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Highly efficient copper-catalysed oxidation of ascorbic acid by peroxynitrite

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The simple salt $CuCl_2{\cdot}2H_2O$, and the new Cu-substituted polyoxometalate (POM), $Na_7[CuCoW_{11}O_{39}]{\cdot}5H_2O$ 1 are highly efficient catalysts for the oxidation of ascorbic acid by peroxynitrite.

The chemistry and reactivity of the biologically important inorganic toxin peroxynitrite† is not yet fully understood.[1,2] Antioxidants are believed to reduce the toxic effects of peroxynitrite[3] and ascorbic acid is the most common antioxidant *in vivo*. The reaction between ascorbic acid and peroxynitrite is relatively slow (k = 42–47 M^{-1} s^{-1} at 25 °C, pH 7.4).[4,5] Transition metals are known to catalyse the decomposition of peroxynitrite and other peroxo species.[1,2,6] There are a number of reports in the literature of copper complexes that catalyse the oxidation of ascorbic acid using dioxygen[7] and peroxo species.[8] We report here the highly efficient catalytic oxidation of ascorbic acid by peroxynitrite in the presence of the simple aqueous cupric ion (henceforth Cu^{2+}), formed by dissolution of $CuCl_2{\cdot}2H_2O$, and the new Cu-substituted Co-centred polyoxometalate (POM), $Na_7[CuCoW_{11}O_{39}]{\cdot}5H_2O$ **1**.

Peroxynitrite was prepared by the reaction of nitrite and acidified hydrogen peroxide solution followed by quenching with NaOH in a simple flow reactor.[9] The concentrations of these solutions were determined by UV–VIS spectroscopy [ε_{302} = 1.7 × 10^3 M^{-1} cm^{-1}].[10] The new POM, **1**, was prepared by the slow addition of $K_9[CoW_{11}O_{39}]{\cdot}14H_2O$[11] to a solution containing an excess of $CuCl_2$.‡

Phosphate buffer solutions (pH 7.4) of Cu^{2+} show highly efficient catalysis of ascorbic acid oxidation by peroxynitrite. We also prepared and characterised **1**, a new type of mixed-metal POM, and compared its catalytic properties to those of Cu^{2+}. The kinetics of peroxynitrite decay are exponential§ and follow the rate law in eqn. (1) where k_{obs} increases linearly with [Cu^{2+}] (Fig. 1).

$$-d[ONOO^-]/dt = k_{obs}[ONOO^-] \qquad (1)$$

The addition of EDTA completely inhibits the catalytic activity of Cu^{2+} and even results in a decrease of the reaction rate in the blank reaction (due to chelation of residual Cu^{2+}). The rate remains constant until [Cu^{2+}] ≈ 1.5 μM (in the presence of EDTA, Fig. 1) and then increases linearly with [Cu^{2+}]. The slope of k_{obs} *vs.* [Cu^{2+}] is the same in the presence and absence of EDTA (Fig. 1). The amount of residual Cu^{2+} in the solutions can be estimated from the interception of the straight lines (shown by the arrow in Fig. 1) at negative [Cu^{2+}]. The intercept of the plot with EDTA and the *y*-axis is linearly proportional to [ascorbic acid]. From this value the reaction rate constant for the bimolecular reaction of peroxynitrite and ascorbic acid is (51 ± 5) M^{-1} s^{-1} and is in good agreement with the literature value of 42–47 M^{-1} s^{-1}.[4,5] Thus, the reaction rate law can be written [eqn. (2)]:

$$-d[ONOO^-]/dt = (k_o + k_a[\text{ascorbic acid}] + k_{cat}[Cu^{2+}])[ONOO^-] \qquad (2)$$

where k_o = the rate constant of unimolecular peroxynitrite self-decay (k_o = 0.4 s^{-1} [1,2]), k_a = the rate constant for the bimolecular reaction of ascorbic acid with peroxynitrite (k_a = 51 M^{-1} s^{-1}), and k_{cat} = the rate constant for the catalytic pathway. k_{cat} values for both Cu^{2+} and **1** depend on [ascorbic acid] (Fig. 2). This dependence is complex, and the activities of the two Cu species are very similar.¶

