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Site-specific modification of albumin by free radicals

Reaction with copper(II) and ascorbate

Gerard MARX*‡ and Mordechai CHEVION†

*Department of Hematology, Beilinson Medical Center, Petah Tikva, Israel, and †Department of Cellular Biochemistry, Hebrew University–Hadassah Medical School, Jerusalem, Israel

Exposure of albumin to Cu(II) (10–100 μM) and ascorbate (0.1–2 mM) results in extensive molecular modifications, indicated by decreased fluorescence and chain breaks. The rate of utilization of molecular oxygen and ascorbate as a function of Cu(II) concentration is non-linear at copper/albumin ratios of > 1. It appears that Cu(II) bound to the tightest albumin-binding site is less available to the ascorbate than the more loosely bound cation. SDS/polyacrylamide-gel electrophoresis reveals new protein bands corresponding to 50, 47, 22, 18 and 3 kDa. For such a cleavage pattern, relatively few (~ 3) and rather specific chain breaks occurred. Repeated addition of portions of ascorbate to the albumin/Cu(II) mixture results in increased intensity of the new bands. The absence of Cu(II) or the presence of metal chelating agents is inhibitory. There was no evidence of intermolecular cross-linking or of the formation of insoluble, albumin-derived, material. A mechanism is proposed wherein the loosely bound Cu(II) participates in a Fenton-type reaction. This generates OH$^{\cdot}$ radicals, which rapidly inter-react with the protein and modify it in a 'site-specific' manner.

INTRODUCTION

Free radicals, generated by the reaction of Cu(II) [or Fe(III) salts] with ascorbate, are capable of modifying proteins in various ways [1–8]; modifications can be measured by the release of NH_3 [1], decreased fluorescence [2], loss of enzyme activity [3, 4], protein coagulation [7] and changes in amino acid composition [5, 6]. In order to develop a general model for the modifications undergone by a plasma protein with chemically generated free radicals, we examined the effect of Cu(II) and ascorbate on albumin.

Cation binding to a few sites on the albumin molecule is an important facet in the binding and physiological distribution of other metabolites or drugs [9,10]. For Cu(II), the tightest binding site on albumin is the *N*-terminal sequence Asp-Ala-His ($K_D = 6.61 \times 10^{-17}$ M^{-1}) [11–16]. Other cation-binding sites have been less well characterized, though His-9 and His-18 are constituents of a second Cu(II)-binding site of peptide 1–24 [15, 16], and Cys-34, which has a free thiol group, has been reported to participate in the binding of heavy metals [17].

Our experiments demonstrate that Cu(II) bound to albumin at the 'loose' binding sites are available for interaction with ascorbate. As a consequence, free radicals are generated via a Fenton reaction and localized modifications, such as specific chain breaks, occur.

MATERIALS AND METHODS

Albumin (human and bovine), ascorbate, $CuSO_4$ and Tris were purchased from Sigma (St. Louis, MO, U.S.A.). Stock solutions of the protein and reagents were prepared in 0.015 M-Tris/0.15 M-NaCl, pH 7.4, and all dilutions were made in this buffer. The reaction between albumin, Cu(II) and ascorbate was carried out in separate test tubes at 37 °C, as follows. Albumin (1 mg/ml; 15 μM) and Cu(II) (< 100 μM) were incubated at 37 °C and a portion of stock ascorbate (5–20 mM in Tris buffer) was added (final concn. 0.1–2 mM). In some experiments, up to ten portions of ascorbate were added at 15 min intervals. After appropriate dilution, the emission fluorescence spectrum (excitation 290 nm, emission 300–400 nm with the maximum at ~ 340 nm) was recorded at pH 7.4 and 4 (Perkin–Elmer model 44 spectrofluorimeter).

To monitor the loss of ascorbate, changes in A_{280} were measured with a Kontron (Zürich, Switzerland) model 801 spectrophotometer. Initial slopes were measured for the minute after addition of ascorbate to a mixture of Cu(II) and albumin, at 25 °C, with an equivalent concentration of albumin in the reference cell, and were recalculated by using a conversion factor of 0.0087 A_{280}/μM-ascorbate.

E.p.r. spectra were recorded on a Varian E-4 spectrophotometer using a modulation amplitude of 1 μT (10 mG) and 10 mW power. Samples were premixed in plastic test tubes at 25 °C and, after the addition of ascorbate, were rapidly (~ 30 s) transferred to Pasteur pipettes sealed at the narrow end and the spectra were recorded at timed intervals. Oxygen-electrode studies were performed at 37 °C (Yellow Springs Instrument Co., Yellow Springs, KY, U.S.A.).

