steps. Ferric ion is more reactive than cupric ion toward the oxidation of the neutral form H_2A. This may be correlated with a much higher probability of formation of a ferric complex of the type FeH_2A^{3+} compared to CuH_2A^{2+}, because of the higher charge of the ferric ion. One would expect a higher entropy increase and higher enthalpy decrease on the formation of the ferric complex.

The enthalpies of activation of the ferric ion are lower than for cupric ion. This seems to correlate with the expected greater binding energy of ferric over that of cupric ion. The lower enthalpies for the neutral form compared to the monoionic species may be due to the difference in the relative stabilities of the metal complexes corresponding to the two species. The neutral form, as compared to the monoionic form, is expected to give a less rigid complex which is easily deformed on electron transfer as a result of the change in hydration energy. This would result in a lowering of $\Delta H^{\pm}$.

Modification of the Radiolytic Oxidation of Ribonuclease Induced by Bound Copper

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Received March 4, 1967

Abstract: The specific effect of cupric ions on the radiolytic oxidative inactivation of enolase and ribonuclease has been investigated. It has been shown that cupric ions bound to the enzymes enhance the radiolytic inactivation induced by OH radicals. The destruction of the amino acid residues was followed in parallel to enzymatic inactivation in the absence and in the presence of Cu^{2+}. It is shown that complexing of Cu^{2+} by RNAase does not alter the *total* yield of radiolytic destruction of amino acid residues, but induces a significant change in the radiolytic yield of individual amino acid residues sensitive to oxidation. A significant increase in the yield of radiolyzed histidyl residues on RNAase in the presence of Cu(II) was observed, accompanied by a corresponding decrease in damaged S–S bonds. A quantitative evaluation of the effect of OH radicals on RNAase in the presence and absence of copper suggests an intramolecular charge transfer in the protein molecule following its reaction with OH radicals. The radiobiological implications of the copper-induced "sensitization" of vital sites in a bipolymer are discussed.

Cupric ions have been shown to enhance the radiolytic decomposition of diamines and amino acids.[1,2] The effect of cupric ions on the radiolytic inactivation of α-amylase and catalase has been subsequently investigated and it was found that binding of Cu(II) to both enzymes resulted in an enhanced radiolytic inactivation of the enzymes by γ irradiation.[3] This enhanced sensitization to ionizing radiation was shown to be due to the action of oxidizing radicals. Other metal ions investigated did not exhibit a comparable effect, thus confirming the suggestions on the possible specific role of copper in radiobiological damage.[4,5] In order to investigate more thoroughly the role of copper in radiation damage to proteins, the Cu(II)–enolase and the Cu(II)–ribonuclease complexes were investigated. In both cases it is known that Cu(II) inhibits enzyme activity.[6,7] For enolase it has been shown that copper ions displace other metal ions essential for the enzymatic activity,[8] suggesting that Cu(II) is bound to a site essential for enolase activity. In the case of RNAase there is accumulating evidence that the Cu(II) interacts with the histidine residues[9–11] which are involved in the catalytic function.[12] Thus, if Cu(II) "sensitizes" its ligands to ionizing radiation,[2] one should expect a preferential radiolytic destruction of the active site in the presence of copper. In the present work we have investigated, in addition to the copper-induced enhancement of radiolytic inactivation of enolase and RNAase, also the effect of bound copper on the radiolytic destruction of individual amino acid residues in the RNAase molecule.

Experimental Section

Reagents and Materials. Crystalline rabbit muscle enolase supplied by Boheringer and Sons, Germany, was used. In order to remove low molecular weight impurities and metal ions the enzyme was dialyzed at 0–4° against 0.02 *M* sodium phosphate buffer, pH 7.0, containing 0.01 *M* EDTA, for 20 hr, and then against phosphate buffer alone (20 hr) to remove the EDTA. This process was shown not to affect the enzymatic activity.

Pure ribonuclease A, lyophilized, phosphate free, obtained from Worthington (lot no. 6508 and 6022), was used without further treatment.