Similar reactions using other transition metal-containing species ($CoCl_2{\cdot}6H_2O$, $K_7[CoAlW_{11}O_{39}]{\cdot}13H_2O$,[12] $Fe(NO_3)_2{\cdot}6H_2O$, $MnCl_2{\cdot}4H_2O$ and $NiNO_3{\cdot}6H_2O$) in aqueous solution at pH 7.4 (25 °C, 75 mM phosphate buffer, 12 mM ascorbic acid and 0.1 mM initial peroxynitrite) were investigated, and the reaction rates (k_{cat}) compared to those catalysed by Cu^{2+} and **1**. While Fe^{2+} and Ni^{2+} are inactive, Mn^{2+} exhibits slight activity ($k_{cat} \leqslant 0.05 \times 10^6$ M^{-1} s^{-1}). Both Co^{2+} and $K_7[CoAlW_{11}O_{39}]$ showed significant catalytic activity[12] (k_{cat} = 0.13 ± 0.03 × 10^6 and 0.15 ± 0.03 M^{-1} s^{-1}, respectively) but were still over 30 times slower than either Cu^{2+} or **1** (k_{cat} = 3.65 ± 0.2 × 10^6 and 3.15 ± 0.2 × 10^6 M^{-1} s^{-1}, respectively). This catalytic system is one of the most efficient reported thus far. At physiological concentrations of ascorbic acid (1 mM), Cu^{2+} catalysis of peroxynitrite-based oxidation proceeds with k_{cat}

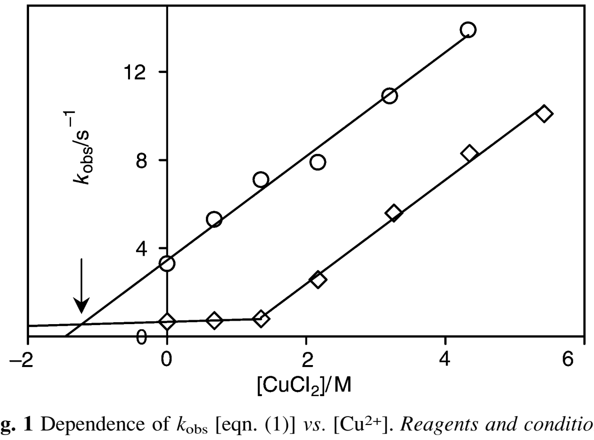

Fig. 1 Dependence of k_{obs} [eqn. (1)] *vs.* [Cu^{2+}]. *Reagents and conditions*: 25 °C, pH 7.4, 75 mM phosphate buffer, 12 mM ascorbic acid and 0.1 mM initial [peroxynitrite]. (○) No EDTA added. (◇) 2.5 μM EDTA added.

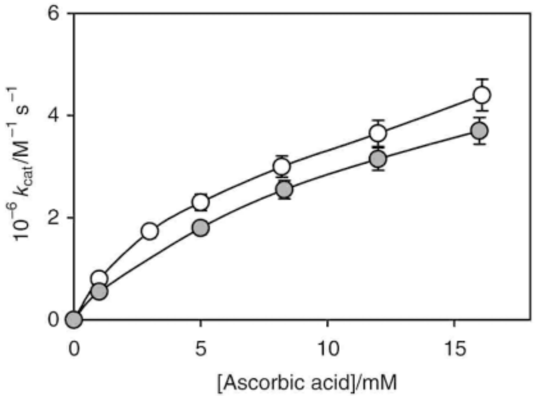

Fig 2 Dependence of k_{cat} [eqn. (2)] *vs.* [ascorbic acid]. *Reagents and conditions*: 25 °C, pH 7.4, 75 mM phosphate buffer and 0.1 mM initial [peroxynitrite]. (○): k_{cat} data for Cu^{2+}. (●): k_{cat} data for **1**.

