Cu(II)-Induced Oxidation of Catechols, Ascorbate and *o*-Phenylenediamine is Promoted by DNA

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Cu(II)-Induced oxidation of catechols (dopa, dopamine and epinephrine) to yield indolic aminochromes is speeded up by at least two orders of magnitude upon addition of DNA. This effect was observed both in aerobic and anaerobic solutions, indicating that it is *not* due to the reductive activation of oxygen with formation of $O_2^{\cdot -}$ and H_2O_2 as oxidant. It is suggested that the unfavourable redox equilibrium of the catechol-Cu(II) complex, ROH-Cu(II) $\rightleftarrows$ Cu(I) + RO$^{\cdot}$ + H^+, to yield Cu(I) and semiquinone, is efficiently driven to the right by DNA due to formation of the strong DNA-Cu(I) complex, for which a stability constant of $K \approx 2 \times 10^9\ M^{-1}$ has been estimated previously. Absorbance changes characteristic to the formation of DNA-Cu(I) appear in fact concomitantly with the 480 nm absorption of aminochrome, derived from the semiquinone RO$^{\cdot}$. Cu(II)-Induced oxidation of ascorbate and *o*-phenylenediamine (leading to 2,3-diaminophenazine) was also speeded up by almost two orders of magnitude in presence of DNA. The observed activation of redox reactions of copper in the vicinity of DNA may have important implications with respect to the toxicological properties of certain bioreductive endogenous and xenobiotic compounds.

Introduction

Already in 1945 it was recognized by McCarty that nucleic acids can be inactivated by treatment with Cu(II) and ascorbate under aerobic conditions [1]. Such transition-metal dependent damage by ascorbate and by other reductants has received growing interest up to the present, and the proposal has been made that transition-metals generally play an important role in oxidative stress induced in aerobic cells by the reductive activation of oxygen ($O_2 \rightarrow O_2^{\cdot -} \rightarrow H_2O_2 \rightarrow {}^{\cdot}OH$) [2–5].

In DNA/Cu(II)/ascorbate model systems the following reactions can be envisaged:

$$Cu(II) + AH^- \rightleftharpoons Cu(I) + A^{\cdot -} + H^+ \quad (1)$$

$$DNA + Cu(I) \rightleftharpoons DNA\text{-}Cu(I) \quad (2)$$

$$2A^{\cdot -} + 2H^+ + O_2 \rightarrow 2A + H_2O_2 \quad (3)$$

$$DNA\text{-}Cu(I) + H_2O_2 \rightarrow DNA^{\cdot}OH + Cu(II) + OH^- \quad (4)$$

$$\downarrow$$

damage.

Cu(I) forms a very strong complex with DNA, reaction (2), with a stability constant in the order of $K_2 \approx 2 \times 10^9\ M^{-1}$ and a rate constant of $k_2 \approx 4 \times 10^7\ M^{-1}\ s^{-1}$ [6, 7]. Hence it can be implied that DNA presents a prominent target for copper-dependent damage by the Fenton-type reaction (4) which generates hydroxyl radicals (or other oxidizing entities) *in situ, i.e.* immediately near the site of Cu(I) fixation in the DNA [6–10]. This is obviously the reason why Cu(II) is much more efficient in mediating damage to DNA than for instance Fe(III) [6, 10]. From the pH-dependent rate of reaction (4) [11] it was implied that HO_2^- ($pK_a(H_2O_2) = 11.9$) is the main reactive entity even at physiological pH. Cu(I) adds preferentially to alternating dG-dC sequences [7], and strand breakage *via* reaction (4) is frequent near guanine residues [12, 13]. Interestingly, the interaction of Cu(I) with DNA leads to a thermal stabilization of the DNA, and to changes in the UV absorbance and circular dichroism spectra [14, 15], resembling those for a conformational B→Z DNA transition [16].