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2-Phosphoglyceric acid (2-PGA), disodium salt, was obtained from Boheringer and Sons, Germany. Yeast RNA was obtained from Schwartz Bio-Research Inc. (lot no. 6502). Ascorbic acid was a product of the British Drug House Ltd. 2,2′-Biquinoline "puriss" was obtained from Fluka, Switzerland. Dowex A-1 chelating resin, 200–400 mesh, was supplied by Bio-Rad Laboratories, purchased from CalBiochem. All metal salts were used in the sulfate form and were of analytical grade. All solutions were in thrice-distilled water, prepared by redistillation from alkaline permanganate and then from phosphoric acid.

Analytical Methods. Enolase. The activity of the enzyme was measured according to Westhead and McLain[13] and Malmstrom[14] except that tris(hydroxymethyl)aminomethane-$HClO_4$ buffer, pH 7.4, was used instead of Tris–HCl since the heavy metal content of $HClO_4$ is lower than that of HCl. The measurements were carried out at 30° using a Zeiss PMQ-II spectrophotometer.

The enzyme concentration was determined by the method described by Lowry, *et al.*,[15] using bovine serum albumin as a standard, or by using the value of 0.885 for the absorbance of 1 mg/ml at 280 mμ, as described by Malmstrom.[14] The specific activity of the enzyme as defined by Malmstrom[14] was found to be 120 ± 10 at 25° indicating that the enzyme used was fully active. The amounts of Cu(II) added to the irradiated samples were shown not to alter the enzymatic activity under the assay conditions.

RNAase. Ribonuclease activity was measured using a modified Kunitz method.[16,17] A 0.1% solution of RNA in 0.1 *M* sodium acetate buffer, pH 5.0, was prepared each day, 0.5–1.5 μg of active enzyme was introduced rapidly into the quartz cuvette containing 3.0 ml of substrate at 30°, and the change of absorbance at 300 mμ was followed. Since under these conditions the enzyme is saturated with substrate for several minutes, the activity could be computed from the linear part of the curve obtained on the recorder. In our assay system a pure enzyme exhibits a change at 300 mμ which amounts to 0.2 absorbance unit/min mμmole of RNAase The ribonuclease concentration was determined using 9600 M^{-1} cm^{-1} as the molar extinction coefficient of the enzyme at neutral pH.[18] Copper ions at concentrations equimolar to RNAase were shown not to affect the enzymatic activity.

Copper Determination. Copper was determined by the 2,2′-biquinoline method according to Felsenfeld.[19]

Irradiation of Samples. Dilute aqueous solutions of the enzymes were irradiated at room temperature by a Co^{60} γ-ray source at a dose rate of 5000 rads/min, determined by the Fricke dosimeter. Samples containing 130 μg of enolase/ml (1.53×10^{-6} *M*) in 0.001 *M* sodium phosphate buffer, pH 7.0, were irradiated.

Two series of 1.0-ml RNAase samples were prepared. The concentration of the enzyme in one series was 1×10^{-4} *M* and in the other 1×10^{-6} *M*. The irradiated and nonirradiated samples containing RNAase at 1×10^{-4} *M* concentration were analyzed also for total amino acid content after complete hydrolysis (see below). The pH of the RNAase solutions were adjusted to 8.0 with NaOH and thus no buffer was used.

Metal ions in micromolar concentrations were added as described under Results. The samples were saturated with N_2O in uniform rubber-stoppered irradiation tubes prior to irradiation.

The samples were assayed for enzymatic activity within 30 min following irradiation. No significant change in enzymatic activity was observed up to 4 hr and over, following irradiation.