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values of 0.8×10^6 or 1.6×10^6 $\mathrm{M^{-1}\,s^{-1}}$ (at 25 and 37 °C, respectively), which is slightly slower than for Mn porphyrins (1.6×10^6 $\mathrm{M^{-1}\,s^{-1}}$ for MnTMPyP at 23 °C).[13]

In order to assess the possible relevance of Cu-catalysed ascorbic acid oxidation by peroxynitrite *in vivo*, the activity of $\mathrm{Cu^{2+}}$ was evaluated in the presence of biologically important Cu-binding chelates, Gly-Gly-His (GGH), bovine serum albumin (BSA), cysteine and ceruloplasmin. The effect of the abiological chelates *o*-phenanthroline and nitrilotriacetic acid was also evaluated. At a 2:1 GGH:Cu ratio ([ascorbic acid] = 12 mM), $k_{\mathrm{cat}} = 3.0 \pm 0.2 \times 10^6$ $\mathrm{M^{-1}\,s^{-1}}$, which is slightly lower than that for the reaction catalysed by $\mathrm{Cu^{2+}}$ and similar to that exhibited by **1**. Further increases in the GGH:Cu ratio decreased the rate until rate was that of the background reaction at a ratio higher than 6:1. BSA also decreased the rate. At a BSA:Cu ratio = 0.5 (the molecular weight of BSA was taken as 66429),[14] the reaction rate dropped to 60% of that in the absence of the chelate. However, at a BSA:Cu ratio > 10 the activity decreased to 10% that of $\mathrm{Cu^{2+}}$. Pure Cu-loaded ceruloplasmin purified from non-specifically bound $\mathrm{Cu^{2+}}$ was catalytically inactive. However, addition of up to 8 μM $\mathrm{Cu^{2+}}$ to this ceruloplasmin is a catalytic system with 50% of the activity of free $\mathrm{Cu^{2+}}$. Cysteine strongly inhibits the catalytic activity of $\mathrm{Cu^{2+}}$ at a cysteine:Cu ratio > 1.0, but the kinetics are more complex than eqn. (1). The non-biological chelates *o*-phenanthroline and nitrilotriacetic acid substantially inhibit the reaction (to approximately the background level) when they are added in a chelate:Cu ratio of 2 and 3, respectively. Most of the biologically important Cu-chelates do not greatly affect the catalytic activity. It is evident that at least one coordination site of the $\mathrm{Cu^{2+}}$ is required for catalytic activity. Significantly, the data suggest that Cu-catalysed peroxynitrite oxidation of ascorbic acid may constitute a minor but probable pathway for ascorbic acid depletion *in vivo*.

Detailed kinetic studies showed the oxidation of $\mathrm{Cu^{I}(GGH)}_n$ to $\mathrm{Cu^{II}(GGH)}_n$ (soluble models of $\mathrm{Cu^{+}/Cu^{2+}}$ suitable for kinetic studies) and subsequent reduction by ascorbic acid are both too slow under our experimental conditions to account for the observed overall reaction rate. The mechanism therefore may involve the formation of copper-peroxynitrito intermediate complex [eqn. (3)] which is subsequently trapped by ascorbic acid [eqn. (4)]:

$$\mathrm{Cu^{II}(H_2O)} + \mathrm{ONOO^-} \rightleftharpoons \mathrm{Cu^{II}(OONO^-)} + \mathrm{H_2O} \qquad (3)$$

$$\mathrm{Cu^{II}(OONO^-)} + \mathrm{H^+} + \mathrm{HA^-} \rightarrow \mathrm{Cu^{II}(H_2O)} + \mathrm{NO_2^-} + \mathrm{A} \qquad (4)$$

where $\mathrm{HA^-}$ is the ascorbate anion and A is dehydroascorbic acid (the product of ascorbic acid oxidation). An alternative mechanism [eqns. (5) and (6)] may involve the formation of a complex between ascorbate and Cu(II) which is then oxidised by peroxynitrite:

$$\mathrm{Cu^{II}(H_2O)} + \mathrm{HA^-} \rightleftharpoons \mathrm{Cu^{II}(HA^-)} + \mathrm{H_2O} \qquad (5)$$

$$\mathrm{Cu^{II}(HA^-)} + \mathrm{H^+} + \mathrm{ONOO^-} \rightarrow \mathrm{Cu^{II}(H_2O)} + \mathrm{NO_2^-} + \mathrm{A} \qquad (6)$$