Reductive activation of oxygen in the Cu(II)/ascorbate system can be summarized by the reaction (3) [17], it probably proceeds *via* formation and disproportionation of $O_2^{\cdot -}$ [18], involving copper. Surprisingly, little is known about the kinetics of the initiating reaction (1). According to the one-

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electron reduction potentials, $E^\circ(Cu^{2+}/Cu^+) = 158$ mV and $E^\circ(A^{\cdot -}/AH^-) = 330$ mV [19], the $A^{\cdot -}$ species is a stronger oxidant than Cu^{2+}, thus the reaction (1) should predominantly equilibrate to the left. In presence of DNA we can expect that fixation of Cu(I) by reaction (2) might pull reaction (1) to the right. Cu(II)-dependent DNA degradation by catecholamines [20] and by other reducing substrates [13, 21] may primarily also involve thermodynamically forbidden substrate oxidations, as in reaction (1), driven by reaction (2), and predisposing the DNA to the deleterious reaction (4) with H_2O_2.

In this paper we present stopped-flow kinetics of Cu(II)-induced oxidation of catechols, ascorbate and *o*-phenylenediamine in the absence and presence of DNA, which confirm that Cu(II)-induced redox reactions can be drastically promoted by DNA.

Materials and Methods

The following commercial products were used as received: deoxyribonucleic acid sodium salt (DNA, salmon testes), dopamine (4-[2-aminoethyl]-1,2-benzenediol), epinephrine (4-[1-hydroxy-2-(methylamino)ethyl]-1,2-benzenediol), poly(I), poly(U), and ribonucleic acid (RNA, yeast) from Sigma Chemie; adenosine-5′-phosphate (AMP), L-ascorbic acid (AH^-), $CuCl_2 \cdot 2H_2O$, dopa (L-3,4-dihydroxyphenylalanine), L-glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine), glutathione disulfide (GSSG), guanosine-5′-phosphate (GMP), poly(A), ribonuclease A (RNase, bov. pancreas, EC 3.1.27.5), and superoxide dismutase (SOD, bov. erythrocytes, EC 1.15.1.1) from Serva Feinbiochemica; *o*-phenylenediamine from Aldrich. Other chemicals such as buffers and salts were of the highest purity available.

Solutions were prepared freshly for each experiment using redistilled water, and anaerobic conditions were obtained by flushing the solutions gently for at least 30 min with high purity N_2. The stopped-flow measurements were performed with a SFA-12 "Rapid Kinetics Stopped-Flow Accessory" (Hi-Tech Scientific Ltd.) coupled with a UV-Vis spectrophotometer (Shimadzu Corporation). The optical cell (1 cm light path) was thermostated at 20 °C.

Results

Cu(II)-Induced oxidation of chatecholamines

Fig. 1 presents time profiles for Cu(II)-induced oxidation of dopa (a), dopamine (b) and epinephrine (c), in each case in the presence (upper trace) and absence (lower trace) of DNA. The stopped-flow kinetics were monitored at 480 nm by formation of aminochromes such as dopachrome (from dopa) and adrenochrome (from epinephrine). Spectra of the absorbance change obtained at various times after mixing are shown in Fig. 1 for dopamine: (d) in the absence of DNA, and (e, f) in the presence of DNA. The broad absorption band at 480 nm (Fig. 1 d and e) can in fact be assigned to aminochrome, which is commonly formed upon one-electron oxidation of various catecholamines by a complex mechanism [22–25], involving disproportionation of the *o*-semiquinone and cyclization of the *o*-quinone to an indolic intermediate which is then oxidized to the aminochrome. The much stronger absorbance changes in the 200–350 nm range (Fig. 1 f) are consistent with the formation of DNA-Cu(I). The difference spectrum between DNA-Cu(I) and DNA-Cu(II) exhibits negative and positive absorbance peaks at 250 nm and 295 nm, respectively, and an isosbestic point around 270 nm [7, 14, 15]; these characteristics can be seen in Fig. 1 f, although weaker absorptions due to the dopamine → dopachrome transformation are superimposed.