Amino Acid Analysis. After irradiation, Cu(II) was removed from the RNAase samples by shaking the samples with regenerated Dowex A-1 (chelex-100) resin, 200–400 mesh, preadjusted to pH 8.0. While the copper was found to be removed from the RNAase solution as checked by the biquinoline method,[19] no protein was absorbed on the resin. Copper-free samples of ribonuclease containing 1.3 mg/ml were hydrolyzed for 22 hr in 6 *N* HCl in vacuum-sealed glass tubes. After removal of HCl (high vacuum over KOH), amino acid analysis was carried out on 0.25–0.35 mg of hydrolysate using a Beckman-Spinco Model 120 amino acid analyzer. The ninhydrin reagent was calibrated against a standard amino acid mixture. Each series of hydrolyzed irradiated RNAase was accompanied by a nonirradiated (native) sample. Glucosamine and norleucine were occasionally incorporated into some samples as internal standards. Since it was found that in samples irradiated under our conditions no alanine or glutamic acid was destroyed, these were used as references and the amount of amino acids in the mixture was calculated assuming the average (Ala + Glu)/2 = 12.0. An accuracy of about ±5% was achieved by this procedure.

Metal Binding. Enolase. The binding of Cu(II) to enolase was investigated by equilibrium dialysis as previously described[8] for the yeast enzyme except that 0.02 *M* veronal buffer, pH 7.0, was used.

Equilibrium dialysis studies of enolase demonstrated that 3–4 cupric ions can be bound to the enzyme without causing precipitation of the protein. Removal of the Cu(II) by passing the Cu(II)–enolase complex through a column of Dowex A-1 chelating resin yielded a fully active enzyme.

The inhibition of the Cu(II)-catalyzed oxidation of ascorbic acid was applied as a measure of the amount of copper bound to a given amount of protein. The results presented in Table I demonstrate that at least six Cu(II) ions can be bound tightly to the protein molecule. Since the determination of Cu(II) binding to muscle enolase was complicated by the fact that increasing concentration of copper led to progressive denaturation, resulting in a loss of activity and even precipitation, it was decided to measure the extent of copper binding in *dilute* enolase solutions at concentrations as close as possible to those of the irradiated samples. Thus the maximum cupric ions per molecule of enolase used in the experiments never exceeded six in order not to complicate interpretation.

Table I. Binding of Copper to Enolase as Measured by the Inhibition of the Cu^{2+}-Catalyzed Oxidation of Ascorbic Acid[a]

Cu^{2+} concn, *M*	Rate of ascorbate oxidation, $A^{265} \times min^{-1}$	
	In the absence of enolase	In the presence of 0.8×10^{-6} *M* enolase
Nil	0.000	0.000
1.6×10^{-6}	0.017	0.000
3.2×10^{-6}	0.035	0.000
4.0×10^{-6}	0.043	0.000
4.8×10^{-6}	0.057	<0.005

[a] The rate of ascorbate oxidation was followed spectrophotometrically at 265 mμ at 25° in the Zeiss PMQ-II spectrophotometer. The system contains 0.5 *M* sodium acetate, pH 5.20, $CuSO_4$, enolase as specified in the table, and 2×10^{-5} *M* ascorbic acid. Initial rates were determined at 25°

RNAase. The binding of Cu(II) to RNAase was investigated by measuring the absorption spectrum within the visible range at pH 7.6. The results were in agreement with those recently reported.[10] The 1:1 and 2:1 complexes were found to be stable for several hours whereas the 3:1 complex began to precipitate after 2 hr. The 3:1 Cu(II)–RNAase complex is thus unstable and probably decomposes to give a mixture of polymolecular aggregates. Potentiometric titrations[10] and optical rotatory dispersion[11] also suggest strong binding of Cu(II) to RNAase.

Results

Radiolytic Inactivation of Enolase and RNAase in the Presence of Metal Ions. The effect of various metal ions on the radiolytic deactivation of the enzymes enolase and RNAase is summarized in Table II in terms of D_{37} (the dose of radiation which brings about 63% inactivation).

It is clearly shown that of all ions investigated only Cu^{2+} has a pronounced effect on the radiolytic inactivation of both enzymes. These results are in accord with our previous observations on α-amylase and catalase.[3] The effects of Cu(II) on the radiolytic inactivation of enolase and RNAase are shown in Figures 1 and 2, respectively.

