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STIMULATORY AND INHIBITORY ACTIONS OF PROTEINS AND AMINO ACIDS ON COPPER-CATALYSED FREE RADICAL GENERATION IN THE BULK PHASE

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The effect of a variety of proteins and amino acids was investigated on oxygen free radical activity as assessed by copper/hydrogen peroxide induced benzoate hydroxylation as well as copper-catalysed ascorbate autoxidation. Serum albumins from a variety of species (human, bovine and dog) had both inhibitory and stimulatory effects depending on the molar copper to protein ratio; low ratios were inhibitory and high stimulatory. Some other proteins tested (lysozyme, soybean trypsin inhibitor and conalbumin) also had dual (inhibitory and stimulatory) effects, as did both histidine and polyhistidine, but all effects occurred at different molar ratios presumably dependent on the relative affinities for the copper ions. In contrast, metallothionein and caeruloplasmin, proteins specialised to bind copper *in vivo* had no stimulatory effects. In this paper we show that in addition to their fairly well documented inhibitory effects, under certain conditions some proteins also stimulate radical reactions. The possible role of this phenomenon *in vivo* is discussed.

KEY WORDS: Serum albumins, lysozyme, soybean trypsin inhibitor, metallothionein, caeruloplasmin, copper-dependent radical generation.

INTRODUCTION

Proteins are known to be modified in a variety of ways by exposure to free radical fluxes.[1,2] Many metal catalysed radical generating systems produce hydroxyl radicals but higher oxidation states of transition metals (which display essentially the same kind of properties) may also be generated.[3] Localisation of damage on the protein molecule when exposed to copper(but not to iron)-catalysed hydroxyl radical fluxes is a result of metal chelation to specific sites.[4,5] For instance, many serum albumins, including bovine[6] (BSA) and human[7] (HSA) have a common specific binding site for copper ions but dog (DSA) does not have the same site.[8] By metal binding, albumin can inhibit generation of free hydroxyl radicals from systems containing copper and hydrogen peroxide. The copper ions bind to albumin, and are rendered less available for reaction,[9] but some of the bound ions are still accessible to hydrogen peroxide, superoxide and ascorbate and they can still cause formation of hydroxyl radicals. However, these radicals immediately attack the albumin molecule itself and are supposedly not available away from the albumin surface.[10] The result is damage to the

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albumin molecule although it should be noted that this is a generalised property of proteins attributed to binding of copper ions.

In contrast to the above, under certain conditions chelation of metal ions can enhance radical reactions in the bulk phase. For instance, the "Good" buffers like Hepes are known to augment copper-catalysed radical fluxes[11] probably by increasing the availability of redox active metal. Whilst we were in the preliminary stages of this work Sutton and Winterbourn[3] pointed out the need for expanding our knowledge of bulk phase reactions when metal is chelated. Therefore, in this paper we have examined how protein, amino acids and certain specialised metal binding proteins affect copper-dependent radical reactions.

MATERIALS AND METHODS

Reagents

Unless stated otherwise, chemicals were of the highest purity available and were obtained from Sigma (St Louis, Missouri, U.S.A). The deionised water used in experiments was obtained from a Sybron Barnstead Nanopure II filtration unit and was "free" of redox active transtion metal using the criterion of Buettner.[12] All reagents were made up fresh as required and kept on ice until use. All the albumins tested were fraction V and were also obtained from Sigma. Metallothionein (MT) from rainbow trout was a gift from Dr John Kay, UWCC, Cardiff, Wales, U.K. and caeruloplasmin was obtained from Calbiochem, San Diego, California, U.S.A. All proteins tested (except caeruloplasmin, approx 46 nmoles/mg protein) contained negligible quantities (< 0.5 nmoles/mg protein) of copper as detected by its complexation with neocuproine hydrochloride (Merck, Darmstadt, FRG) using the methodology of Malkin *et al.*[13] The proteins tested with known metal binding sites (caeruloplasmin, BSA and MT) were homogenous and uncontaminated ($< 5\%$ coomassie blue-detectable protein outside the main protein band) as assayed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE).

