The Reaction of Superoxide Radicals with Metal Picolinate Complexes

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Received July 22, 1982

α-Picolinic acid, a metabolite of tryptophan, forms stable complexes with copper, iron and zinc. The copper(II) complex was found to dismute superoxide though at a much lower rate than copper/zinc superoxide dismutase. The iron(III) picolinate was found to enhance the rate of formation of hydroxyl radicals through the Haber–Weiss reaction. The zinc(II) picolinate did not react with superoxide. The role of iron(III) picolinate as a possible intracellular catalyst of the Haber-Weiss reaction is discussed.

Introduction

α-Picolinic acid is an intermediate in the metabolic degradation of tryptophan [1]. It forms stable complexes with essential metals such as copper, iron and zinc at physiological pH [2]. It is present in solution as a pyridinyl α-carboxylate anion and binds metal ions as a bidentate ligand coordinating via the pyridinyl nitrogen and the α-carboxylate oxygen.

Both copper(II) and zinc(II) picolinate complexes are of the metal(ligand)$_2$ type. The association constants are high: copper(picolinate)$_2$, log K = 23 and zinc(picolinate)$_2$, log K = 14.9 [3]. In aqueous solution the metal(ligand)$_2$ complex is the predominant form. The iron(III) picolinate, however, forms as a metal(ligand)$_3$ complex with an association constant of log K = 26.2 [3]. The iron(III) picolinate is isolated as the iron(picolinate)$_3$ complex. However, in aqueous solution at pH 7.0, the predominant form is the Fe^{III}(picolinate)$_2$OH complex up to a concentration of 100 μM. Above this concentration, the complex forms a hydroxyl bridged dimer [4].

α-Picolinic acid has been shown to produce changes in cell shape and also induce mitochondrial swelling. Enhanced lipid peroxidation in the presence of α-picolinic acid has been reported to occur in mitochondria [5]. Other *in vitro* investigations have shown that α-picolinic acid inhibits mitosis [6]. Although some controversy appears to exist regarding the role of α-picolinic acid in trace metal transport [7, 8] the observations described appear to suggest that it enhances free radical formation after metal, possible iron, chelation. Fernandez-Pol [9] noted that exogenous α-picolinic acid at levels of 3 m*M* was observed to have both bacteriostatic and bacteriocidal effects in cell cultures.

The interaction of superoxide radicals with copper, iron and zinc picolinate complexes are reported in this investigation. Whilst the copper(II) picolinate complex catalysed the dismutation of superoxide radicals, the iron(III) picolinate complex catalysed a Haber–Weiss reaction resulting in the production of hydroxyl radicals. The zinc(II) picolinate did not react with superoxide radicals.

Experimental

α-Picolinic acid was obtained from British Drug Houses Ltd., Dorset, England. All other reagents were of the best grade available. The spin trap 5,5-dimethyl-pyrroline-N-oxide (DMPO) was prepared and purified by the method of Bonnet *et al.* [10]. Superoxide dismutation was assayed either by following the reduction of cytochrome *c* by the xanthine–xanthine oxidase system, or by following the disappearance of superoxide generated by pulse radiolysis at 250 nm in nitrous oxide/oxygen (4:1) equilibrated solutions containing 100 m*M* formate. A 4 MeV linear accelerator and associated equipment for kinetic spectroscopy and computer analysis of data described elsewhere [11, 12] were used. The radiation dose was of the order of 5–10 J Kg^{-1} following a pulse of 0–2 μs.

Electron paramagnetic resonance (Epr) spectra were recorded on a Varian E104 X-band Epr spectrometer with a Varian E900-3 data acquisition system. Generation of superoxide radicals, O_2^-, and hydroxyl radicals, OH′, by the xanthine/xanthine oxidase system can be followed by spin trapping using the spin trap DMPO [13]. Superoxide reproduction was observed as the superoxide spin adduct of DMPO, 5,5-dimethyl-2-hydroperoxypyrrolidino-1-oxyl (DMPO-OOH) and hydroxyl radical production was

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TABLE I. Composition of Metal Picolinate Complexes.