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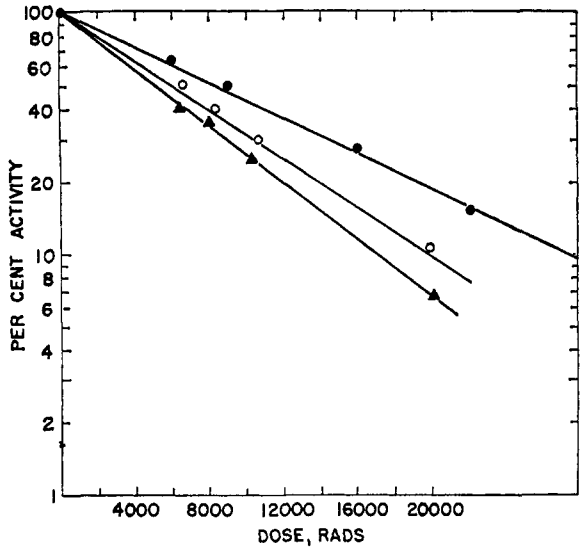

Figure 1. The radiolytic deactivation of enolase by γ irradiation: ●, 1×10^{-6} *M* enzyme; ○, 1×10^{-6} *M* enzyme + 2×10^{-6} *M* $CuSO_4$; ▲, 1×10^{-5} *M* enzyme + 4×10^{-6} *M* $CuSO_4$. Other details are given under Experimental Section.

Since the effect of Cu(II) in the case of ribonuclease was evident already for the 1:1 complex while the effect of Cu(II) on enolase, although significant, was not as pronounced under the given conditions, it was decided to investigate the ribonuclease system more thoroughly.

Table II. The Effect of Metal Ions on the Radiolytic Inactivation of Enolase and RNAase Ribonuclease[a]

Metal ion added	D_{37}, rads	
	Enolase	Ribonuclease
None	12,400	10,000
Cu^{2+}	8,000	4,500
Zn^{2+}	12,000	...
Mg^{2+}	11,600	9,800
Mn^{2+}	12,000	10,000
Fe^{2+}	12,000	...
Co^{2+}	11,800	9,800

[a] The enolase solution contained 2×10^{-6} *M* enzyme, 1×10^{-5} *M* of the metal sulfate, and 0.001 *M* phosphate buffer, pH 7.0. The RNAase solution contained 1×10^{-6} *M* enzyme and 1×10^{-5} *M* $MeSO_4$; the pH solution was adjusted to 8.0 before irradiation. All irradiations were performed under nitrous oxide.

In order to find out which amino acid residues are destroyed during irradiation, the samples to be irradiated had to contain a higher concentration of ribonuclease. The enhanced radiolytic inactivation of ribonuclease has again been demonstrated for irradiated samples containing 1×10^{-4} *M* enzyme (Figure 3). It should be noted that in contrast to the more dilute RNAase solutions the binding of a second cupric ion to the ribonuclease molecule does not enhance further the radiolytic inactivation of the enzyme (Figure 3). The only amino acid residues which were found to undergo measurable radiolytic destruction were: lysine, histidine, cystine, and proline. From the results summarized in Table III it can be seen that the *total* amount of residues destroyed for a given dose is the same for the copper complex as for the copper-free enzyme. However, a significant change is observed in the yield of radiolysis of the individual amino acid