The most remarkable effect seen from the time profiles in Fig. 1 and from the rates, given in Table I for dopamine, is that the Cu(II)-induced oxidation of catechols is speeded up by a factor of more than 100 upon addition of DNA. In the absence of DNA the oxidation takes place in a time scale of hours and aminochrome, formed in the initial phase (Fig. 1 d), is further oxidized, probably by catalytic reactions of copper, to give polymeric melanins which absorb over the whole visible spectrum. In the presence of DNA, aminochrome is formed within minutes (Fig. 1 e), and it persists over hours without further oxidation to melanins. The rate of Cu(II)-induced catechol oxidation in presence of DNA appears to increase slightly when going from aerobic to anaerobic solution (Table I), though catecholamines are not oxidized by Cu(II) under anaerobic conditions in the absence of DNA [22]. There was little effect of ionic

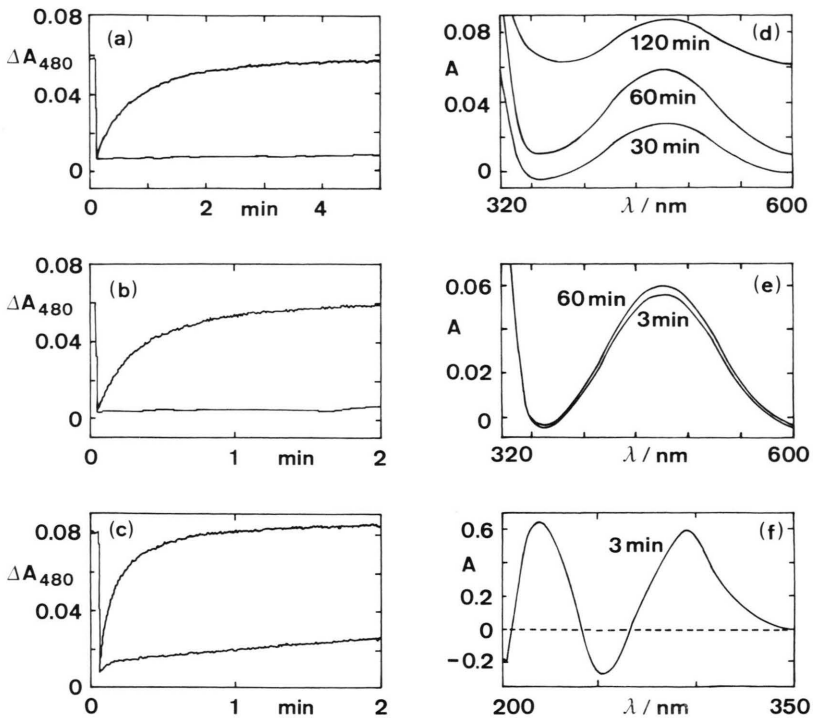

Fig. 1. Cu(II)-Induced oxidation of various catechols in absence and presence of DNA, monitored by the formation of characteristic product absorptions (dopachrome, adrenochrome, DNA-Cu(I)). The results were obtained by rapid 1:1 (v:v) mixing of solutions containing 75 μM catechol derivative with solutions of 150 μM $CuCl_2 \pm 250$ mg/l DNA at 20 °C; all components were air-saturated and buffered with 2 mM TRIS (pH 7.0). *Left panels:* Stopped-flow time profiles of the change in 480 nm absorbance for dopa (a), dopamine (b), and epinephrine (c); lower traces in absence and upper traces in presence of DNA. Similar time profiles were obtained by mixing freshly prepared (catechol + $CuCl_2$)-solutions with DNA solutions. *Right panels:* Spectra of the absorbance change for the dopamine system in absence of DNA (d), and in presence of DNA (e) and (f). Spectra were recorded *versus* a blank without Cu(II); the time after mixing of the components is indicated.

