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Hydroxyl radical oxidation of guanosine 5'-triphosphate (GTP): requirement for a GTP-Cu(II) complex

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Abstract

Levels of oxidized guanosine (G) base in DNA have become a hallmark biomarker in assessing oxidative stress implicated in a variety of disease and toxin-induced states. However, there is evidence that the G in the nucleotide triphosphate pool (GTP) is more susceptible to oxidation than Gs incorporated into nucleic acids and this causes a substantial amounts of the oxidized product, 8-oxoguanosine 5'-triphosphate (oxo⁸GTP), to accumulate in cell-free and in cell culture preparations. Electron paramagnetic resonance (EPR) spectroscopy and direct EPR analysis of free radical production by copper sulfate and L-ascorbic acid demonstrates that the hydroxyl radical ([•]OH) is produced via oxidation of Cu⁺ to Cu²⁺ while in a complex with GTP. This [•]OH production is dependent on the availability of oxygen and the presence of GTP in the reaction milieu. Verification of free radical-mediated production of oxo⁸GTP is presented using HPLC with electrochemical (EC) detection and matrix-assisted laser desorption/ionization linear time-of-flight mass spectrometry (MALDI-LTOF-MS). The sum of these results is presented in a novel mechanism of GTP oxidation by Cu²⁺ and L-ascorbic acid. A better understanding of the chemistry involved in this oxidative modification of GTP facilitates a more comprehensive understanding of its potential physiological consequences.

Keywords

oxidative stress; free radicals; guanosine triphosphate; GTP; guanosine; hydroxyl radical; copper; ascorbate

Introduction

Oxidative stress is defined as the deleterious impact on cell function or viability as a consequence of the loss in balance between reactive oxygen species (ROS) and antioxidants in the cellular milieu ¹. ROS are formed continuously as a result of normal cellular respiration, enzymatic metabolism, and exogenous insults ². An excess of ROS is known to induce damage to DNA and the cellular nucleotide pool, yielding DNA strand breaks as well as specific oxidized bases in DNA. 8-oxo-7,8-dihydro-2'-deoxyguanosine (oxo⁸dG) is the most prevalent form of oxidative base modifications produced by the reaction of a hydroxyl radical ([•]OH) at the C8 position of 2'-deoxyguanosine (dG) incorporated into DNA or in the guanine incorporated in the cytosolic nucleotide triphosphate form (dGTP and GTP)³⁻⁵. The nucleotide

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pool has recently been investigated as a significant target for oxidative stress given that GTP is more accessible for OH[•]-mediated oxidative modification than the deoxynucleoside form, dG, incorporated into DNA⁶⁻⁸. Substantial evidence has been presented that much of the biologically relevant oxidation occurs in the cytosol at the nucleotide pool level⁷.

GTP comprises nearly 25% of the total intracellular nucleotide triphosphate pool, it acts as a versatile nucleotide participating in many critical physiological functions including RNA synthesis, cell signaling through activation of GTP-binding proteins, as well as the production of the second messenger cyclic guanosine monophosphate (cGMP)⁹. It has been previously shown that OH[•] is produced by electron transfer by Fe(II), Fe(III), and Cu(II) complexed with nucleotide triphosphates such as GTP in the presence of the abundant cellular reductant ascorbate¹⁰. Additionally, such Cu complexes are known to affect signal transduction by interfering with second messenger systems, and depending on their concentration and the target, these Cu complexes may be a major factor influencing cell survival or cell death responses^{11, 12}. Oxidation to the GTP pool produces the oxidized adduct 8-oxoguanosine 5'-triphosphate (oxo⁸GTP) (Figure 3). It is unknown how the increase in OH[•] by Cu complexes such as Cu(II)-GTP in the presence of reductants might effect the various cellular targets of GTP either directly or indirectly due to the oxidation of GTP itself. Despite the high cellular abundance of GTP and the availability of Cu, the specific mechanism of OH[•] production by GTP in the presence of Cu(II) and ascorbate has not been investigated.

Copper is contained within all respiring tissues as an essential trace element, a micronutrient essential for cell survival, as well as a cofactor for several metalloenzymes (e.g. Cu/Zn-SOD, cytochrome c oxidase)^{13, 14}. However, in some pathological conditions, copper homeostasis is lost and the toxicological consequences have been ascribed to its potential to act as a catalyst for oxidative damage by redox cycling between univalent (Cu(I)) and divalent (Cu(II)) oxidation states¹⁵⁻¹⁷. In particular, redox cycling in the presence of partially reduced oxygen species such as hydrogen peroxide (H₂O₂) and superoxide anion (O₂^{•-}) is believed to result in the generation of the highly reactive and damaging OH[•] via the Haber-Weiss cycle (eq. 1-3).

Hence, the copper ion has been highly implicated in ROS-mediated damage in the high concentrations that occur in diseases such as Alzheimer's Disease, familial amyotrophic lateral sclerosis, and Creutzfeldt-Jacob Disease¹⁵.

The goal of this study is to identify the free radical chemistry of OH[•] production by Cu(II) complexed with GTP in the presence of ascorbate. In order to assess the mechanism by which copper ions catalyze the oxidation of GTP in the presence of L-ascorbic acid, we detected the generation of ROS by EPR spin trapping. We also evaluated the influence of pH, copper and GTP concentration, and oxygen consumption on the production of OH[•] and oxo⁸GTP along with verification of the Cu(II)-GTP complex formed during the oxidation process. Our results are compiled to propose a mechanism for this reaction.

