSA appeared linked to the transport of metal ions by HSA since the treatment of this protein with DDTC, a metal chelator, suppressed this enhancing effect (Figure 5).

Effect of copper and iron. Iron (Fe^{2+}) and copper (Cu^{2+}) both generated ascorbyl radicals from ascorbate (Figure 6). We observed an enhancing effect of Fe^{2+} until 2×10^{-5} M; at higher concentrations, the ESR signal intensity decreased. The addition of increasing concentrations of Cu^{2+} resulted in a progressive increase of the ESR signal. At 5×10^{-5} M, the action of the two metal ions was similar.

AOA of human transferrin. The effects of chelexation were monitored by spectrophotometry at 280 nm and 465 nm; we observed the disappearance of absorbance at 465 nm indicating that the extraction of iron was complete (or nearly complete). In the absence of transferrin, an ESR signal was observed, and the addition of crude transferrin increased this ESR signal in a dose-dependent manner. But, when transferrin was chelexed for removing all the metal ions, a slight decrease of signal was observed, proportional to the concentration of treated transferrin (Figure 7).

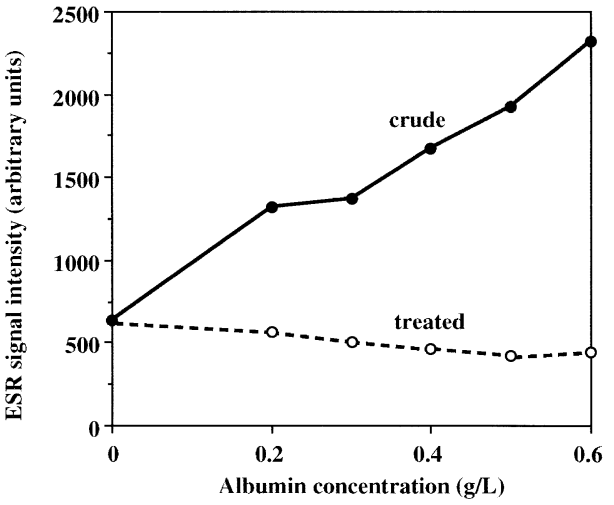

Figure 5. Effect of increasing human serum albumin addition on the ascorbate oxidase-like activity of ceruloplasmin (measured by the height of ESR signal of ascorbyl radicals) generated from 1×10^{-4} M ascorbate. Comparison between crude and metal free albumin (treated with DDTC).

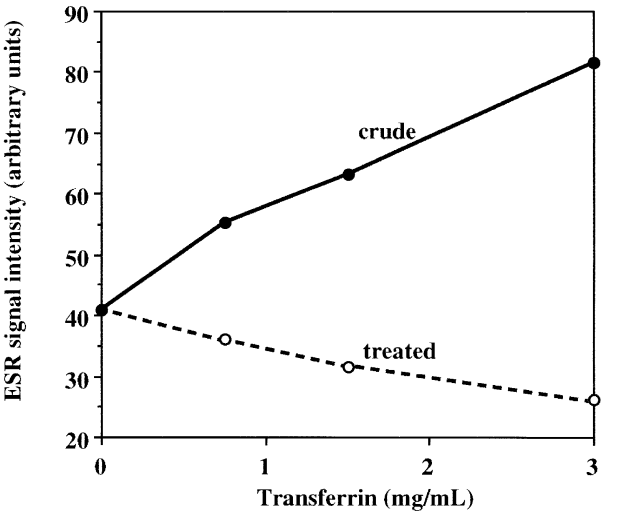

Figure 7. Ascorbate oxidase-like activity of crude or chelexed human transferrin on 1×10^{-4} M ascorbate.

Discussion

Until now, no data were published, reporting the capacity of isolated plasma to oxidize ascorbate. After overloading of isolated plasma with exogenous ascorbate, we observed a significant enhancement of the ESR signal of the ascorbyl radical. From these observations, we thus concluded that the plasma had its own ascorbate oxidizing activity. We also observed that the intensity of the spontaneous signal of ascorbyl in plasma varied from one donor to another and with the nature of the anticoagulant used for blood sampling (the ESR signal was lowered in citrated plasma), and that the overloading with ascorbate always led to a variable increase of the ascorbyl signal without correlation with the initial intensity of the signal before the overloading (Table 1 and Figures 1 and 2). The addition of Cu(II) enhanced the signal, and thus the ascorbate oxidation in plasma, underlining the preeminent role of this metal ion. On the contrary, the addition of iron-free transferrin to plasma diminished the importance of the ascorbate oxidation, an inhibitory effect that was confirmed after ascorbate overloading.