strength, of pH in the range pH 6.8 to 7.5, and of concentration of the individual components on the rate of oxidation in the presence of DNA. Denatured DNA and poly (A) were less efficient, poly(I) was even more efficient, but poly(U) was completely inefficient in speeding up Cu(II)-induced catechol oxidation (Table I). When DNA was replaced by poly(I) the Cu(II)-induced oxidation of dopamine to depachrome concomitantly produced the characteristic absorption spectrum of poly(I)-Cu(I), with a peak at 315 nm [7, 26]. The final yield of aminochrome in presence of DNA was found to increase linearly with Cu(II) concentration under the conditions [Cu(II)] < [dopamine] and DNA(base)/Cu(II) > 4.

The results presented are consistent with the proposal (see Introduction) that an unfavourable redox interaction, here the reaction (5) between catechol (ROH) and Cu(II), can be driven to the right by DNA due to fixation of Cu(I), reaction (2), and with concomitant formation of oxidation products (aminochrome):

$$\text{ROH-Cu(II)} \rightleftharpoons \text{Cu(I)} + \text{RO}^{\cdot} + \text{H}^{+} \quad (5)$$

$$\text{DNA} + \text{Cu(I)} \rightleftharpoons \text{DNA-Cu(I)} \quad (2)$$

$$\text{RO}^{\cdot} \rightarrow \rightarrow \rightarrow \text{aminochrome.} \quad (6)$$

In the absence of DNA reaction (5) is expected to equilibrate mainly to the left, like reaction (1), since the *o*-semiquinone ($RO^{\cdot}$) is a fairly strong oxidant ($E^{\circ}(RO^{\cdot}/ROH) = 530$ mV at pH 7 [25]). That reaction (5) cannot be driven by poly(U) (Table I) is consistent with its very low affinity for Cu(I) [7]. We also recognized that neither the individual DNA bases nor AMP and GMP are able to

Table I. First-order rates of Cu(II)-induced dopamine oxidation in presence of various additives[a].

[Cu(II)] [μM]	[dopamine] [μM]	Additive, Buffer[b]	Rate[c] k [s^{-1}]
50	100	no additive, H or T	$\approx 4 \times 10^{-4}$
50	100	100 mg/l DNA, H	5.2×10^{-2}
50	100	100 mg/l DNA, H (anaerobic)	7.8×10^{-2}
50	100	100 mg/l DNA + 0.1 M NaCl, H	5.9×10^{-2}
100	50	80 mg/l DNA, H	5.8×10^{-2}
100	100	80 mg/l DNA, H	6.3×10^{-2}
100	100	80 mg/l DNA (denatured), H	2.5×10^{-2}
100	100	80 mg/l poly(A), H	$\approx 1 \times 10^{-2}$
50	100	100 mg/l poly(U), H	$\approx 4 \times 10^{-4}$
75	37.5	125 mg/l DNA, H or T	5.8×10^{-2}
75	37.5	125 mg/l poly(I), T	9.3×10^{-2}
75	37.5	125 mg/l RNA, T	$\approx 6 \times 10^{-2}$
75	37.5	125 mg/l RNase, T	$\approx 5 \times 10^{-4}$
75	37.5	125 mg/l RNase + 125 mg/l DNA, T	3.5×10^{-2}
75	37.5	250 μM GSSG, T	$< 10^{-4}$
75	37.5	250 μM GSH, T	$< 10^{-4}$

[a] Concentrations after mixing are given; solutions were airsaturated, except experiment 3.
[b] Buffers: H = 5 mM HEPES (pH 6.8), T = 2 or 5 mM TRIS (pH 7.4).
[c] Rates were estimated from the half-life ($k \approx \ln 2/t_{1/2}$) of dopachrome formation; in absence of DNA the reactions were non-exponential, enabling only rough estimates.

promote Cu(II)-induced catecholamine oxidation (data not shown). Poly(I) exhibits an even higher affinity for Cu(I) than DNA [7] and is, as expected, also most efficient in promoting reaction (5) (see Table I).