Materials and methods

Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO), Merck & Co. Inc. (Whitehouse Station, NJ) or Fisher Scientific (Pittsburgh, PA) and were analytical grade or better. 8-oxoguanosine-5'-triphosphate (oxo⁸GTP) was purchased from TriLink Biotechnologies (San Diego, CA). 8-oxoguanosine (oxo⁸G) was obtained from Cayman Chemical (Ann Arbor, MI). Cu(II) isatin-diimine complex [Cu(Imine)] used in the control reaction was synthesized by condensation reaction of the amine ligand 2-aminoethyl pyridine with isatin, followed by metallation with Cu(II) ions added as perchlorate salt, according to previously described procedures¹⁸. All buffers were pre-treated with Chelex-100 to remove metal ion contamination. All solutions were prepared with distilled water purified in Milli-Q UF-Plus apparatus (Millipore Corp., Bedford, MA).

EPR Spin-Trapping Instrument Conditions

Electronic spectra were recorded on a Shimadzu 1500 spectrophotometer with temperature regulated cell compartment. EPR spectra were recorded in a Bruker EMX Plus Electron-Spin Resonance Spectrometer System instrument, operating at X-band frequency. We utilized standard Wilmad quartz tubes maintained at 77 K for frozen solutions or a flat 200 μ L quartz cell for spin trapping experiments at room temperature. DPPH (α,α' -diphenyl- β -picrylhydrazyl) was used for calibrating frequency ($g = 2.0036$) with samples in a frozen water solution at 77 K. Typical conditions used in these measurements were 2.00×10^4 gain, and 10 G modulation amplitude.

Reaction Conditions for EPR Analysis—To examine the production of radicals during the incubations of copper, GTP and ascorbic acid, EPR spin trapping experiments were performed utilizing 5,5-dimethylpyrroline N-oxide (DMPO) as a spin trap. A typical incubation mixture contained 10 μ M copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), 1 mM GTP, and 1 mM L-ascorbic acid maintained in phosphate buffered acid solution (pH 4). Reactions were diluted in 10mM phosphate buffered saline (PBS) with the addition of 80 mM DMPO, pH 7.4. The reaction mixtures were monitored over several time points between one and 30 minutes. The spectra were recorded at room temperature ($22 \pm 1^\circ\text{C}$) on a Bruker EMX Plus Electron-Spin Resonance Spectrometer System instrument at X-Band equipped with a high sensitivity cavity (4119HS). The magnetic field and the quantification of radical production were measured with a standard aqueous solution of 4-hydroxy-2,2,6,6-tetramethyl-1-piperidiniloxyl (TEMPOL, $g = 2.0056$). All EPR spin trapping experiments were conducted in replicates of three.

Oxymetry Conditions

Oxygen measurements were carried out in a GILSON oxygraph apparatus (Medical Electronics Inc. USA). A Clark platinum electrode was used as an O_2 probe with an internal reference probe containing a Ag/AgCl solution and a YSI membrane (Yellow Spring Instruments, Yellow Springs, OH). A saturated KCl solution was maintained between the electrode and the membrane. A 1.5 mL volume standard cell was utilized in these experiments enclosed with a capillary cap, ensuring no oxygen was exchanged with the atmosphere, and incubated in a water bath maintained at $22.0 \pm 0.2^\circ\text{C}$ with constant stirring. Sodium dithionite was used to calibrate the oxygraph. Oxymetry experiments were conducted in replicates of three.

Quantification of Oxo⁸GTP by HPLC-EC

Oxo⁸GTP was quantified using HPLC-EC with preparative dephosphorylation as described previously⁶. A typical reaction contained 10 μ M Cu(II) sulfate, 1mM GTP, and 1 mM L-Ascorbic Acid diluted in PBS, pH 7.4 exactly as described above. Incubations were maintained

at 37°C for four hours. Oxo⁸GTP was then dephosphorylated to its nucleoside form, 8-oxoguanosine (oxo⁸G), for detection via HPLC-EC. On ice, 25 Units of alkaline phosphatase (dissolved in Tris-HCl pH 8.0), 1.8 mM sodium acetate, and 100 mM Tris-HCl, were added to 10 µL of sample in total volume of 20 µL. After incubation at 37°C for 1 hour, the dephosphorylation reaction was stopped by placing on ice. This was followed by filtering through Ultrafree-MC (30-kD) tubes (Millipore Corp., Bedford, MA). Detection of the generated nucleoside oxo⁸G was carried out by injecting 10 µL of the filtrate into the HPLC. Oxo⁸G was resolved by HPLC with a reverse phase YMC basic column (4.6 × 150 mm; particle size 3-micron) (YMC Inc., Wilmington, NC) and quantified using a CoulArray electrochemical detection (EC) system (ESA, Inc., Chelmsford, MA). An isocratic mobile phase consisting of 100 mM sodium acetate, pH 5.2, 4% Methanol (HPLC Grade) diluted in water polished with C18 Sep-Pak cartridges (Waters Corp., Milford, MA) was utilized to elute oxo⁸G from the column. The mobile phase was filtered using 0.2 µm nylon filters and degassed by sonication before use with the HPLC. Potentials of the twelve coulometric analytical cells of the CoulArray system, placed in series, were as follows: 50, 125, 175, 200, 250, 380, 500, 700, 785, 850, 890, 900 mV. Data were recorded, analyzed, and stored using CoulArray for Windows data analysis software (ESA Inc., Chelmsford, MA). Oxo⁸G was monitored in the 250 mV channel and injected amounts were graphed relative to peak area. A calibration curve for oxo⁸G was generated from known quantities ranging from 10 picomoles to 500 picomoles. Oxo⁸G quantified in reactions were reported as oxo⁸GTP.