Radical Generation

Unless stated otherwise, experiments were conducted at various copper to protein ratios with a fixed concentration of 50 μM copper in 10 mM sodium phosphate buffer (pH 7.4). All potential radical targets (volumes as required from a 10 mg/ml stock in water) were present from the start of the experiment (before radical generation) and all experiments were conducted at room temperature. The following indices of radical generation were used.

Benzoate Hydroxylation

Hydroxylation of benzoate (1 mM) was determined by a modification of the method of Gutteridge.[14] Hydrogen peroxide (5 mM) was included in the incubation mixture and radical generation initiated by the addition of copper ions. Fluorescent products were determined by excitation at 308 nm with emission at 410 nm in a model F-4010 Hitachi fluorescence spectrometer. Results are expressed in terms of equivalent salicylic acid produced on hydroxylation of benzoic acid during the reaction. Standard curves for a range of concentrations of salicylic acid were constructed.

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Ascorbate Consumption

This was determined by a modification of the method of Buettner[12] in which the consumption of ascorbate was monitored spectrophotometrically at 265 nm in a model U-3210 Hitachi double beam spectrophotometer. Radical generation was started by the addition of 6 ul of ascorbic acid (0.1 mM final) to the experimental beam cuvette (water was added to the reference beam cuvette). Results are expressed as the relative linear rate of change of absorbance over the first minute of the reaction (which is monitored for a total of 3 minutes) after the addition of ascorbate.

Oxygen Consumption

This was determined by a modification of the method of Marx and Chevion.[9] A change in rate of oxygen consumption was detected by a Clark type model 5300 YSI oxygen monitor (Yellow Springs Inst. Co., Yellow Springs, Ohio, USA) when ascorbate (0.17 mM final) was added to the radical generating system described above. Results were expressed as the initial rate of oxygen consumption (nmols oxygen/min) over the first minute of the reaction after the addition of ascorbate.

RESULTS

All data presented are representative of at least three separate experiments and points were normally performed in duplicate within each experiment. The degrees of variation for the benzoate and oxygen consumption assays were essentially the same, the maximum standard deviation being 5% of the mean. Thus any points at least 10% different were considered significantly different. Ascorbate consumption was more precise, with the standard deviation being 2% of the mean.

Radical Reactions of Copper Chelated to:

(a) *Serum albumins* In all the systems tested (ascorbate consumption, oxygen consumption and benzoate hydroxylation), BSA has both a stimulatory effect (at high copper concentrations relative to BSA) and an inhibitory effect (at low copper concentrations) on copper-catalysed radical generation. Data of hydroxyl radical generation detected by benzoate hydroxylation is shown in Figure 1. Almost exactly the same results were obtained using the deoxyribose degradation means of detecting hydroxyl radicals[14] (data not shown). The higher the protein concentration in the system the more copper is needed to overcome the inhibitory component of copper binding to protein so that the addition of BSA becomes stimulatory (in comparison with the situation without protein present). The inhibitory effect is seen with constant protein concentration (whilst varying copper) suggesting that radical scavenging by BSA is not the reason for the effect (Figure 1).

Other albumins display essentially the same property with slightly different dependence on copper to protein ratios (Figure 2). For instance, BSA, HSA and DSA became stimulatory (ascorbate consumption became greater than in the absence of protein) at 6.5, 7.5 and 5.5 molar ratio (assessed by interpolation) of copper to protein respectively. The ratio of copper to protein needed to provide stimulatory conditons, which is a useful internal comparison, will from now on be referred to as the stimulatory ratio (SR). In addition to the inhibitory effects at low levels of copper, all

