

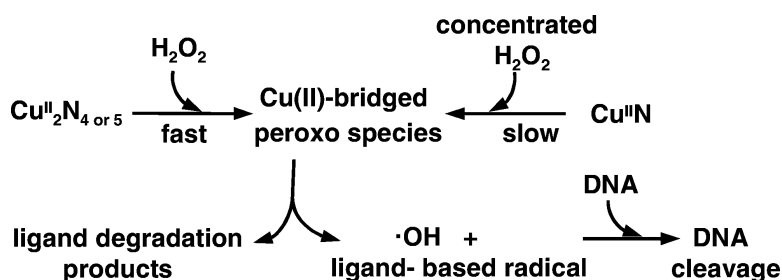
Communication

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## Hydrogen Peroxide and Dioxygen Activation by Dinuclear Copper Complexes in Aqueous Solution: Hydroxyl Radical Production Initiated by Internal Electron Transfer

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Dinuclear Cu(II) complexes  $[\text{Cu}^{\text{II}}_2(\text{N}_n)\text{Y}_2]^{2+}$  (Figure 1), where  $n = 4$  ( $\text{Cu}^{\text{II}}_2\text{N}_4$ ) or 5 ( $\text{Cu}^{\text{II}}_2\text{N}_5$ ), were recently found to cleave DNA specifically and effectively in the presence of a reducing thiol and  $\text{O}_2$  or in the presence of  $\text{H}_2\text{O}_2$  alone.<sup>1</sup> In contrast, the closely related  $[\text{Cu}^{\text{II}}_2(\text{N}_3)\text{Y}_2]^{2+}$  complex ( $\text{Cu}^{\text{II}}_2\text{N}_3$ ) and the mononuclear analogue,  $[\text{Cu}^{\text{II}}(\text{MePY}_2(\text{CH}_3\text{CN})(\text{ClO}_4^-))^{1+}(\text{Cu}^{\text{II}}\text{N})$  (Figure 1), exhibited no selective cleavage under either condition.<sup>1</sup> The origin of these differences and of the nature of the reactive intermediate(s) initiating the cleavage remains unclear, not only for these dinuclear complexes but also for other Cu-based nucleases in general.<sup>2</sup> Here we show that addition of  $\text{H}_2\text{O}_2$  to aqueous solutions of  $\text{Cu}^{\text{II}}_2\text{N}_4$ ,  $\text{Cu}^{\text{II}}_2\text{N}_5$  and  $\text{Cu}^{\text{II}}\text{N}$  produces an intermediate that subsequently decays with the concomitant generation of the hydroxyl radical.

Within minutes after the addition of low concentrations of  $\text{H}_2\text{O}_2$  ( $<100 \mu\text{M}$ ) to either aerobic or anaerobic aqueous solutions of  $\text{Cu}^{\text{II}}_2\text{N}_4$  and  $\text{Cu}^{\text{II}}_2\text{N}_5$  at near neutral pH, a new absorption band with a maximum at 376 nm was observed (Figure 2); this band was not formed with  $\text{Cu}^{\text{II}}_2\text{N}_3$  and  $\text{Cu}^{\text{II}}\text{N}$  under these same conditions but was formed slowly with  $\text{Cu}^{\text{II}}\text{N}$  under much higher  $\text{H}_2\text{O}_2$  (and Cu) concentrations (Figure 2).<sup>3</sup> Under conditions in which this band was formed maximally ( $[\text{H}_2\text{O}_2] \geq 500 \mu\text{M}$  for  $\text{Cu}^{\text{II}}_2\text{N}_4$  and  $\text{Cu}^{\text{II}}_2\text{N}_5$ , and  $[\text{H}_2\text{O}_2] \approx 50 \text{ mM}$  and  $[\text{Cu}] \approx 500 \mu\text{M}$  for  $\text{Cu}^{\text{II}}\text{N}$ ), molar absorptivities at 376 nm, calculated on the basis of  $[\text{Cu}]$ , were very similar not only for  $\text{Cu}^{\text{II}}_2\text{N}_4$  ( $2630 \text{ M}^{-1} \text{ cm}^{-1}$ ) and  $\text{Cu}^{\text{II}}_2\text{N}_5$  ( $2900 \text{ M}^{-1} \text{ cm}^{-1}$ ) but also for  $\text{Cu}^{\text{II}}\text{N}$  ( $2690 \text{ M}^{-1} \text{ cm}^{-1}$ ), suggesting that this band arises from a bridged peroxo intermediate (see below).