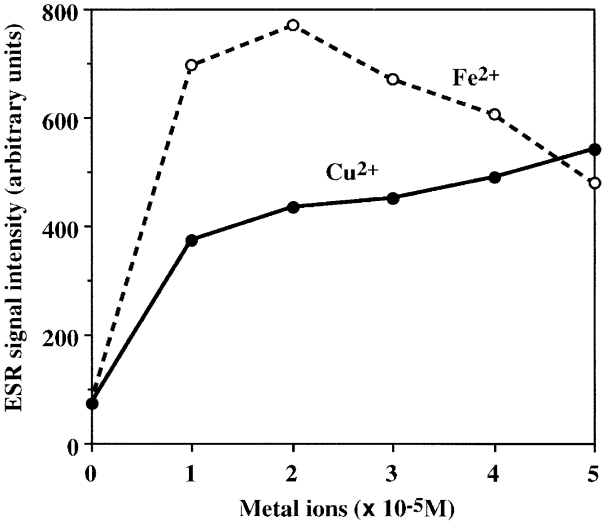

Figure 6. Effect of iron (Fe^{2+}) and copper (Cu^{2+}) on the intensity of ESR signal of ascorbyl radicals generated from a 1×10^{-4} M ascorbate solution.

This concept of an 'ascorbate oxidase-like' activity (AOA) of plasma was first suggested by Curzon Young (1972) who considered that the acute phase protein Cp was an animal ascorbate oxidase. Lohmann (1981) agreed with this opinion, and later, Sasaki *et al.* (1985) attributed the AOA of plasma to albumin and Cp.

By measuring the oxygen consumption as a witness of ascorbate oxidation, Lovstad (1995) recently demonstrated that this oxidation is mediated by a catalytic redox system formed by copper atoms bound to Cp and iron atoms complexed by albumin. He also observed that citrate acted as a chelator in this system, lowering the kinetics of

ascorbate oxidation and that holotransferrin strongly inhibited the catalytic system. Our ESR experiments with pure compounds (albumin, Cp, copper, iron and transferrin) afforded data similar to those obtained by Lovstad with electrometric measurements. We also observed that the ESR signal of ascorbyl radical rapidly disappeared in the absence of oxygen.

Oxygen is directly reduced in water by an addition of four electrons in one step, a process which avoids the production of excited oxygen species intermediates (Malmström 1982):

$$4\ Cu^{+} \rightarrow 4\ Cu^{2+} + 4e^{-} \quad [1]$$

$$O_2 + 4e^{-} + 4H^{+} \rightarrow 2H_2O \quad [2]$$

Lovstad (1995) suggested that concerted redox reactions occurred between Cu bound to Cp, Fe bound to albumin or another ligand, and ascorbate reducing Fe^{3+} and oxygen. In these concerted actions, the ferroxidasic activity of Cp played a master role: $4\ Fe^{2+} + 4\ Cu^{2+} \rightarrow 4\ Cu^{+} + 4\ Fe^{3+}$.

The proteolytic digestion of Cp and albumin by trypsin decreased the ascorbate-like oxidase activity (Figure 4). Near 50% of Cp-dependent AOA and 90% of HSA dependent AOA were lost after proteolysis. Ortel *et al.* (1983) demonstrated that trypsination of Cp released a 19 kDa fragment bearing three copper atoms. It is generally admitted that the oxygen activating site of Cp is constituted by a cluster of three copper atoms (Calabrese *et al.* 1988, 1989). But, for Linder (1991b) and Fox & Karlin (1995), this site would be a four copper atom oxidative unit, as for the other blue oxidases (laccase and ascorbate oxidase) which also completely reduce oxygen. The lowering effect of trypsination on AOA could thus be explained by the destruction of this oxidative unit.

The AOA of plasma could thus be attributed to the concerted reactions that are mentioned above, variable from one volunteer to another. Moreover, in one volunteer, we observed that the AOA of the plasma fell after oral administration of a non-steroidal anti-inflammatory drug (meclofenamate; data not shown).

The individual variations of plasmatic AOA can be explained by *in vivo* limited levels of ascorbate, Cp, and redox active forms of iron. The plasma level of Cp is particularly dependent from many factors : cytokines (TNFα and IL1), oestrogens and corticosteroids modify the Cp production by hepatic cells (Linder 1991b). From the observations that plasma from healthy human volunteers can produce a strong enhancement of the ESR signal of ascorbyl after overloading with ascorbate, it ensues that an inflow of vitamin C (coming from the digestive tract, by example) will contribute to the amplitude of ESR signal.

In conclusion, it appears that the plasma possesses its own AOA, which is a redox system formed by the copper atoms cluster of Cp, by iron bound to albumin and by oxygen. This AOA thus depends on several individual factors, which themselves depend on humoral factors and on the presence of siderophores. The results of AOA measurement will thus largely vary with the origin of plasma.

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