Complex	% Carbon		% Hydrogen		% Nitrogen		Structure
	Expt.	Calc.	Expt.	Calc.	Expt.	Calc.	
Iron(II)	48.17	48.21	2.86	2.67	9.44	9.38	$Na^+[Fe(II)L_3]^-$
Iron(III)	50.64	50.82	2.79	2.82	9.71	9.88	$Fe(III)L_3$
Copper(II)	46.53	40.49	2.60	2.58	9.01	9.04	$Cu(II)L_2$
Zinc(II)	41.29	41.42	2.33	2.30	8.07	8.05	$Zn(II)L_2$

L = picolinate ligand.

observed as the DMPO hydroxyl spin adduct, 5,5-dimethyl-2-hydroxylpyrrolidino-1-oxyl (DMPO-OH). Changes in epr spin adduct spectral intensities with time were followed by setting the field for the top of the downfield hyperfine component in the spectrum and monitoring the signal intensity. Spin adduct concentrations were calculated from a calibrated double integral. The effect of the metal picolinate complexes on the free radical production from the xanthine/xanthine oxidase reaction was investigated by spin trapping. All reaction mixtures contained 1 mM α-picolinic acid to chelate adventitious trace metal ions.

Copper(II), iron(III) and zinc(II) picolinate complexes were prepared by mixing aqueous solutions of the chloride salts (0.2 *M*) and α-picolinate acid (1 *M*). The precipitated metal picolinate complex was filtered, washed with distilled water and ethanol and finally dried. The iron(II) picolinate because of its high chloride (0.2 *M*) and α-picolinic acid (0.59 *M*). Excess metal ion was removed from solution with a small amount of Chelex-100 resin (Bio-Rad Laboratories, California, U.S.A.). The complex precipitated following concentration by freeze-drying. The precipitate was collected, washed with ethanol and dried. Analytical data for the metal picolinate complexes (Table I) are consistent with the predicted structures.

Metal analysis by atomic absorption spectroscopy resulted in the following metal ion content for a 0.1 m*M* solution of each complex: copper(II) 6.4 ppm; iron(II) 5.6 ppm; iron(III) 5.6 ppm and zinc(II) 6.4 ppm. These are again consistent with structures predicted in Table I.

The epr spectrum of a 0.1 m*M* solution of the copper complex recorded at 77 °K gave parameters ($g_\parallel$ = 2.233 and $g_\perp$ =2.075) characteristic of an axial copper(II) spectrum [14].

Results

The production of superoxide and hydroxyl radicals by the xanthine–xanthine oxidase reaction in the presence and absence of metal picolinate complexes is shown in Fig. 1. Copper(II) picolinate appears to scavenge superoxide radicals (Fig. 1b) whilst zinc(II) picolinate caused a slight decrease in the production of both radicals (compare Figs. 1a and 1d). In the presence of iron(III) picolinate, the superoxide signal decreased with a concomitant increase in the hydroxyl signal (Fig. 1c).