Verification of Oxo⁸GTP formation by MALDI-LTOF

GTP reactions and standards were analyzed using negative-mode, matrix-assisted laser desorption/ionization linear time-of-flight mass spectrometry (MALDI-LTOF-MS) as previously described with minor modifications¹⁹. Standards and reaction mixtures diluted in methanol were mixed in a 1:1 ratio with 9 mg/mL of 9-aminoacridine (9-AA) matrix in acetone for deposition. A 1 µL aliquot of these mixtures was deposited onto a single spot on a 96-well, stainless steel (SS), MALDI sample plate and allowed to dry at room temperature (Applied Biosystems, Foster City, CA). Analysis was performed using a Voyager-DE Pro MALDI-TOF mass spectrometer using the negative linear mode of operation (Applied Biosystem, Foster City, CA). The following settings were used for each analysis: accelerating voltage of 20,000V, acquisition mass range between 60 and 600 Dalton, laser intensity of 2000, and laser repetition rate of 20.0Hz. Data was acquired with 50 laser shots/spectrum. Spectra were analyzed using Data Explorer Version 4.0.0.0 (Applied Biosystem, Foster City, CA). The oxo⁸GTP analyte was confirmed by comparing spectra to standard preparations of purified oxo⁸GTP and GTP.

Statistical Analysis

All data were analyzed using GraphPad 4.0 software (GraphPad Software Inc., San Diego, CA). A one-way ANOVA with a Newman-Keuls multiple comparison test post-hoc analysis was performed on the oxo⁸GTP data presented; values denoted with an asterisk are significantly different from their corresponding control ($p < 0.05$).

Results

Production of [•]OH by Cu(II)-GTP

Figure 1 shows representative EPR spectra from reactions of GTP (1 mM) and copper sulfate (10 µM) diluted in PBS at pH 7.4 in the presence of 1 mM L-ascorbic acid. A constant ascorbyl radical background signal was detected in control experiments containing only L-ascorbic acid and DMPO (Figure 1, spectra A-B). Interestingly, in reactions under our experimental conditions (copper/GTP/L-ascorbic acid) the signal from the ascorbyl radical increased in the first 2-5 minutes and then decreased over the course of 30 minute incubation (Figure 1, spectra

C to H). We also confirmed the initial formation of DMPO/•OH adduct, characterized by $a_N = a_H$ EPR hyperfine parameters of 14.9 G (Figure 1, spectra C). After 10 minutes, this signal is no longer detected (Figure 1, spectrum E). The relatively small signal from DMPO/•OH is due to the low concentration of GTP and Cu(II) ion. We also performed experiments under low oxygen conditions by bubbling Argon into the solution during the 30 minute incubation as well as into all stock solutions included in the reactions (Figure 1, spectra C' to H'). This resulted in a lower concentration of DMPO/•OH adduct at the early time points of incubation, and greater ascorbyl radical detection at later time points as compared to PBS and stock solutions saturated in atmospheric oxygen (Figure 1, spectrum C' to H'). This finding suggests that oxygen participates directly in the reaction mechanism. Low oxygen concentration resulted in a slower extinction of the ascorbyl radical and a decrease in •OH production as compared to reactions saturated in higher, atmospheric oxygen.

Hydroxyl radicals react with dimethyl sulfoxide (DMSO) at a high velocity with second order rate constant of about $10^9 \text{ M}^{-1} \text{ s}^{-1}$, generating a methyl radical trapped by DMPO (DMPO/•CH₃) (Figure 2)²⁰. To confirm the formation of •OH in our copper/GTP/L-ascorbic acid system, DMSO was added into the incubations mixtures and the DMPO/•CH₃ adduct ($a_N = 16.4 \text{ G}$ and $a_H = 23.4 \text{ G}$) was detected with a signal that increased over time, while the ascorbyl radical signal remained unchanged (Figure 2).

To compare the differences in the radical generation during GTP oxidation between Cu(II) ion free in solution and Cu(II) complexed with GTP, an organic Cu(II) complex [Cu(Imine)] was used as model of a stable Cu(II) complex in the reaction. In these experiments, the Cu(II) sulfate solution was substituted with Cu(Imine) which contains an imine ligand derivate from the oxindole isatin^{12, 18}. The resulting EPR spectra from spin trapping experiments were very similar to those using Cu(II) sulfate as a catalyst. This suggests that the Cu(II) ion in this reaction is complexed and the electron recycling between Cu(II) and Cu(I) is facilitated while in this GTP-Cu(II) complex (data not shown).

A series of experiments were performed to identify the individual influence of copper, ascorbic acid, and GTP on the production of •OH (Figure 3). In reactions without the reducing agent L-ascorbic acid, production of the DMPO/•OH adduct starts at time points beginning at 10 minutes, rises slowly, and persists for about 1 hour (Figure 3, spectra A to G). This is in contrast to the fast DMPO/•OH signal seen with the complete system (Figure 1). This suggests a requirement for L-ascorbic acid, most likely to regenerate the Cu(II) species from Cu(I) formed during the reaction. In the absence of Cu(II) ions, there is no detectable •OH formed, and the time course and amplitude of the ascorbyl radical is exactly as in control reactions containing L-ascorbic acid in PBS solution alone (Figure 3, spectra H to L and Figure 1, spectra A). Without the substrate GTP, we observed only the high-amplitude ascorbyl radical peak due to recycling with the copper ions. This diminishes over time in a similar fashion as reactions containing GTP (Figure 3, spectra M to P and Figure 1, spectra D to H).