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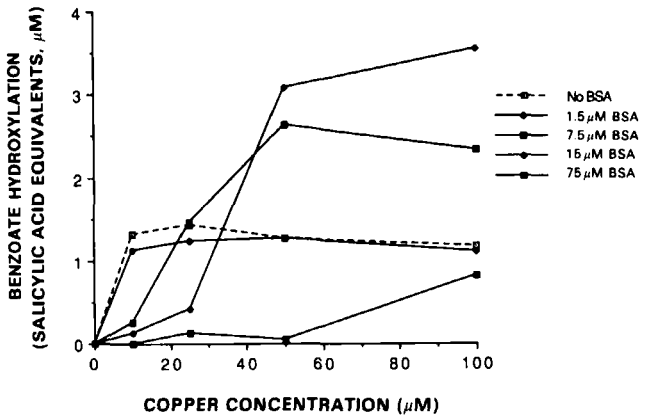

FIGURE 1 Effect of BSA on the copper-catalysed hydroxylation of benzoic acid (1 μM) in 10 μM sodium phosphate buffer (pH 7.4). Note: 75 μm BSA and 500 μM copper gave 2.27 μM salicylic acid equivalents: (data not shown on the graph).

albumins tested significantly stimulated ascorbate consumption at 10:1, 20:1 and 30:1 copper:protein molar ratio (Figure 2). The peak of stimulation with BSA (in terms of oxygen consumption) occurred at approximately 15:1 (Figure 3a). In figures 2–5, copper:protein ratios are varied by changing protein concentration (in contrast to Figure 1). Thus at high protein concentrations (i.e. low copper:protein ratios), both protein binding of copper and protein scavenging of radicals may influence the reaction.

Denaturation (85°C, 10 min) of albumins removes all stimulatory effects (no radical reactions greater than those in the absence of protein) and actually enhanced the inhibitory capacity (inhibitory effects were displayed at higher copper to protein ratios). For instance, a Cu:BSA ratio of 10:1 increases oxygen consumption from 152.7 $\pm$ 6.3 to 192.6 $\pm$ 6.8 and denaturation of the protein brings the rate down to

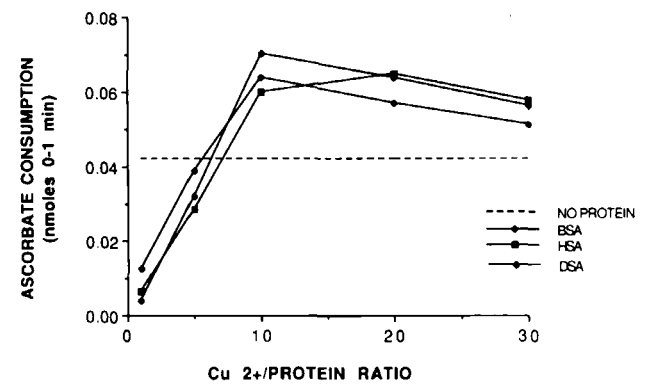

FIGURE 2 Effect at various copper to protein ratios of different albumins on the copper (50 μM)-catalysed consumption of ascorbate (0.1 mM) in 10 mM sodium phosphate buffer (pH 7.4). The dotted line reprensents the rate of ascorbate consumed at a fixed amount (0.1 mM) and copper (50 μM) in the absence of protein.

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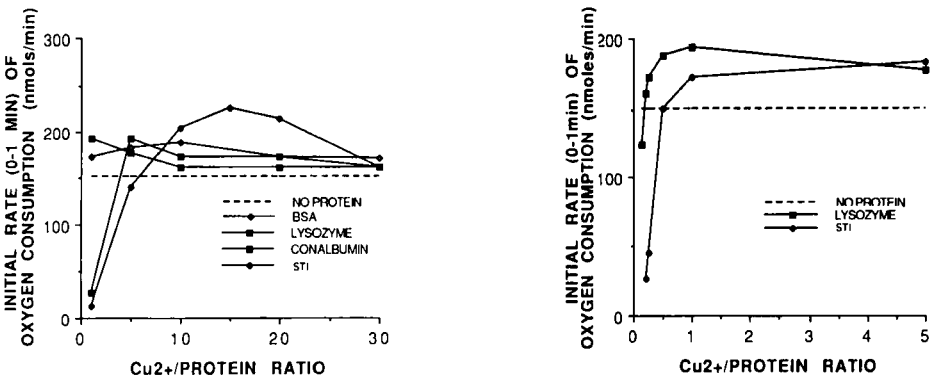