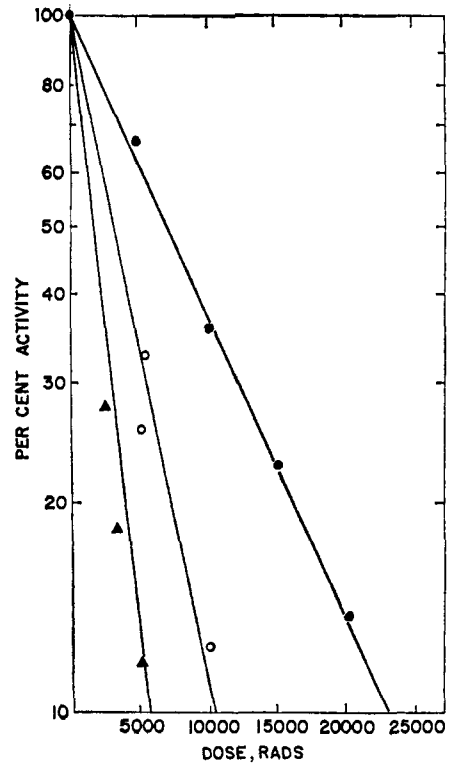

Figure 2. The radiolytic deactivation of dilute ribonuclease solution by γ irradiation: ●, 1×10^{-6} *M* enzyme; ○, 1×10^{-6} *M* enzyme + 1×10^{-6} *M* $CuSO_4$; ▲, 1×10^{-6} *M* enzyme + 2×10^{-6} *M* $CuSO_4$. Further details are given under Experimental Section.

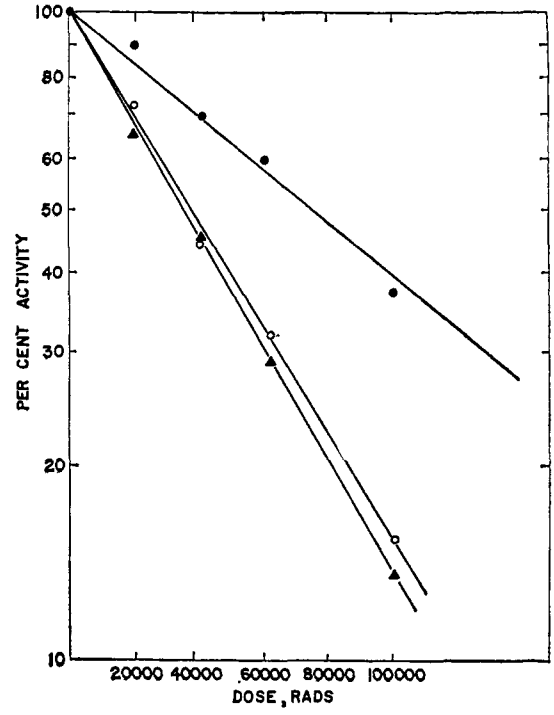

Figure 3. The radiolytic deactivation of 10^{-4} *M* RNAase solutions: ●, 1×10^{-4} *M* enzyme; ○, 1×10^{-4} *M* enzyme + 1×10^{-4} *M* $CuSO_4$; ▲, 1×10^{-4} *M* enzyme + 2×10^{-4} *M* $CuSO_4$. Further details are given under Experimental Section.

residues. Histidine, lysine, and proline were found to undergo extensive radiolysis in the presence of copper whereas the radiolytic destruction of cystine is diminished. In the presence of copper, 2:1, the total damage

Table III. The Effect of Copper Ions on the Radiolytic Decomposition of Four Amino Acids of RNAase[a]

Amino acid residues	Dose, rads: Nil[b]	50,000 Cu-RNAase molar ratio: 0	50,000 Cu-RNAase molar ratio: 1	50,000 Cu-RNAase molar ratio: 2	100,000 Cu-RNAase molar ratio: 0	100,000 Cu-RNAase molar ratio: 1	100,000 Cu-RNAase molar ratio: 2
Lys	10.20 ± 0.10	10.05 ± 0.20	9.55 ± 0.20	9.40 ± 0.10	9.50 ± 0.25	9.40 ± 0.20	9.50 ± 0.10
His	3.90 ± 0.10	3.90 ± 0.10	3.55 ± 0.10	3.30 ± 0.10	3.50 ± 0.05	3.35 ± 0.10	3.10 ± 0.10
Pro	4.15 ± 0.10	4.20 ± 0.10	3.70 ± 0.05	3.70 ± 0.05	4.07 ± 0.10	3.60 ± 0.20	3.70 ± 0.10
Cys	7.5 ± 0.10	5.05 ± 0.70	6.20 ± 0.15	6.70 ± 0.40	5.20 ± 0.20	6.00 ± 0.20	6.30 ± 0.20
Total amino acid residues destroyed	0.0	2.65	2.70	2.60	3.43	3.15	3.10

[a] The other amino acids showed no measurable destruction at the given doses of irradiation. The values are the number of amino acid residues normalized to (Ala + Glu)/2 = 12. Each value is an average of six independent analyses. [b] Native ribonuclease.

to all four residues is somewhat lower although a corresponding destruction of additional amino acids was not detectable within the experimental error.