With the aim of evaluating the effect of pH on •OH formation, we also analyzed reactions at pH 7.0 and pH 5.7 (Figure 4). The production of both the ascorbyl radical and the DMPO/•OH adduct were decreased by lowering the pH of the PBS in the reaction solution from control, pH 7.4, to pH 7.0 and pH 5.7 (Figure 1, Figure 4, spectra A to E and N to R). We also evaluated the effects of varying Cu(II) and GTP concentrations in solution on radical formation. Spectra F to I and J to M are from reactions containing 1 mM L-ascorbic acid and 2 mM GTP/30 μM Cu(II) sulfate and 3 mM GTP/40 μM Cu(II) sulfate respectively (Figure 4, Spectra F to I and J to M). Increased concentrations of GTP and Cu(II) sulfate allowed for the reactions to proceed faster as compared to reactions with 1mM GTP and 10 μM Cu(II) sulfate, as demonstrated by the disappearance of the ascorbyl radical after 20 minutes (Figure 4, Spectra I and M).

Cu(II)-GTP complex

We performed direct EPR and UV-Vis analysis of Cu(II) sulfate in PBS solution and Cu(II) sulfate combined with GTP and detected changes in the Cu(II) center which indicate a complexation of Cu(II) with GTP (Figure 5). EPR spectra of Cu(II) sulfate in PBS solution and Cu(II) sulfate combined with GTP were collected from frozen solutions of each and showed a characteristic profile of an axial environment around the Cu(II) center, as shown by the relationship of the electron g-factors ($g_{\parallel} > g_{\perp}$) (Figure 5, A-B). However, it was observed that a great structural change on the Cu(II) center took place in the presence of GTP in solution. Specifically, the EPR hyperfine parameters $A_{\parallel}$, $g_{\parallel}$ and $g_{\perp}$ changed from $132 \times 10^{-4} \text{ cm}^{-1}$, 2.42 and 2.1217 respectively, in an aqueous solution of Cu(II) sulfate, to $149 \times 10^{-4} \text{ cm}^{-1}$, 2.37 and 2.0736 respectively, in solution where GTP was present (Figure 5, A-B). Additionally, electronic spectra of Cu(II) sulfate alone and in the presence of GTP carried out under the same conditions of EPR analysis, showed a slight change on d-d absorption band (data not shown).

Oxygen consumption

To confirm the participation of molecular oxygen in the GTP oxidation we monitored oxygen consumption in reaction of GTP, Cu(II) sulfate, and L-ascorbic acid using a Clark electrode. Figure 6 shows representative curves of O_2 consumption when either Cu(II) sulfate or Cu(II) imine are added as the Cu(II) catalysts in solutions containing GTP and L-ascorbic acid. Oxygen consumption over time was very similar for the two Cu(II) compounds which indicates that Cu(II) sulfate acts as a stable complex in solutions with GTP.

Total consumption of O_2 was observed within approximately 20 minutes. This corroborates the EPR spin trapping results presented in Figure 1 which shows that the DMPO/ $\bullet\text{OH}$ adduct formed during the reaction is undetectable and the ascorbyl radical is at background levels after 20 minutes.

Oxo⁸GTP production

It has been established that the reaction of $\bullet\text{OH}$ with guanine (G) base in DNA leads to a two electron oxidation at the C-8 position. In order to verify that generation of $\bullet\text{OH}$ in the Cu(II)/GTP/L-ascorbic acid system would have preferential reactivity towards the G base, we quantified the levels of oxo⁸GTP produced by HPLC-EC, and verified its formation using MALDI-TOF. Figure 7 includes representative chromatograms and spectra from four-hour incubations at 37°C of 1mM GTP under control conditions (1 mM L-ascorbic acid), incubations of GTP with 200 μM Cu(II) sulfate only, and incubations of GTP with both 1mM L-ascorbic acid and 10 μM Cu(II) sulfate.

The highest levels of oxo⁸GTP, approximately 430 picomoles, were produced with a four-hour incubation of 1 mM GTP with both 1 mM L-ascorbic acid and 10 μM Cu(II) sulfate (Figure 7C and 8C). L-ascorbic acid alone was able to oxidize GTP due to the presence of the ascorbyl radical confirmed by EPR analysis although the oxo⁸GTP produced was four times lower than incubations that included 10 μM Cu(II) sulfate (Figure 7A and 8C). However, oxo⁸GTP is undetectable in incubations of GTP with 200 μM Cu(II) sulfate alone in the μAmpere (μA) scale used to report oxo⁸GTP under control and 1 mM-ascorbic acid/10 μM Cu(II) sulfate conditions (Figure 7B). When quantified using the nanoampere (nA) scale, oxo⁸GTP levels in incubations with 200 μM Cu(II) sulfate without 1 mM L-ascorbic acid are detectable although three orders of magnitude lower than those that include the reductant in the presence of 10 μM Cu(II) sulfate (Figure 8C). These results confirm the EPR analysis of GTP incubations where the highest levels of the most oxidizing radical $\bullet\text{OH}$ were observed in reactions that included both L-ascorbic acid and copper. MALDI-TOF spectra also confirmed the presence of oxo⁸GTP in these four-hour incubations (Figure 7, D to F). Mass to charge (m/z) ratios for standard mixtures of GTP and oxo⁸GTP were 522.20 and 538.33 respectively, which were also

detected in our incubations of GTP with 1 mM L-ascorbic acid alone and 1 mM L-ascorbic acid/10 μ M Cu (II) sulfate conditions.