Either of these mechanisms results in the rectangular hyperbolic rate law (7):

$$-\mathrm{d}[\mathrm{ONOO^-}]/\mathrm{d}t = a[\mathrm{Cu(II)}][\mathrm{ONOO^-}][\mathrm{HA^-}]/(b + c[\mathrm{HA^-}]) \qquad (7)$$

where $a = k_3k_4$, $b = k_{-3}$, $c = k_4$ for eqns. (3) and (4), or $a = k_6K_5$, $b = 1$, $c = K_5$ for eqns. (5) and (6). Eqns. (3) and (4) are likely to operate at low [ascorbic acid], while eqns. (5) and (6) are likely to operate at high [ascorbic acid] which is consistent with the data in Fig. 2.

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Notes and references

† The term peroxynitrite is used to refer to the peroxynitrite anion $\mathrm{O{=}NOO^-}$, and peroxynitrous acid, ONOOH, unless otherwise indicated. The IUPAC recommended names are oxoperoxonitrate(−1) and hydrogen oxoperoxonitrate, respectively.

‡ *Synthesis* of $\mathrm{Na_7[CuCoW_{11}O_{39}]\cdot 5H_2O}$ **1**: $\mathrm{CuCl_2\cdot 2H_2O}$ (0.12 g, 0.7 mmol) was dissolved in $\mathrm{H_2O}$ (20 mL) and $\mathrm{K_9[CoW_{11}O_{39}]\cdot 14H_2O}$[11] (2.0 g, 0.6 mmol) was added over 30 min in small aliquots with each being allowed to dissolve before further POM was added. The resulting brown solution was stirred for a further 20 min, and KCl (10 g, 0.13 mol) was added. The solution was cooled at 5 °C overnight and the resulting brown precipitate filtered off, washed with cold $\mathrm{H_2O}$ (3 × 25 mL) and purified by dissolving in warm $\mathrm{H_2O}$ and passing a 0.1 M solution 3 times through an Amberlite® resin ion-exchange column charged with 1 M NaCl. The solution was concentrated and cooled overnight at 5 °C and the resulting solid was filtered and dried *in vacuo*. Analytical data: Calc. (found) for $\mathrm{Na_7CuCoW_{11}O_{44}H_{10}}$: Na, 5.3 (5.2); Cu, 2.1 (2.1); Co 2.0 (2.0); W 67.0 (66.8%); IR data ($\mathrm{cm^{-1}}$): 942m, 878s, 773vs, 750s, 697m, 530w, 450m.

§ Kinetics were monitored at 302 nm using a SF-61 stop flow instrument (Hi-Tech Scientific, UK). A deviation from exponential decay for the first 5–10% conversion of peroxynitrite was observed. The reaction proceeded more slowly than expected. In consequence, the first 10–15% of the kinetic curve was omitted for fitting the data. There are three possible explanations for this rate retardation. First, $\mathrm{Cu^{2+}}$ could be reduced by ascorbic acid to $\mathrm{Cu^{+}}$ (in the stock solution), and subsequent reoxidation to the catalytically active $\mathrm{Cu^{2+}}$ is slow. Second, at high [peroxynitrite] the equilibrium in eqn. (3) can be shifted to the right and thus all the $\mathrm{Cu^{2+}}$ is in the form of the peroxynitrite complex. In this case, the reaction rate is zero-order with respect to peroxynitrite and proceeds slower than the projected first-order reaction. Third, at high [peroxynitrite], eqn. (5) can be rate-limiting again resulting in zero-order with respect to peroxynitrite.

¶ In aqueous solution **1** may dissociate to $\mathrm{Cu^{2+}}$ and lacunary POM ($\mathrm{POM_{lac}}$). However, the observed activity of **1** is *not* due to this dissociation. The catalytic activities of mixtures of $\mathrm{Cu^{2+}}$ and $\mathrm{POM_{lac}}$ were investigated. The addition of $\mathrm{POM_{lac}}$ to $\mathrm{Cu^{2+}}$ slightly decreased the reaction rate. However, at a $[\mathrm{POM_{lac}}] : [\mathrm{Cu^{2+}}] > 1$ the catalytic activity was the same as for solutions of **1**. Moreover, the addition of $\mathrm{POM_{lac}}$ to **1** did not inhibit its activity.

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