The band at 376 nm decayed exponentially over the course of  $\sim 15$  min to produce a product spectrum that clearly differed from that of the original complexes (Figure 2). First-order rate constants for the decay of this intermediate at 26.5 °C for  $\text{N}_4$  ( $(8.1 \pm 0.4) \times 10^{-3} \text{ s}^{-1}$ ),  $\text{N}_5$  ( $(7.9 \pm 0.1) \times 10^{-3} \text{ s}^{-1}$ ), and  $\text{N}$  ( $(7.7 \pm 1.0) \times 10^{-3} \text{ s}^{-1}$ ) were indistinguishable within the experimental uncertainties and were independent of  $[\text{H}_2\text{O}_2]$ , initial  $[\text{Cu}]$ , and the level of absorption at 376 nm, indicating that the intermediate does not react with itself, the original complex, or  $\text{H}_2\text{O}_2$ . To test whether the formation of the intermediate was reversible, catalase (1.5 units/mL) was added to remove  $\text{H}_2\text{O}_2$  following complete intermediate formation. Catalase addition did not accelerate the loss of the intermediate formed from  $\text{Cu}^{\text{II}}_2\text{N}_4$  and  $\text{Cu}^{\text{II}}_2\text{N}_5$ , implying that the  $\text{H}_2\text{O}_2$  off rate is slow with respect to intermediate decay.

The decay of the intermediate formed from  $\text{Cu}^{\text{II}}_2\text{N}_4$  and  $\text{Cu}^{\text{II}}_2\text{N}_5$  was not accelerated substantially by the addition of putative electron or hydrogen atom donors<sup>4</sup> including guanine (300  $\mu\text{M}$ ), glucose (40 mM), *N,N*-dimethylaniline (9 mM),<sup>5</sup> 3-amino-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (3-ap) (1.5 mM),<sup>5</sup> DMSO (40 mM), and

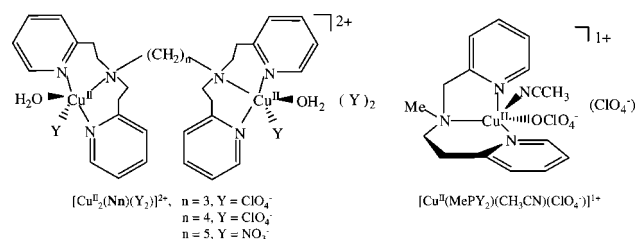


Figure 1. Structure of Cu(II) complexes.

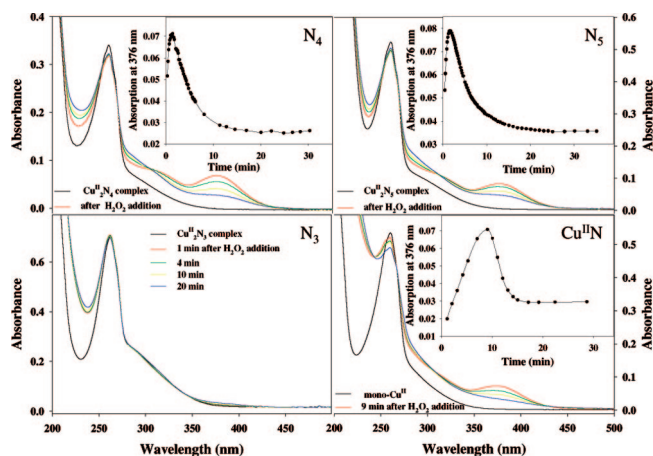


Figure 2. Absorption spectra of  $\text{Cu}^{\text{II}}_2\text{N}_{3-5}$  and  $\text{Cu}^{\text{II}}\text{N}$  before and after addition of  $\text{H}_2\text{O}_2$  in 10 mM phosphate at pH 6.8.  $\text{Cu}^{\text{II}}_2\text{N}_4$  (20  $\mu\text{M}$ ),  $\text{Cu}^{\text{II}}_2\text{N}_5$  (20  $\mu\text{M}$ ),  $\text{Cu}^{\text{II}}_2\text{N}_3$  (30  $\mu\text{M}$ ), or  $\text{Cu}^{\text{II}}\text{N}$  (50  $\mu\text{M}$ ) was dissolved in 10 mM phosphate buffer at pH 6.8.  $\text{H}_2\text{O}_2$  (80  $\mu\text{M}$  for  $\text{Cu}^{\text{II}}_2\text{N}_{4-5}$  or 3 mM for  $\text{Cu}^{\text{II}}_2\text{N}_3$  and  $\text{Cu}^{\text{II}}\text{N}$ ) was added to the corresponding solution to initiate the reaction under anaerobic conditions. Time dependence of the absorption spectra following  $\text{H}_2\text{O}_2$  addition is provided, along with the time dependence of the absorption at 376 nm (insets), with lines drawn through the data points only to highlight the temporal variation.