L-ascorbic acid alone was able to increase oxo⁸GTP levels in a dose-dependent manner during one-hour incubations at 37°C with the highest oxidation seen at 1 mM and a decrease in oxo⁸GTP observed at a concentration of 10 mM (Figure 8A). Increasing concentrations of Cu (II) sulfate in the presence of 1 mM L-ascorbic acid also dose-dependently increased oxo⁸GTP levels reaching the highest values at 10 μ M with no additional oxidation seen at 100 μ M (Figure 8B).

To emphasize the specificity of our oxo⁸GTP-generating system, we included another endogenous biological oxidant, the aggregated peptide found in the senile plaques of AD brains, amyloid beta peptide (A β ₁₋₄₂). Our results included in Figure 8 indicate that A β ₁₋₄₂ alone is not capable of producing ROS at a level that oxidizes GTP to a detectable degree.

Mechanism proposed

The results presented herein indicate a mechanism involving ROS and the ascorbyl radical in the oxidation of GTP by copper. As shown in the proposed mechanism in Figure 9, key factors in this oxidative modification of GTP is the requirement for oxygen and the complexation of Cu(II) with GTP. The $\bullet$ OH produced in our solutions, via Fenton reactions involving Cu(II) is likely responsible for the production of oxo⁸GTP because of its high oxidation potential ($E^\circ = 2.31$ V, pH 7.0)². In the first step of our proposed mechanism, the Cu(II) ion forms a complex with GTP and in step 2, is reduced to Cu(I)-GTP via electron transfer from the oxidation of ascorbic acid to the ascorbyl radical. This is followed by step 3 in which the Cu(I)-GTP reacts with molecular oxygen present in the solution to generate O₂ $\bullet$ by electron transfer, and Cu(II)-GTP is regenerated and enters the cycle to produce another ascorbyl radical. The generated O₂ $\bullet$ is subsequently reduced ($E^\circ = 0.94$ V) in the presence of the ascorbyl radical to H₂O₂ in step 4. The ascorbyl radical is also converted back to ascorbate in this step ($E^\circ = 0.28$ V). The reaction of the ascorbyl radical and O₂ $\bullet$, generating ascorbate and H₂O₂ has a second order rate constant of 2.3×10^8 M⁻¹ s⁻¹²¹. The other important reaction that can happen in this system is the oxidation of ascorbic acid by O₂ $\bullet$ which proceeds with a second order rate constant of about 2.7×10^5 M⁻¹ s⁻¹²². Thus, the ascorbyl radical produced in the first step of this reaction acts as an important agent in the reduction of O₂ $\bullet$ to H₂O₂ which enters the Fenton reaction with Cu(I)-GTP in step 5 to produce $\bullet$ OH. Finally, this generated $\bullet$ OH goes on to oxidize GTP to oxo⁸GTP in step 6. The reaction stops when available oxygen is consumed in the reactions or all available GTP is oxidized. Based on the results presented in this work, we propose a $\bullet$ OH-mediated mechanism for GTP oxidation by Cu(II) in the presence of L-ascorbic acid.

Discussion

We have previously demonstrated that the Cu/L-ascorbic acid system leads to the formation of oxo⁸GTP in cell-free preparation as well as in cells in culture⁶. It has been proposed that oxidation of the G moiety in DNA is mainly due to attack by $\bullet$ OH²³. In order to verify the chemical generation of $\bullet$ OH and consequent oxidation of GTP by Cu(II)-GTP complexes, we investigated free radical production by the copper/GTP/L-ascorbic acid system. In general, EPR spin trapping experiments confirmed the production of $\bullet$ OH and the ascorbyl radical in reactions containing GTP, Cu(II), and ascorbate (Figure 1). The results also suggest that copper (II) catalyzes the formation of $\bullet$ OH while complexed, stabilizing the copper ion. The empirical ratio $g_{\parallel}/A_{\parallel}$ is frequently used to evaluate tetrahedral distortions in tetragonal structures of Cu (II) compounds. In Figure 5, a ratio $g_{\parallel}/A_{\parallel}$ close to 100 cm indicates a square planar or tetragonal structure around the Cu(II) ion, and values from 175 to 250 cm are indicative of a distorted tetrahedral symmetry²⁴. Based on this designation, our data revealed a change to a more tetragonal structure when Cu(II) is combined with GTP in solution and strongly suggests the

complexation of Cu(II) with GTP. The maximum wavelength for Cu(II) sulfate in aqueous PBS solution decreased from 750 nm to 830 nm in presence of GTP thus corroborating the changes on the Cu(II) center observed by EPR. The formation of a Cu(II) complex with GTP favors the formation of a more efficient catalyst for the observed oxidation of GTP, by facilitating Cu(II)/Cu(I) redox potential changes²⁵. When a redox active metal like copper is bound to a biological molecule such as GTP, known to be susceptible to oxidation, the oxidative-reduction reaction between ion states of the metal can generate ROS locally that modify the molecule to which the metal is bound most efficiently. Our finding that L-ascorbic acid alone generates detectable levels of the ascorbyl radical and consequently leads to the oxidation of GTP in the absence of copper is not unusual. The dual-role of L-ascorbic acid as an oxidant at lower concentrations and antioxidant at higher concentrations has been previously reported and is due to the decrease in the rate of antioxidant reactions by ascorbate at low concentrations as compared to the rate of radical chain reactions initiated by catalytic metals such as copper²⁶.

The •OH-producing system of GTP/1 mM L-ascorbic acid/ 10 μ M Cu(II) sulfate presented in this study has been shown to generate significant levels of oxo⁸GTP in cell culture⁶. Interestingly, the increase in the percent of oxo⁸GTP produced relative to GTP in HEK 293T cells exposed to 1 mM L-ascorbic acid/ 10 μ M Cu(II) sulfate as compared to control is nearly twice as high as that observed in cell-free preparations of GTP like those reported here. This is likely due to higher availability of oxygen for this •OH-producing system in a respiring cell as compared to a cell-free, closed reaction with high oxygen demand but limited oxygen supply.