Complexes of Cu(II) with catecholamines, ROH-Cu(II), are stable under anaerobic conditions [22, 27], and recently it has been suggested that they interact with DNA to form ternary complexes, DNA-ROH-Cu(II), capable of catalyzing the reduction of O_2 and H_2O_2 [20]. Spectral changes resembling those in Fig. 1 f were actually attributed to the DNA-ROH-Cu(II) complex [20]. However, since fast Cu(II)-induced aminochrome formation in the presence of DNA, poly(A) and poly(I) is unequivocal (*e.g.* Fig. 1 e) and evidence is seen for concomitant formation of the respective Cu(I) complex, we suppose that the ternary DNA-ROH-Cu(II) complex is rather unstable. The DNA-ROH-Cu(II) complex may certainly be formed as an intermediate, and as an alternative to the coupled equilibria (5) and (2) we may consider a fast irreversible decay of this complex, to give the same final products:

$$\text{DNA-ROH-Cu(II)} \rightarrow \text{DNA-Cu(I)} + \text{RO}^{\cdot} + \text{H}^{+}. \quad (7)$$

The insignificant effect of concentration of the individual components on the rate of dopachrome formation (Table I) might be taken as evidence in favour of the unimolecular reaction (7). Also the reduction potentials of ROH and Cu(II) could be changed by going from ROH-Cu(II) to the ternary complex [20], making the redox reaction more feasible. With the formation of DNA-Cu(I), as already mentioned, the system becomes predisposed to the deleterious reaction (4). DNA cleavage mediated by catecholamines in the presence of both Cu(II) and H_2O_2 has previously been demonstrated [20], and it should be pointed out in this context that copper acts as a catalyst in the coupled reactions (4) and (7), when H_2O_2 is available as oxidant.

That oxygen has no effect on the rate of Cu(II)-induced oxidation of dopamine in the presence of DNA (Table I, exp. 3), indicates that reductive activation of O_2 with formation of $O_2^{\cdot -}$ and H_2O_2 is not prerequisite to oxidation. The slow oxidation in absence of DNA (Table I) is on the other hand oxygen dependent [22, 27], but oxygen is apparently much less efficient than DNA in promoting reaction (5) by interacting with the products Cu(I) and $RO^{\cdot}$. The further oxidation of dopa-

chrome to yield melanins absorbing throughout the visible spectrum (Fig. 1 d) is actually inhibited in presence of DNA (Fig. 1 e), indicating that the formation of DNA-Cu(I) reduces the catalytic activity of copper. The DNA-Cu(I) complex is in fact rather stable even in oxygenated solution [7, 11, 14, 15] and reaction of DNA-Cu(I) with O_2 to give Cu(II) and $O_2^{\cdot-}$ seems to be sluggish due to its reverse; it can be promoted however by SOD (Prütz, unpublished result), *i.e.* by the removal of $O_2^{\cdot-}$ from the equilibrium, to yield O_2 and H_2O_2. The biochemical and toxicological properties of catecholamine oxidation products, and mechanisms of concomitant formation of damaging oxygen-derived species, have been discussed in a recent review [25].

Only few other compounds were tested for their ability to promote, like nucleic acids, the Cu(II)-induced oxidation of catecholamines. GSH and GSSG, as shown in Table I, strongly retard the oxidation of dopamine. This effect is most likely due to inhibition of reaction (5) by complexation of Cu(II). The pronounced activity of DNA in promoting Cu(II)-induced redox reactions obviously derives from the high affinity of DNA for Cu(I), combined with a low affinity for Cu(II). RNase did not activate dopamine oxidation by Cu(II) nor did it inhibit the promoting activity of DNA (Table I). This is consistent with previous observations which have shown that proteins like RNase and serum albumine do not interfere with reaction (2) [7].