The reaction products were analysed by SDS/10%-(w/v)-polyacrylamide-slab-gel electrophoresis. Gels were stained with Coomassie Blue and densitometry was performed with a Helena Laboratories Quick Scan densitometer.

RESULTS

The rapid decrease in A_{280} is a measure of the loss of ascorbate (Fig. 1). For a fixed concentration of Cu(II),

‡ To whom all correspondence and reprint requests should be sent.

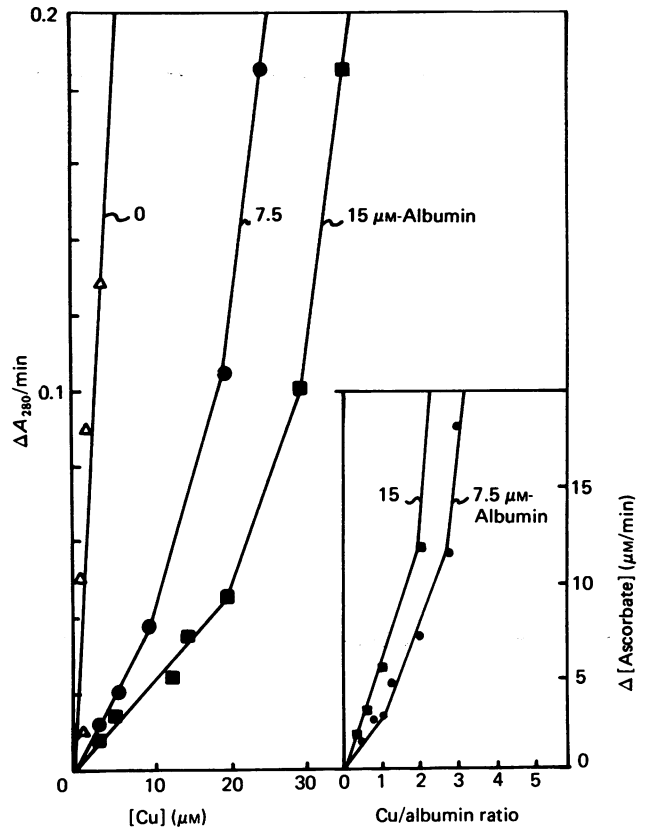

Fig. 1. Rate of decrease of A_{280} at 7.5 μM- and 15 μM-albumin and increasing [Cu(II)]

The inset shows same data as rate of ascorbate consumption versus Cu(II)/albumin ratio.