In order to evaluate this mechanism of oxidation in regards to a disease pathology known to be associated with oxidative stress, we decided to include the aggregated peptide A β ₁₋₄₂ found in senile AD plaques²⁷. There has been some debate as to whether aggregated A β ₁₋₄₂ can spontaneously produce ROS^{28, 29}. Our results indicate that A β ₁₋₄₂ alone is not capable of producing ROS at a level that oxidizes GTP to a detectable degree. Our results would rather suggest that A β deposits could serve as a hot spot where Fenton-reactive metals such as copper could react with endogenous cellular components such as GTP and be the source of ROS in pathologies such as AD³⁰⁻³³. This is supported by the finding of increased oxidative damage to areas surrounding A β deposits, where Cu concentrations are estimated to be as high as 400 μ M in AD brain.

Conclusions

In this study we report that GTP oxidation by copper is promoted by the production of ROS (summarized in Figure 9). Complexation of Cu(II) with GTP is an integral step in this mechanism of oxidation to produce oxo⁸GTP as is the availability of a cellular reductant such as L-ascorbic acid and oxygen. An endogenous environment that fulfills these oxidative requirements is found in the brains of patients with neurodegenerative disease pathologies such as AD where copper, L-ascorbic acid, GTP, and oxygen are all within the range used in the cell-free experiments presented herein. Insight into the mechanism of GTP oxidation could lend important clues as to how oxidative damage from aging or disease pathologies might impair neuronal cell processes necessary for proper brain function. The influence of pH on the level of the generated radicals suggest that H₂O₂ is involved in the •OH production. This H₂O₂ is likely formed by the reaction of O₂^{•-}, generated initially by copper in the presence of O₂, with available hydrogen in the reaction milieu.

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References

1. Sies H. Role of reactive oxygen species in biological processes. *Klin Wochenschr* 1991;69(2123):965–8. [PubMed: 1798292]
2. Halliwell B, Gutteridge JM. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch Biochem Biophys* 1986;246(2):501–14. [PubMed: 3010861]
3. Einolf HJ, Schnetz-Boutaud N, Guengerich FP. Steady-state and pre-steady-state kinetic analysis of 8-oxo-7,8-dihydroguanosine triphosphate incorporation and extension by replicative and repair DNA polymerases. *Biochemistry* 1998;37(38):13300–12. [PubMed: 9748338]
4. Hayakawa H, Taketomi A, Sakumi K, Kuwano M, Sekiguchi M. Generation and elimination of 8-oxo-7,8-dihydro-2'-deoxyguanosine 5'-triphosphate, a mutagenic substrate for DNA synthesis, in human cells. *Biochemistry* 1995;34(1):89–95. [PubMed: 7819228]
5. Russo MT, Blasi MF, Chiera F, Fortini P, Degan P, Macpherson P, et al. The oxidized deoxynucleoside triphosphate pool is a significant contributor to genetic instability in mismatch repair-deficient cells. *Mol Cell Biol* 2004;24(1):465–74. [PubMed: 14673178]
6. Bolin C, Cardozo-Pelaez F. Assessing biomarkers of oxidative stress: analysis of guanosine and oxidized guanosine nucleotide triphosphates by high performance liquid chromatography with electrochemical detection. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007;856(12):121–30.
7. Haghdoust S, Sjolander L, Czene S, Harms-Ringdahl M. The nucleotide pool is a significant target for oxidative stress. *Free Radic Biol Med* 2006;41(4):620–6. [PubMed: 16863995]
8. Svoboda P, Harms-Ringdahl M. Protection or sensitization by thiols or ascorbate in irradiated solutions of DNA or deoxyguanosine. *Radiat Res* 1999;151(5):605–16. [PubMed: 10319734]
9. Strader CD, Fong TM, Tota MR, Underwood D, Dixon RA. Structure and function of G protein-coupled receptors. *Annu Rev Biochem* 1994;63:101–32. [PubMed: 7979235]
10. Biaglow JE, Held KD, Manevich Y, Tuttle S, Kachur A, Uckun F. Role of guanosine triphosphate in ferric ion-linked Fenton chemistry. *Radiat Res* 1996;145(5):554–62. [PubMed: 8619020]
11. Filomeni G, Rotilio G, Ciriolo MR. Disulfide relays and phosphorylative cascades: partners in redox-mediated signaling pathways. *Cell Death Differ* 2005;12(12):1555–63. [PubMed: 16151458]
12. Filomeni G, Cerchiaro G, Da Costa Ferreira AM, De Martino A, Pedersen JZ, Rotilio G, et al. Pro-apoptotic activity of novel Isatin-Schiff base copper(II) complexes depends on oxidative stress induction and organelle-selective damage. *J Biol Chem* 2007;282(16):12010–21. [PubMed: 17327230]
13. Kaim, Wa, JR. *Angew Chem Int Ed Engl* 1996;35:43–XX.
14. Solomon EI, Sundaram UM, Machonkin TE. Multicopper Oxidases and Oxygenases. *Chem Rev* 1996;96(7):2563–2606. [PubMed: 11848837]
15. Rossi L, Arciello M, Capo C, Rotilio G. Copper imbalance and oxidative stress in neurodegeneration. *Ital J Biochem* 2006;55(34):212–21. [PubMed: 17274527]
16. Rossi L, Lombardo MF, Ciriolo MR, Rotilio G. Mitochondrial dysfunction in neurodegenerative diseases associated with copper imbalance. *Neurochem Res* 2004;29(3):493–504. [PubMed: 15038597]
17. Klomp LW, Lin SJ, Yuan DS, Klausner RD, Culotta VC, Gitlin JD. Identification and functional expression of HAH1, a novel human gene involved in copper homeostasis. *J Biol Chem* 1997;272(14):9221–6. [PubMed: 9083055]
18. Cerchiaro G, Aquilano K, Filomeni G, Rotilio G, Ciriolo MR, Ferreira AM. Isatin-Schiff base copper (II) complexes and their influence on cellular viability. *J Inorg Biochem* 2005;99(7):1433–40. [PubMed: 15878622]
19. Edwards JL, Kennedy RT. Metabolomic analysis of eukaryotic tissue and prokaryotes using negative mode MALDI time-of-flight mass spectrometry. *Anal Chem* 2005;77(7):2201–9. [PubMed: 15801754]
20. Zhang H, Joseph J, Felix C, Kalyanaraman B. Bicarbonate enhances the hydroxylation, nitration, and peroxidation reactions catalyzed by copper, zinc superoxide dismutase. Intermediacy of carbonate anion radical. *J Biol Chem* 2000;275(19):14038–45. [PubMed: 10799477]