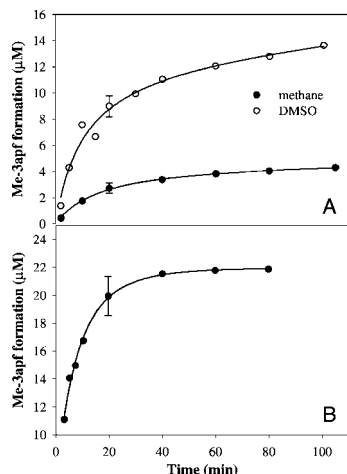
benzoic acid (1.1 mM), providing further evidence that the intermediate itself is not highly reactive.

Evidence for the formation of hydroxyl radical (OH) during the decay of the intermediate was acquired using a selective and quantitative chemical trapping technique<sup>6</sup> (Figure 3). In this technique, either DMSO<sup>7</sup> or  $\text{CH}_4$ <sup>8</sup> is employed to react with OH and produce a methyl radical, which is trapped in turn by an amino nitroxide (3-ap) to form a stable *O*-methylhydroxylamine adduct.<sup>6,8</sup> Derivatization with fluorescamine forms a highly fluorescent product (Me-3apf),<sup>9</sup> which is separated by HPLC and quantified fluorometrically.<sup>6,8,10</sup>

In the presence of either DMSO or  $\text{CH}_4$  under anaerobic conditions, Me-3apf was generated concomitantly with the decay of the  $\text{N}_4$  intermediate (Figures 2 and 3A) as well as with the decay of the  $\text{N}_5$  and  $\text{N}$  intermediates (Figure S1). These data and the

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**Figure 3.** Formation of Me-3apf in the presence of DMSO or methane. In panel A, Cu<sup>II</sup><sub>2</sub>N<sub>4</sub> complex (10.0 μM), DMSO (1.5 mM), or methane (1.5 mM, solubility in water at room temperature) and 3-ap (50 μM in DMSO experiment, 20 μM in methane experiment) were dissolved in phosphate buffer (10 mM, pH 6.8). H<sub>2</sub>O<sub>2</sub> (100 μM) was added to initiate the reaction under anaerobic conditions. Reaction was terminated by derivatization with fluorescamine under aerobic conditions, with Me-3apf then separated and analyzed by HPLC. Lines are based on a polynomial fit only to highlight the trend of Me-3apf formation. In panel B, Cu<sup>II</sup><sub>2</sub>N<sub>4</sub> complex (20.0 μM), DMSO (10 mM), and 3-ap (500 μM) were dissolved in phosphate buffer (10 mM, pH 6.8). H<sub>2</sub>O<sub>2</sub> (80 μM) was then added to initiate the reaction under anaerobic conditions. Catalase (1.5 units/mL) was added when intermediate formation was complete. Error bars represent ±1 standard deviation about the mean of three independent experiments.

observation that the ratio of Me-3apf yields for DMSO and CH<sub>4</sub> are consistent with the expected reactivity of OH at the concentrations of reactants reported in Figure 3A (see Supporting Information) provide exceedingly strong evidence that OH is generated in this step. At longer times, a much slower accumulation of Me-3apf was observed (Figure 3A) and attributed to secondary reaction of excess H<sub>2</sub>O<sub>2</sub> with the decomposition product(s) of the intermediate. Addition of catalase to remove excess H<sub>2</sub>O<sub>2</sub> following complete formation of the intermediate eliminated this slow phase of Me-3apf formation (Figure 3B), in accord with this explanation.

Under conditions of quantitative radical trapping (Figure 3B), the stoichiometry of OH formation to intermediate loss was close to one for N<sub>4</sub> (1.1:1) and N<sub>5</sub> (1.1:1) based on complex concentration (Figure 3B and Figures S3 and S4). In contrast, this ratio was ~0.5 for N (Figure S5),<sup>11</sup> providing additional support for a Cu-bridged peroxy intermediate.