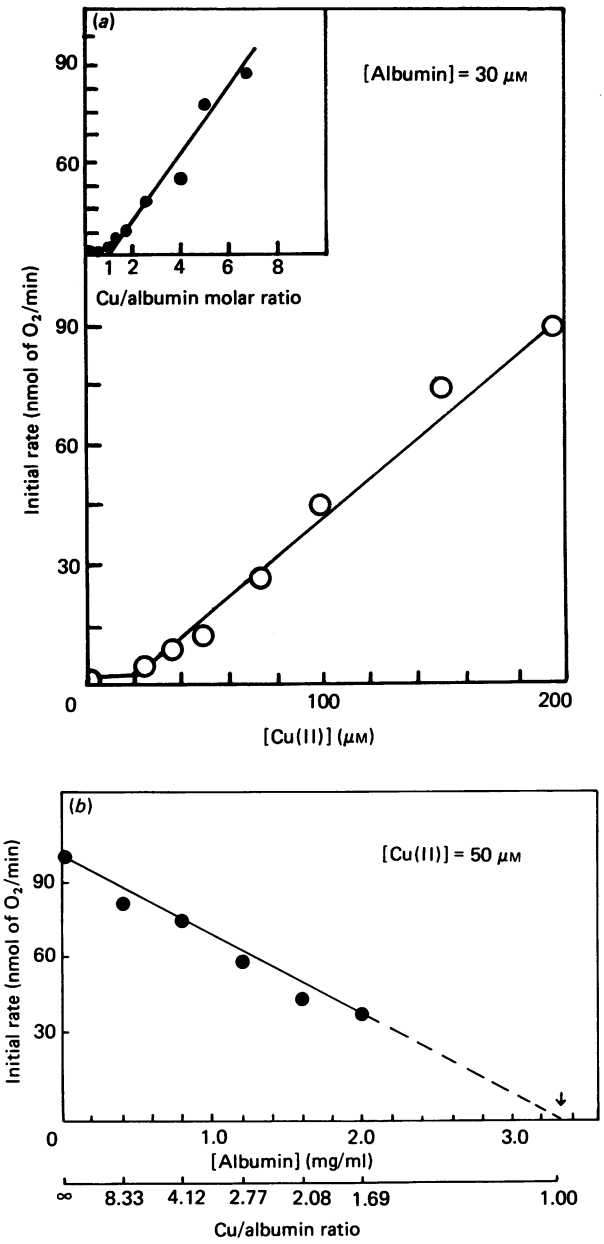

Fig. 2. Initial rates of oxygen consumption

(*a*) Initial rate of oxygen consumption versus [Cu(II)] at an albumin concentration of 30 μM. The inset shows the same data with the abscissa showing the Cu(II)/albumin molar ratio. (*b*) Initial rate of oxygen consumption versus [albumin] at a Cu(II) concentration of 50 μM and an ascorbate concentration of 0.4 mM. The arrow at the extrapolated intercept is at a Cu(II)/albumin molar ratio of 1.

increasing concentrations of albumin slow down the loss of ascorbate in a non-linear fashion. At 7.5 μM- and 15 μM-albumin, the initial losses were 1.4 and 2.8 nmol of ascorbate/min per μmol of Cu(II) respectively. At Cu/albumin ratios of more than 1, the rate of ascorbate decay is non-linear and eventually approaches that of free Cu(II) (290 nmol of ascorbate/min per μmol of Cu(II)). Apparently, the reduction rate of Cu(II) bound to different sites on the albumin molecule is variable, the Cu(II) bound to the 'tightest' binding site being least available.

Albumin exposed to one portion of ascorbate (0.4 mM final concn.) at various concentrations of Cu(II) exhibited decreased 300–400 nm emission fluorescence as a consequence of the reaction (results not shown). For a given initial level of Cu(II) (50 μM), the fluorescence intensity decreased with the number of portions of ascorbate added. Similar results were obtained with both human and bovine albumin.

For a fixed concentration of protein and ascorbate (0.4 mM), the initial rate of utilization of oxygen is a function of the Cu(II) concentration (Fig. 2*a*). Alternately, for a fixed concentration of ascorbate (0.4 mM) and Cu(II) (50 μM), the reaction rate decreases with increasing concentrations of albumin (up to 2 mg/ml; Fig. 2*b*). Protein-bound Cu(II) reacts more slowly than free Cu(II). Chelating agents (1 mM-EDTA or citrate) inhibit the reaction (results not shown). The initial oxygen-consumption rate at > 30 μM-Cu(II) is 0.5 nmol of O_2/min per μM-Cu(II). For a fixed concentration of Cu(II) (50 μM) and increasing concentrations of albumin (< 2.0 mg/ml, 30 μM), the extrapolated curve intercepts at a Cu(II)/albumin molar ratio of 1, below which the rate of oxygen consumption is much slower (Fig. 2*b*).

The ascorbyl radical (Asc$^{\cdot}$) is generated by the reduction of Cu(II) and has a doublet hyperfine splitting value of 218 μT (2.18 G), $g = 2$ (not shown) [18]. The net level of the Asc$^{\cdot}$ signal and its rate of decay are greatest with free Cu(II), are decreased by binding to albumin, and