21. Scarpa M, Stevanato R, Viglino P, Rigo A. Superoxide ion as active intermediate in the autoxidation of ascorbate by molecular oxygen. Effect of superoxide dismutase. *J Biol Chem* 1983;258(11):6695–7. [PubMed: 6304051]
22. Nishikimi M. A function of tetrahydropteridines as cofactors for indoleamine 2,3-dioxygenase. *Biochem Biophys Res Commun* 1975;63(1):92–8. [PubMed: 1125028]
23. Breen AP, Murphy JA. Reactions of oxyl radicals with DNA. *Free Radic Biol Med* 1995;18(6):1033–77. [PubMed: 7628729]
24. Muller J, Felix K, Maichle C, Lengfelder E, Strahle J, Weser U. *Inorg Chem Acta* 1995;233:11–19.
25. Azzellini MA, Bagatin IA, Ferreira AM. Di-imine copper(II) complexes as redox mediator and modulator in 2-deoxy-D-ribose oxidative damage. *Redox Rep* 2006;11(1):25–37. [PubMed: 16571273]
26. Buettner GR, Jurkiewicz BA. Catalytic metals, ascorbate and free radicals: combinations to avoid. *Radiat Res* 1996;145(5):532–41. [PubMed: 8619018]
27. Gaggelli E, Kozlowski H, Valensin D, Valensin G. Copper homeostasis and neurodegenerative disorders (Alzheimer's, prion, and Parkinson's diseases and amyotrophic lateral sclerosis). *Chem Rev* 2006;106(6):1995–2044. [PubMed: 16771441]
28. Hensley K, Carney JM, Mattson MP, Aksenova M, Harris M, Wu JF, et al. A model for beta-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease. *Proc Natl Acad Sci U S A* 1994;91(8):3270–4. [PubMed: 8159737]
29. Turnbull S, Tabner BJ, El-Agnaf OM, Twyman LJ, Allsop D. New evidence that the Alzheimer beta-amyloid peptide does not spontaneously form free radicals: an ESR study using a series of spin-traps. *Free Radic Biol Med* 2001;30(10):1154–62. [PubMed: 11369506]
30. Huang X, Atwood CS, Hartshorn MA, Multhaup G, Goldstein LE, Scarpa RC, et al. The A beta peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction. *Biochemistry* 1999;38(24):7609–16. [PubMed: 10386999]
31. Huang X, Cuajungco MP, Atwood CS, Hartshorn MA, Tyndall JD, Hanson GR, et al. Cu(II) potentiation of alzheimer abeta neurotoxicity. Correlation with cell-free hydrogen peroxide production and metal reduction. *J Biol Chem* 1999;274(52):37111–6. [PubMed: 10601271]
32. Lovell MA, Robertson JD, Teesdale WJ, Campbell JL, Markesbery WR. Copper, iron and zinc in Alzheimer's disease senile plaques. *J Neurol Sci* 1998;158(1):47–52. [PubMed: 9667777]
33. Dikalov SI, Vitek MP, Mason RP. Cupric-amyloid beta peptide complex stimulates oxidation of ascorbate and generation of hydroxyl radical. *Free Radic Biol Med* 2004;36(3):340–7. [PubMed: 15036353]

Figure 1. EPR spectra of GTP, L-ascorbic acid, and Cu(II)

A) and B) EPR spectra of control experiments in PBS, pH =7.4, 1 mM L-ascorbic acid, and 80mM DMPO incubated for 2 min (A) and 30 min (B). C) to H) EPR spectra of reactions of 1 mM GTP, 1 mM L-ascorbic acid, 10 μ M Cu(II) sulfate, and 80mM DMPO in air-saturated PBS, pH 7.4 solution. C') to H') EPR spectra of reactions exactly as in C to H, with the exception that the PBS pH 7.4 solution is devoid of oxygen by saturation with argon for 30 minutes. All spectra were collected at room temperature (22 $^{\circ}$ C) and plotted on the same scale (magnetic field and intensity of EPR) for comparison. EPR conditions: receiver gain 5.02×10^4 , modulation amplitude 1 G, time constant 163.84 ms, frequency 9.687 GHz, power 20 mW and 1024 points resolution.