FIGURE 3 Initial oxygen consumption rate during the copper (50 μM)-catalysed autoxidation of ascorbic acid (0.17 mM) in 10 mM sodium phosphate buffer (pH 7.4) at copper to lysozyme, soybean trypsin inhibitor and conalbumin ratios of (a) 1–30 and (b) 0–5 (for clarity BSA and conalbumin data are not shown). The dotted line represents the rate of oxygen consumed with a fixed amount of ascorbate (0.17 mM) and copper (50 μM) in the absence of protein.

109.0 $\pm$ 11.2. However, it should be noted that the effect of denaturation was generalised for all other albumins (human, dog, horse and rat) tested. The stimulatory effect seen with native albumin could not be reproduced with the denatured form even with copper to protein ratios of up to 80:1 (data not shown).

(b)*Other proteins* Lysozyme and soybean trypsin inhibitor (STI) displayed similar effects on radical reactions (in terms of oxygen consumption) to the albumins (Figure 3a) but the SR was much lower (0.5 and 0.75 respectively, Figure 3b). Conalbumin displayed properties intermediate between those of BSA and the other proteins tested (Figure 3a). Unlike the albumins tested, denaturation had no significant effect on the behavior of lysozyme or STI (data not shown).

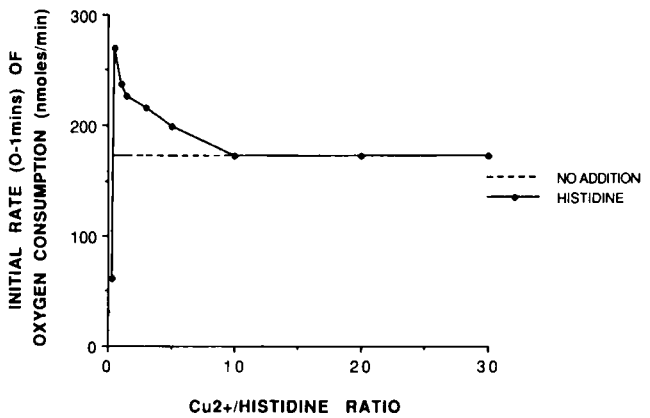

FIGURE 4 Effect of various copper to histidine ratios on the initial rate of oxygen consumption during the copper (50 μM)-catalysed autoxidation of ascorbic acid (0.17 mM) in 10 mM sodium phosphate buffer (pH 7.4). The dotted line represents the rate of oxygen consumed with a fixed amount of ascorbate (0.17 mM) and copper (50 μM) in the absence of histidine.

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(c)*Amino and polyamino acids* Polyhistidine and polyaspartic acid also showed biphasic responses similar to those already described and at relatively high copper:-protein ratios; SRs were 70:1 and 22:1 respectively (data not shown). Indeed, histidine alone also showed both stimulation and inhibition but at a much lower copper/amino acid molar ratios (Figure 4). There also seemed to be a much more stringent dependency on the copper to chelator ratio: significant stimulation occurred at ratios up to approximately 5:1, is maximal at 1:1, but ratios less than equimolar lead to inhibtion.

(d)*Specialised copper binding proteins* In contrast to the proteins and amino acids tested, the specialised metal binder MT showed no significant stimulatory effects and was purely inhibitory at relatively high levels of copper (up to approximately 30:1, Figure 5). Caeruloplasmin behaved in a similar but slightly less effective manner reducing ascorbate consumption by 29% at a 10:1 ratio of copper to protein.