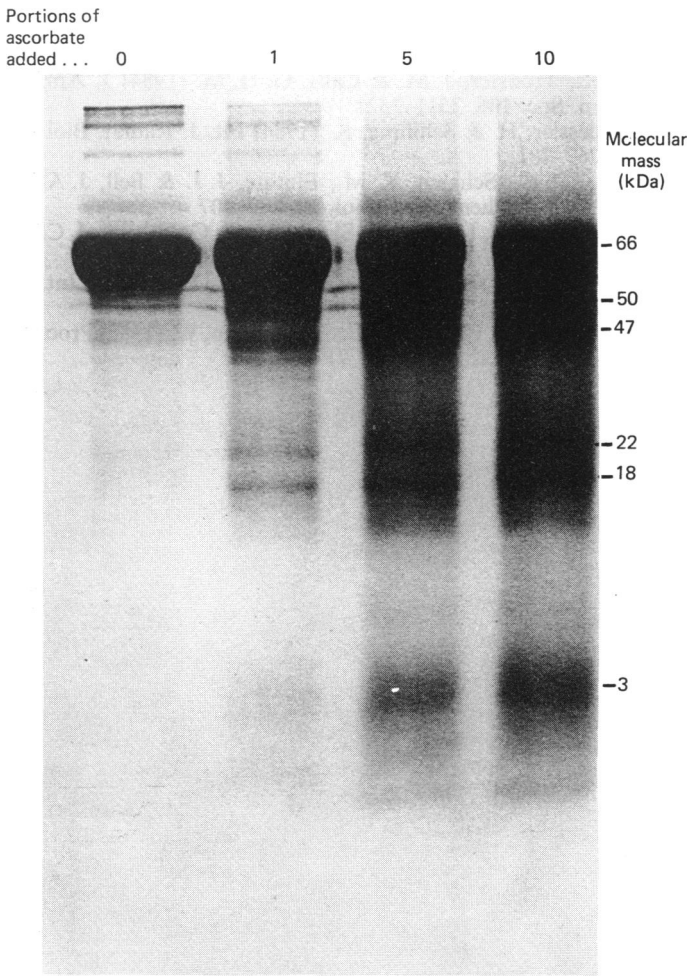

Fig. 3. SDS/polyacrylamide-gel electrophoresis of the products of the reaction between human albumin, Cu(II) (50 μM) and ascorbate (0.2 mM), multiple portions of which were added at 15 min intervals (third and fourth lanes)

are quite low in the absence of Cu(II) or with chelating agents (not shown).

SDS/polyacrylamide-gel-electrophoretic analysis of human albumin subjected to between one and ten portions of ascorbate (0.2 mM each, giving 0.1–2.0 mM final concn.) in the presence of 50 μM-Cu(II) is shown in Fig. 3. Coomassie Blue-stained gels reveal that degradation of the starting albumin (M_r 66000) was a function of the number of portions of ascorbate added, with new bands observed at 50, 47, 22 and 18 kDa. After five portions, a peptide fragment at 3 kDa became more visible (Fig. 3, lanes 3 and 4). In the absence of Cu(II) or in the presence of 1 mM chelating agent (EDTA or citrate), such degradation does not occur (results not shown). The gradual decrease of the band intensity of the starting native albumin and the increased band density at lower-molecular-mass regions is evident. The high-M_r albumin multimer bands which are initially visible (Fig. 3, lane 1) disappear with no evidence of chain cross-linking or formation of high-M_r material (Fig. 3, lanes 3 and 4).

Under the above-described reaction conditions, mixtures of bovine or human albumin (< 2 mg/ml) and Cu(II) (< 0.5 mM) subjected to a single or multiple portions of ascorbate do not give rise to insoluble albumin-derived products.

DISCUSSION

Modifications of albumin induced by the reaction between bound Cu(II) and ascorbate lead to a significant decrease in fluorescence and in chain fragmentation. Ascorbate and oxygen consumption rates are more rapid with Cu(II) bound to the 'loose' albumin-binding sites (Cu/albumin ratio > 1; Figs. 1 and 2). Decreased fluorescence reflects changes in the aromatic-side-chain composition, probably a loss of tryptophan, tyrosine, phenylalanine and histidine groups [1, 2].

The SDS/polyacrylamide-gel-electrophoresis experiments reveal that, as a result of the reaction, the single-chain albumin (M_r 66000) is cleaved into discrete fragments with molecular-mass values averaging 50, 47, 22, 18 and 3 kDa (Fig. 3). The fragmentation pattern suggests that three major chain breaks occur and reflects the location of loosely bound Cu(II). However, it is not clear whether each fragment represents an *N*- or a *C*-terminal. Thus each chain break could occur at a pair of locations, as follows: the 50 kDa fragment from a break at sequence 475–485 or 115–125; the 47 kDa fragment from a break at sequence 435–445 or 150–160; and the 3 kDa fragment from a break at about sequence 20–25 or 560–565 (total 585 amino acids).

The specificity of the chain cleavages is relative. For example, irradiation of albumin, wherein OH· radicals are generated homogeneously, gives rise to some ten individual peaks with molecular masses ranging from 62 to 26 kDa [18]. The fewer and less diffuse bands for the reaction mediated by Cu(II) bound to albumin (Fig. 3) reflect a considerable increase in the degree of specificity for the Fenton reaction. Considering the high reactivity of the OH· radical, it would not diffuse far from the site of its generation. Thus the fragmentation pattern reveals the location of the three 'loose' Cu(II)-binding sites.

There may be physiological implications in the reaction between copper and ascorbate relevant to albumin metabolism. Indirect evidence suggests that oxygen radicals can increase albumin permeability across endothelial cells [20] and may contribute to the formation of senile cataract [21]. In normal blood plasma, ascorbate is loosely bound to the albumin ($n = 4 \pm 2$ binding sites, $K_D = 1200\ M^{-1}$) [21], and micromolar levels of Cu(II) are present. We suggest that the above-described reaction may occur *in vivo* and might be pertinent to the 'aging' of albumin and its physiological catabolism.

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