Figure 2. EPR spectra of GTP, L-ascorbic acid, and Cu(II) with DMSO

Experiment conducted in PBS, pH =7.4, 1mM L-ascorbic acid, and 80 mM DMPO incubated for 1 min (A) or 3 min (B). EPR conditions: receiver gain 5.02×10^4 , modulation amplitude 1 G, time constant 163.84 ms, frequency 9.687 GHz, power 20 mW and 1024 points resolution.

Figure 3. EPR spectra of GTP and Cu(II) or L-ascorbic acid

A) to G) EPR spectra of the reaction of 1mM GTP, 10 μ M Cu(II) sulfate, and 80 mM DMPO in air-saturated PBS, pH=7.4 solution. **H) to L)** EPR spectra of the reaction of 1 mM GTP, 1 mM L-ascorbic acid, and 80 mM DMPO in air-saturated PBS, pH 7.4 solution. **M) to P)** EPR spectra of the reaction of 1mM L-ascorbic acid, 10 μ M Cu(II) sulfate, and 80 mM DMPO in air-saturated PBS, pH=7.4 solution. All spectra were collected at room temperature (22 °C) and plotted on the same scale (magnetic field and intensity of EPR) for comparison. EPR conditions: receiver gain 5.02×10^4 , modulation amplitude 1 G, time constant 163.84 ms, frequency 9.687 GHz, power 20 mW and 1024 points resolution.

Figure 4. Effects of pH and reactant concentrations on free radical generation

A) and N) EPR spectra of 1 mM L-ascorbic and 80 mM DMPO in PBS, pH 7.0 (A) and PBS, pH 5.7 (N); B) to E) EPR spectra of the reaction of 1 mM GTP, 1 mM L-ascorbic acid, 10 μ M Cu(II) sulfate, and 80 mM DMPO, in air-saturated PBS, pH 7.0 solution. F) to I) EPR spectra of the reaction of 2 mM GTP, 1 mM L-ascorbic acid, 30 μ M Cu(II) sulfate, and 80 mM DMPO, in air-saturated PBS, pH=7.0 solution. J) to M) EPR spectra of the reaction of 3 mM GTP, 1mM L-ascorbic acid, 40 μ M Cu(II) sulfate, and 80 mM DMPO, in air-saturated PBS, pH 7.0 solution. O) to P) EPR spectra of the reaction of 1 mM GTP, 1 mM L-ascorbic acid, 10 μ M Cu(II) sulfate, and 80 mM DMPO, in air-saturated PBS, pH 5.7 solution. All spectra were collected at room temperature (22 $^{\circ}$ C) and plotted on the same scale (magnetic field and intensity of EPR) for comparison. EPR conditions: receiver gain 5.02×10^4 , modulation amplitude 1 G, time constant 163.84 ms, frequency 9.687 GHz, power 20 mW and 1024 points resolution.

Figure 5. Direct EPR analysis of Cu(II) and GTP

A) EPR spectrum of 2 mM Cu(II) sulfate. **B)** EPR spectrum of 500 μ M Cu(II) sulfate with 2 mM of GTP. All solutions diluted in water, PBS, and 5% glycerol. EPR conducted at 77K.

Figure 6. Oxygen consumption by GTP, Cu(II), and L-ascorbic acid

Oxygen measurements using a Clark electrode of the reaction of 1 mM GTP, 1 mM L-ascorbic acid, 10 μ M Cu(II) sulfate ($\bullet$) or Cu(Imine) ($\blacktriangle$), in air-saturated PBS, pH 7.4 solution in a total cell volume of 1.5 mL at $(22.0 \pm 0.2)^\circ\text{C}$.

Figure 7. Oxo⁸GTP formation analysis by HPLC-EC and MALDI-TOF

A) and D) are HPLC-EC chromatogram and MALDI-TOF spectra of oxo⁸G (reported as oxo⁸GTP, see Methods) and GTP respectively, in a four-hour incubation of 1 mM GTP and 1 mM L-ascorbic acid at 37°C. Inset of D) is from a standard solution containing 1:1 GTP:Oxo⁸GTP (m/z GTP:522.21; m/z oxo⁸GTP: 539.41). **B) and E)** is a four-hour incubation of 1mM GTP and 200 μM Cu(II) sulfate at 37°C. **C) and F)** is a four-hour incubation of 1mM GTP, 1mM L-ascorbic acid, and 10 μM Cu(II) sulfate at 37°C. HPLC-EC chromatograms show traces collected at 250 mV EC channel. MALDI-TOF spectra are normalized to % Intensity of signal and baselines were corrected for noise.

Figure 8. Oxo⁸GTP in GTP, Cu(II), L-ascorbic acid and Aβ₁₋₄₂ reactions

A) Oxo⁸GTP levels quantified by HPLC-EC in one-hour incubations of 1mM GTP and increasing concentrations of L-ascorbic acid. **B)** Oxo⁸GTP levels quantified by HPLC-EC in four-hour incubations of 1 mM GTP, 1 mM L-ascorbic acid, and increasing concentrations of Cu(II) sulfate. **C)** Oxo⁸GTP levels quantified by HPLC-EC in four-hour incubations of 1 mM GTP and 1 mM L-ascorbic acid, 200 μM Cu(II) sulfate, 1mM L-ascorbic acid/ 10 μM Cu(II) sulfate, 100 nM Aβ₁₋₄₂, and 10 μM Cu(II) sulfate/100 nM Aβ₁₋₄₂ at 37°C. Data represent mean ± SEM. *n* = 2-9. * *p* < 0.05 applying a one-way ANOVA with Newman-Keuls Multiple Comparison Test post-hoc analysis.

Figure 9. Mechanism proposed for the oxidation of GTP by Cu(II) and L-ascorbic acid