Discussion

It has been demonstrated that binding of Cu(II) to enolase and RNAase enhances the radiolytic inactivation of both enzymes when compared to the radiation-induced inactivation of the metal-free proteins. Furthermore, in analogy with our previous results on α-amylase and catalase,[3] it has been demonstrated (Table II) that of all cations tested, Cu^{2+} is the only one which induces the radiosensitization of the metal-carrying enzyme. In the case of RNAase we have carried out a study of the radiolytic damage to the individual amino acid residues. It was found (Table III) that binding of Cu(II) to RNAase does not change significantly the *total* amount of amino acid residues destroyed, but it induces significant changes in the relative radiosensitivity of individual amino acid residues. It was clearly shown that the radiolysis of histidyl and prolyl residues was enhanced in the presence of copper while that of cystinyl residues was diminished.

A quantitative evaluation of the radiolytic destruction of amino acids in our N_2O-saturated 10^{-4} *M* RNAase solutions gives *G*(amino acids) = 5, for an absorbed dose of 50,000 rads (Table III). This *G* value decreases with increasing absorbed dose. The *G* value of inactivation of the enzyme increases with its concentration from 0.06 at 10^{-6} *M* to 0.6 at 10^{-4} *M*. Our findings of *G*(inactivation) fairly agree with values previously reported,[20] or calculated from data in the literature[21,22] for the same concentration range.

The increase in *G*(inactivation) of RNAase with concentration follows a pattern characteristic of the radiolytic behavior of various enzymes in aqueous solution.[23] This behavior, which has been extensively discussed,[23] may be due to the irreversible denaturation of damaged protein molecules on interaction with nondamaged molecules, leading eventually to the formation of aggregates of inactivated enzyme molecules.[24]

From the extent of damage to the individual amino acids (Table III) and the extent of inactivation of the enzyme, both in concentrated and in dilute solutions, it may be inferred that enzyme molecules which underwent radiolysis of one or more of their amino acid residues still retain their enzymatic activity. This activity may, however, be lost irreversibly if the damaged molecules interact with another protein molecule in solution before undergoing some stabilization, perhaps by intramolecular disproportionation or recombination of radicals.

The radiolytic inactivation of RNAase in the concentration range up to 10^{-4} *M* in the N_2O-saturated neutral solutions is primarily due to the action of OH radicals. In view of the specific rates of N_2O[25] and RNAase[26] with hydrated electrons, all the hydrated electrons are scavenged by N_2O (2×10^{-2} *M*) and converted to OH radicals even in the presence of 10^{-4} *M* RNAase. Hydrogen atoms may, however, contribute to the radiolytic damage of the enzyme, in spite of their relatively low yield under our experimental conditions: $G(H) \sim 0.1G(OH)$.[27]

The specific effect of copper ions on the radiolytic behavior of the enzyme may be connected primarily with the action of OH radicals. This has been inferred by us in other enzymatic systems[3] and may also be deduced from the over-all *G* value of destruction of amino acid residues, modified by copper ions, which is of the order of 5 ($G(OH) = G_{OH} + G_{e^-_{aq}} = 5.2$)[27] for RNAase irradiated with a dose of 50,000 rads (Table III).