DISCUSSION

It is fairly well accepted that in certain circumstances low molecular weight chelators such as EDTA (with Fe) or Hepes (with Cu) enhance the availability of metals for redox reactions involved in radical generation. However, proteins have usually been considered to be purely inhibitory in such copper-catalysed reactions, supposedly by a combination of their metal binding and radical scavenging properties.[15,16] In this paper we find that this is not always the case and that stimulation of radical reactions by increased metal availability is a generalised phenomenon. Our results are consistent with previous observations, but extend the range metal:protein ratios studied.

With albumin (and in particular BSA) as test protein there is a fairly well documented inhibitory effect of the protein on copper-catalysed radical reactions. This is thought to be by tight metal binding to specific high affinity site(s). We demonstrated this effect at low copper to protein ratios. However, when the copper to protein ratio is increased sufficiently, the inhibitory effects of this binding are overcome and the metal associated with the protein participates more in redox reactions as judged by the stimulation of copper-dependent radical reactions. This is probably a loosely

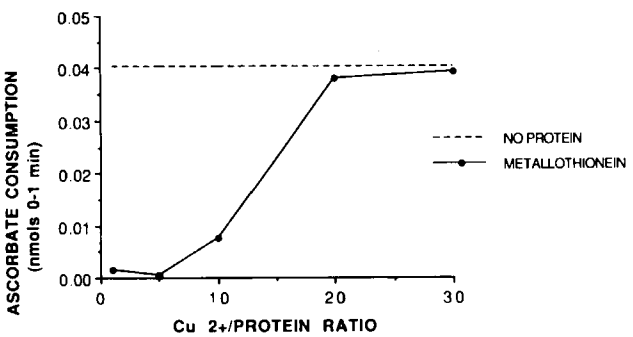

FIGURE 5 Effect of various copper to protein ratios of metallothionein on the copper (50 μM)-catalysed consumption of ascorbic acid (0.1 mM) in 10 mM sodium phosphate buffer (pH 7.4). The dotted line represents the rate of ascorbate consumed with a fixed amount of ascorbate (0.1 mM) and copper (50 μM) in the absence of metallothionein.

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attached metal pool perhaps more like that occurring in the situation previously documented with Hepes buffer[11]; radical reactions are probably promoted by a number of factors including increased solubility of the metal and/or altered redox potential of the reduced/oxidised metal couple. Although only the mercaptalbumin complexes described by Suzuki[17] are formed in our experiments the copper to protein ratio at which stimulation exceeds inhibition is similar for different albumins. This observations is quite surprising considering that DSA does not have the specific site described above. However, DSA does seem to have some other preferred sites for copper ions[8] which may account for its similar behavior in our systems.

Although albumins have been studied here in detail, the dual stimulatory and inhbitory actions on copper-dependent reactions are a generalised property of many proteins. However, with albumins the situation is more complicated. Denaturation of BSA removes the stimulatory effect and actually enhances the inhibitory capacity. The inhibitory role could be attributed to protein unfolding exposing more amino acids able to bind copper and in so doing lowering the copper to chelator ratio greatly (and to a point where inhibition alone is seen). Possibly, the high affinity site (destroyed by denaturation) in most albumins has both stimulatory and inhibitory roles. However, our data also shows that there is another non-specific and similar dual effect displayed by other proteins (Lysozyme and STI) which is independent of structure or conformation. Lack of specificity of this property is also indicated by the fact that both polyamino acids and indeed histidine alone also showed biphasic properties probably by a similar mechanism to Hepes buffer[11] already described. However, and in agreement with the denaturation effect on albumins, the increased SR required for polyhistidine compared to histidine alone may well be a conformational phenomenon.

Although the situation described is complex it is clear that there are at least two different pro-oxidative effects; one is dependent on albumin structure and the other a much more generalised effect of all proteins and their constituent amino acids. Both effects are similar qualitatively but occur at copper to chelator ratios which reflect the relative affinities of interaction.[15] These are in addition to the antioxidative behaviour of specialised copper binding proteins tested.