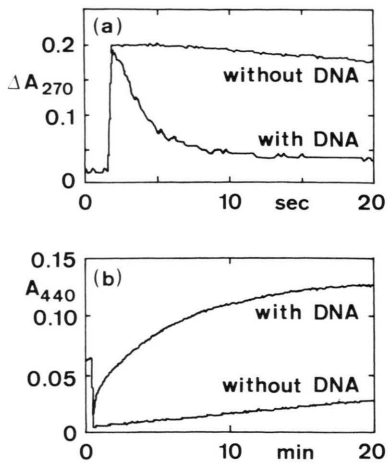

Fig. 2. Stopped-flow time profiles for Cu(II)-induced oxidation of ascorbate (a), and of *o*-phenylenediamine (b) in absence and presence of DNA. The results were obtained by rapid 1:1 (v:v) mixing of solutions containing 75 μM substrate with solutions of 150 μM $CuCl_2 \pm 250$ mg/l DNA at 20 °C under aerobic conditions. (a) Ascorbate oxidation in 5 mM phosphate buffer (pH 6.8) was monitored by the loss of ascorbate absorption at 270 nm, *i.e.* the isosbestic point of the DNA-Cu(II) → DNA-Cu(I) transition (*cf.* Fig. 1 f). (b) *o*-Phenylenediamine oxidation in 2 mM TRIS buffer (pH 7.0) was monitored by the formation of the main oxidation product 2,3-diaminophenazine at 440 nm.

Cu(II)-induced oxidation of ascorbate and o-phenylenediamine

Ascorbate has commonly been applied as reductant to generate the DNA-Cu(I) complex [6, 14, 15] and to induce oxidative DNA cleavage [1, 6, 8–10, 28], but little is known about the kinetics of reaction (1) when coupled to reaction (2). Fig. 2 a shows that Cu(I)-induced oxidation of AH^- is speeded up (by a factor of ≈ 30) upon addition of DNA. As in the case of catecholamines, the oxidation was promoted also by poly(I) but not by poly(U). We have furthermore noted a marked buffer effect, in that AH^- oxidation, both in the presence and absence of DNA, was much faster in HEPES, MES and PIPES buffer than in phosphate, TRIS and phosphate + HEPES at corresponding pH's. The reaction (1) is apparently slowed down when Cu(II) is complexed by phosphate or TRIS; HEPES, MES and PIPES, on the other hand, have only a low affinity for Cu(II) [29]. There was no such effect of buffering in the case of catecholamines, which themselfes behave as strong Cu(II) chelators. The effect of DNA on the rate of AH^- oxidation by Cu(II) (Fig. 2 a) can again be explained, as outlined in the Introduction, by reaction (2) pulling reaction (1) against thermodynamic equilibration.

It must be mentioned that the promotive effect of DNA on Cu(II)-induced ascorbate oxidation at $[AH^-] < [Cu(II)]$ (Fig. 2 a) does not necessarily involve a catalytic action of copper. On the contrary, we have recognized that copper-catalyzed, oxygen-dependent oxidation of ascorbate under the condition $[AH^-] \gg [Cu(II)]$, in the time scale of 30 min at 10 μM Cu(II), is efficiently inhibited by DNA. This effect can be explained with the fixation of Cu(I) by DNA, reaction (2), which interferes with

the catalytic sequence of reactions (1), (3), (4a) and (9),

$$Cu(I) + H_2O_2 \rightarrow {}^{\cdot}OH + Cu(II) + OH^- \quad (4a)$$
$$AH^- + {}^{\cdot}OH \rightarrow A^{\cdot -} + H_2O. \quad (9)$$

The reaction (4a), $k_{4a} = 4.1 \times 10^3$ M^{-1} s^{-1} [30], which regenerates Cu(II), is in fact orders of magnitude faster than the corresponding reaction (4) in presence of DNA, $k_4 \approx 2$ M^{-1} s^{-1} [11].