In view of the specific rates of reaction of OH radicals with organic compounds[28] and with Cu^{2+} ions[29] it is most unlikely that a direct reaction takes place between an attacking OH radical and a single copper ion bound to a protein macromolecule. Still it is evident that the copper ion is *directly* involved in the radiolytic damage as it was shown that its ligands, namely, histidyl residues,[10,11] undergo preferential radiolysis. The only plausible explanation for the specific effect of copper ions is as follows. An OH radical reacts with the biopolymer at some nonspecific site, most probably by a charge-transfer mechanism. It has been shown that OH radicals may react with negatively charged organic molecules carrying deactivated methylene groups by a charge-transfer mechanism rather than by H abstraction.[28] The electron

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vacancy produced by the protein + OH charge-transfer reaction is then filled by an electron originating from the copper which migrates very rapidly through the macromolecule, leaving the copper at its oxidized tervalent state. The rate of intramolecular electron migration in the presence of copper must be high as it has to compete with a proton transfer to the solvent (*i.e.*, $>10^{10}$ sec^{-1})[30] and may be thus considered as a kind of intramolecular electron conductivity. The formation of Cu(III) by the action of OH radicals within a Cu(II)–polylysine complex, containing one copper atom per 44,000 molecular weight, has been directly demonstrated by pulse radiolysis.[31] Tervalent copper was found to be formed in this case within less than 10^{-6} sec. At a subsequent stage, which proceeds most probably at a much slower rate,[32] Cu(III) oxidizes its ligands.[1,2]

Intramolecular electron migration in proteins has been demonstrated in the solid state[33] and in the hydrated form.[34] Electron conductivity has not been demonstrated up to date in proteins in solution. The behavior of our system as well as the specific reduction of Fe(III) within the cytochrome *c* macromolecule by H atoms[35] strongly suggest the existence of intramolecular electron conductivity in proteins in solution. The intramolecular electron migration is most probably not limited to the metal-containing biopolymers. It may be responsible for the extensive damage of certain amino acid residues over and above the statistical value predicted by the specific rates of reaction of the individual amino acids with OH radicals. If the intramolecular electron mobility is impaired in a molecule that underwent radiolytic damage, a second interaction between a damaged molecule and a free radical will be less specific. This may explain the decrease in the radiolytic damage to specific sites with increasing total dose of radiation. It is inferred, therefore, that OH radicals, in spite of their extremely high reactivity, may induce specific damage in a protein molecule.

The mechanism proposed for the copper-induced modification of protein radiolysis may thus be

$$\text{protein} \xrightarrow[\text{diffusion controlled}]{OH\ OH^-} \text{protein}^+ \xrightarrow{\text{slow}} \text{oxidized protein}$$

and in the presence of bound copper

$$\text{protein–Cu(II)} \xrightarrow[\text{diffusion controlled}]{OH\ OH^-} \text{protein}^+\text{–Cu(II)} \xrightarrow[\text{fast}]{\text{very}}$$

$$\text{protein–Cu(III)} \xrightarrow[\text{specific}]{\text{slow}} \text{modified oxidized protein–Cu(II)}$$

Since the loss of enzymatic activity on irradiation of the Cu(II)–RNAase complex is parallel with the loss of histidyl residues, it is attractive to assume that histidine-12 and histidine-119 which are part of the RNAase active site are also the ligands which bind the Cu^{2+}. This suggestion is corroborated by the independent physicochemical studies of the Cu(II)–RNAase complex.[10,11] The proximity of prolyl residues to the copper binding site would explain its increased radiolytic destruction in the presence of copper. Thus Pro-117 is very close to His-119 which is probably bound to Cu(II). It may also be assumed that other proline residues remote in the sequence are brought into the vicinity of the Cu(II) by the new conformation[11] induced by copper binding.

Our results corroborate previous suggestions that copper ions may have a specific role in radiobiological damage.[1,2] Moreover, it is shown that cupric ions change the *pattern* of radiolytic damage; thus protection and sensitization might occur simultaneously. Furthermore, if the Cu(II) is bound in biological systems to nonessential sites it may exhibit a net protective effect by competition for the reactive species. In this relation one should mention the observation[2] of a decrease in the radiosensitivity of living species with increasing total copper content.

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