In our systems, there seem to be gradation of function and effect; MT and caeruloplasmin are specialised for tight metal binding and are as a consequence non productive with respect to copper-dependent radical generation. Albumins represent the half way stage: molecules which may transport copper *in vivo* and appear to be able to bind relatively large levels of metal before stimulation of potentially damaging and deleterious radical reactions became significant. Lastly, many other proteins and amino acids bind copper loosely and seem to have stimulatory effects at relatively low levels of catalytic metal.

The stimulatory effects described in this paper seem to be common to most proteins (although some proteins specifically designed to deal with metals do not display this property). In these experiments albumin displays complex interactions. However, the effects with albumin are probably not relevant in plasma but may be in a few *in vivo* situations.

The ratios of copper to protein necessary before stimulation is significant are not attained in normal blood plasma[18] and low molecular weight pools of redox active metal cannot be detected there.[19] Total copper to albumin molar ratios are of the order of 0.02, total copper in human plasma is around 17 μM, and virtually all the copper is bound to caeruloplasmin.[19] Similar ratios apply in the rat, under a variety

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of dietary regimes, as shown by detailed studies of Taylor *et al.*[20] Damaging radical reactions dependent on copper bound to protein do occur in a site specific manner on the albumin molecule but because of the fast turnover of albumin it can be considered a sacrificial antioxidant in plasma.[21]

However, the ratios of copper to protein needed for stimulation of radical reactions with other proteins may well occur in some extracellular fluids. For instance, in bile (the major excretory route for copper), copper concentrations are much greater than those in plasma[22] (in man, hepatic bile contains approximately 25 uM copper[23]). In addition, the protein concentration of hepatic bile is low (albumin is at approximately 0.63 mg/ml, 8–9 μM) so ratios in favour of redox active metal are feasible *in vivo*. There are also very large variations in the relative proportions of these bile components so that ratios of 10–15:1 are possible. In agreement with the possibility that high copper to protein ratios occur in bile, much biliary copper is not protein bound.[22]

In certain inflammatory sites as represented by exudate fluids, copper concentrations may reach levels much higher than in normal plasma, while proteins concentrations in the vicinity are much lower, so that rather high copper:protein molar ratios may occur there also.[24]

Within the vasculature as a whole, copper may occasionally exceed protein-binding capacity. For example, copper concentrations in brain capillaries of rats vary significantly with age.[25] Similarly, people exposed to metals occupationally, may have elevated levels of copper and other metal in plasma.[26] Furthermore, Harman[27] (reviewed by Feher[28]) has argued, on the basis of limited published data, that elevated plasma copper is associated with increased occurrence of atherosclerosis, and some further data consistent with this idea have been published since.[29] Epidemiological studies[30] reveal increased deaths from cardiovascular diseases (and cancer) associated with serum copper concentrations in the highest quintile (greater than 1.43 mg/l; corresponding to greater than 25 μM) in Dutch persons. These associations probably do not indicate that plasma copper exceeds available protein binding, but they may indicate that within the vessel wall, or in other sequestered sites, it can.

The autosomal recessive disease, Wilson's diseases is associated with lowered plasma copper, and elevated tissue copper in liver, brain and other tisues, due to failure of biliary excretion.[31] Wilsons disease is also accompanied by cardiovascular damage including atherosclerosis.[32] Thus, there may be a damaging elevated copper:-protein ratio in some intracellular sites (perhaps lysosomes). It is already known that sudden necrosis of the liver can release sufficient free copper into circulation to overcome the capacity of plasma to bind copper in a redox inactive state, and hence can lead to intravascular hemolysis.

Thus binding of copper to proteins at molar ratios which facilitate its redox participation, may occur in certain pathologies.

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Accepted by Prof. J.M.C. Gutteridge

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