A further example for the promotion of Cu(II)-induced redox reactions by DNA is the oxidation of *o*-phenylenediamine, shown in Fig. 2b. The main oxidation product, 2,3-diaminophenazine, which was identified by the absorption band at 440 nm [31], is formed *via* a complex mechanism [32], but clearly the reaction is efficiently speeded up by DNA also in this case. Formation of DNA-Cu(I) by the interaction of GSH with Cu(II) in the vicinity of DNA will be described in a separate paper.

Discussion

Copper, as an essential trace element in biological systems, is considered to be important particularly in the antioxidative defense against oxygen-derived free radicals, for instance as reaction center in superoxide dismutases [2]. It has been pointed out, on the other hand, that copper, in terms of its ability to mediate oxidative damage to DNA, is an extremely dangerous metal [7, 10]. Copper may actually play a functional role in cell nuclei, as a ligand involved in structural organization of the chromatin [33, 34], thus there is reason to be concerned about possible deleterious effects of copper in this most fundamental cell compartment.

The present results demonstrate that DNA can drastically promote Cu(II)-induced oxidation of catecholamines, ascorbate and phenylenediamine, taken just as examples of oxidizable endogenous and xenobiotic compounds. It is likely that the observed activity of Cu(II) in combination with DNA is a more general phenomenon. This activity is thought to be due to the very high affinity of DNA for Cu(I), which is formally equivalent to an increase of the reduction potential of Cu(II).

With formation of the rather stable DNA-Cu(I) complex [7], the DNA itself becomes predisposed as a target for oxidative damage *in situ* by H_2O_2, reaction (4), and it has been established that ·OH-scavengers are rather inefficient in protecting the DNA against this type of damage [6, 10, 20, 35]. DNA degradation by H_2O_2 *via* the reaction sequences (1)–(2)–(4) and (5)–(2)–(4) is catalytic, thus the DNA/Cu(II)/AH^- and DNA/Cu(II)/ROH systems behave like a suicidal peroxidase. The reaction (4) is certainly very slow [11], but we have pointed out that with formation of the DNA-Cu(I) complex the catalytic activity of copper with respect to oxidation of other substrates like AH^- is strongly inhibited (at $[AH^-] \gg [Cu(II)]$).

It is commonly assumed that reducing compounds (H-donors) in general terms behave as antioxidants against free radical damage. The present results indicate, however, that such compounds can also act as prooxidants in combination with copper and in the vicinity of DNA. In addition to the formation of DNA-Cu(I), a variety of reactions can be activated in the vicinity of the DNA by the products of Cu(II)-induced oxidation. H_2O_2 can be generated by interactions of oxygen, *i.e.* reaction (3) in the case of ascorbate, and from semiquinones ($RO^{\cdot}$) in the case of catecholamines [25]. Benzoquinones, for instance, are thought to be responsible for antitumor activity and other toxicological properties of catecholamines [25, 36–38] and hydroquinones [39]. With DNA as activator of Cu(II)-induced generation of benzoquinones the cell nucleus is likely to be a preferred compartment of action of such compounds. The fairly simple mechanism of activation, as demonstrated for the DNA/copper system, may certainly also apply to other target molecules in combination with other transition metal ions, and as a general rule it can be assumed that the activating biomolecule must have a high affinity specifically for the reduced form of the redox active metal ion.

Another aspect is that the strong interaction of Cu(I) with DNA, with preference for alternating dG-dC sequences [7], leads to a conformational change [14, 15], possibly a B-to-Z DNA transition. If copper is an essential chromatin component, as has been suggested [33, 34, 40], one can imagine that compounds, capable of reducing Cu(II) specifically in the vicinity of DNA, can act as a trigger to the change of conformation, which may either be a deleterious event [33, 34] or, like Z-DNA formation [16], a regulatory biological process. Glutathione is a possible endogenous compound which may act in this way as a trigger. In a recent

study on GSH-mediated electron transfer [41] we have found evidence that Cu(II) reduction by GSH does not proceed *via* formation of potentially damaging free GS· radicals.

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