Electron paramagnetic resonance spectroscopy (EPR) revealed that, in the absence of H<sub>2</sub>O<sub>2</sub>, Cu<sup>II</sup><sub>2</sub>N<sub>4</sub> and Cu<sup>II</sup><sub>2</sub>N<sub>5</sub> both exhibited spectra characteristic of noninteracting Cu(II) centers (Figures S11 and S12). Addition of H<sub>2</sub>O<sub>2</sub> largely eliminated this signal (Figures S11 and S12), indicating that the intermediate is EPR silent, consistent with the formation of a peroxy-bridged adduct.

Our results thus indicate that H<sub>2</sub>O<sub>2</sub> reacts with these complexes to generate an intermediate that likely corresponds to a Cu(II)-bridged peroxy species of as yet unidentified structure. This intermediate is not reactive with itself, H<sub>2</sub>O<sub>2</sub>, or a series of electron or hydrogen atom donors but does decay slowly to form OH in a 1:1 stoichiometric ratio (or 1 OH for every 2 coppers). These results lead us to conclude that the intermediate decays through a rate-

limiting, inner-sphere electron transfer from the ligand to the bridged center to produce OH and ligand radicals. Product analyses fully support this conclusion (Table S2 and Figures S6–S10); all ligand degradation products so far identified can be explained as arising from an initial oxidation of the coordinating, tertiary alkylamine moiety (Figure 1).

To our knowledge, these results provide the first evidence for the formation of a rather stable dicopper–peroxide intermediate in aqueous solution that decays through an internal electron transfer from the ligand to produce a strong oxidant (OH) and not through a direct metal redox reaction, electron transfer from organic donors, or a direct reaction with organic compounds. On the basis of absorption spectra, preliminary experiments indicate that this same intermediate is also formed through reaction of the Cu(I) complexes of N<sub>4</sub> and N<sub>5</sub> with dioxygen.<sup>12</sup> The reactivity profile of these complexes in water mirrors past DNA cleavage results,<sup>1</sup> suggesting that OH or a ligand-based radical initiates cleavage.

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**Supporting Information Available:** Experimental and characterization data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- (3) Formation of this band from Cu<sup>II</sup><sub>2</sub>N<sub>4</sub> and Cu<sup>II</sup><sub>2</sub>N<sub>5</sub> was found to be first order with respect to the concentration of both the copper complex and H<sub>2</sub>O<sub>2</sub>; second-order rate constants for reaction at 26.5 °C were 250 and 110 M<sup>-1</sup> s<sup>-1</sup> for Cu<sup>II</sup><sub>2</sub>N<sub>4</sub> and Cu<sup>II</sup><sub>2</sub>N<sub>5</sub>, respectively. In contrast, the kinetics of formation from Cu<sup>II</sup>N was slower and more complex, consistent with an intermolecular reaction of two Cu<sup>II</sup>N with H<sub>2</sub>O<sub>2</sub>.
- (4) Cu<sup>II</sup><sub>2</sub>N<sub>4-5</sub> complexes were dissolved in phosphate buffer (10 mM, pH 6.8). H<sub>2</sub>O<sub>2</sub> was added to initiate the reaction under anaerobic conditions at room temperature. Different electron donors were added when the maximum absorption at 376 nm was reached. Control experiments were performed in the same manner except that phosphate buffer was injected instead of the electron donor.
- (5) Slight increases in the decay rate were observed for the intermediate generated from Cu<sup>II</sup><sub>2</sub>N<sub>5</sub> in the presence of 9 mM *N,N*-dimethylaniline (from 0.32 to 0.56 min<sup>-1</sup>) and 1.5 mM 3-ap (from 0.32 to 0.55 min<sup>-1</sup>).
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- (11) Because of the high concentration of H<sub>2</sub>O<sub>2</sub> needed to form the intermediate from Cu<sup>II</sup>N, benzoic acid was employed to determine OH yield under aerobic conditions (see Supporting Information).
- (12) Solutions of the Cu(II) complexes were purged with N<sub>2</sub> for 20 min, with the Cu(II) complexes then reduced stoichiometrically to Cu(I) by additions of a deoxygenated Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> solution. Following reduction, the solution was purged with air, and absorption spectra were recorded over time. Addition of catalase (1.5 units/mL) prior to O<sub>2</sub> addition did not affect intermediate formation, indicating that this species is formed through direct reaction of the Cu(I) complexes with O<sub>2</sub>.

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