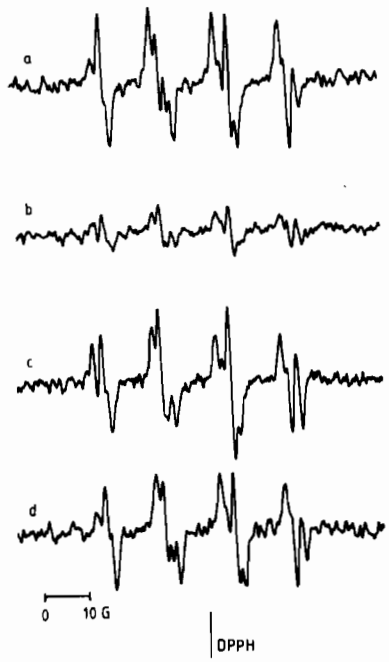

Fig. 1. Spin-trapping of the intermediates produced by the xanthine/xanthine oxidase reaction in the presence of metal picolinate complexes. The reaction mixture contained (a) 40 n*M* xanthine, 0.08 U/ml xanthine oxidase, 10 m*M* DMPO and 1 m*M* picolinic acid in 50 m*M* phosphate buffer, pH 7.8; (b) plus 100 μ*M* copper(II) picolinate; (c) plus 100 μ*M* iron-(III) picolinate and (d) plus 100 μ*M* zinc(II) picolinate. The final picolinic acid concentration in the reactions (b) to (d) was adjusted to 1 m*M*. The instrument settings were: field set 3385 G, field scan 100 G, modulation frequency 100 kHz, modulation amplitude 1.0 G, time constant 0.128 sec, scan time 60 sec from t = 30 to t = 270 sec, microwave power 10 mW, microwave frequency 9.478 GHz.

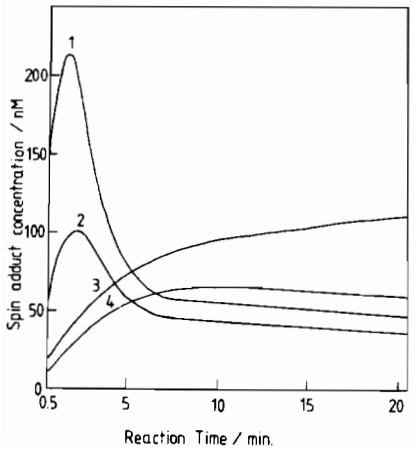

Fig. 2. Rate of production of DMPO-OOH and DMPO-OH from the xanthine/xanthine oxidase reaction in the presence and absence of iron(III) picolinate. Curves represent DMPO-OOH production in the absence (1) and presence (2) of 100 μM iron(III) picolinate and DMPO-OH production in the presence (3) and absence (4) of 100 μM iron(III) picolinate. The reaction conditions for (1) and (4) were the same as in Fig. 1(a) and for (2) and (3) as in Fig. 1(c).

The superoxide dismutase activity of the copper-(II) picolinate complexes was assayed by following the inhibition of the reduction of cytochrome c generated from the xanthine/xanthine oxidase reaction. Analysis of the data obtained by the method described by Sawada and Yamazaki [15] showed that the observed rate of the reaction between superoxide radicals and copper(II) picolinate was first order with respect to both superoxide radicals and the complex $-d[O_2^-]/dT = k[\text{copper(II)picolinate}][O_2^-]$. A bimolecular rate constant of $2 \times 10^6\ M^{-1}\ s^{-1}$ was calculated. From pulse radiolysis experiments the observed rate of disappearance of superoxide radicals by a copper(II) picolinate catalysed route was bimolecular $-d[O_2^-]/2dT = k[O_2^-]^2$ with a rate constant of $1.4 \times 10^8\ M^{-1}\ s^{-1}$.

The enhanced formation of hydroxyl radicals (DMPO-OH) by iron(III) picolinate was also investigated in the presence of various concentrations of the complex. A ten fold increase in DMPO-OH formation occurred with a 10-fold increase in the iron(III) picolinate concentration. The possibility that iron-(III) picolinate catalyses a Fenton-type Haber–Weiss reaction was considered. Both superoxide dismutase and catalase were found to inhibit hydroxyl radical spin adduct formation clearly implicating superoxide radicals and hydrogen peroxide. Hydroxyl spin adduct formation could also be inhibited by mannitol, a well known inhibitor of hydroxyl radicals. The course of superoxide and hydroxyl spin adducts formation by the xanthine/xanthine oxidase reaction in the presence and absence of iron(III) picolinate is shown in Fig. 2. A two-fold increase in hydroxyl spin adduct formation is seen to take place

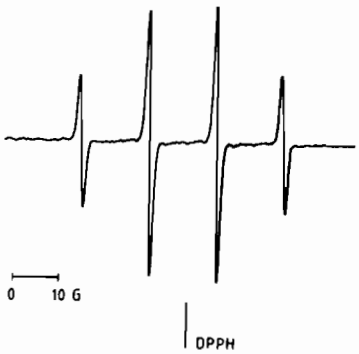

Fig. 3. Production of hydroxyl radicals by iron(II) picolinate. The reaction mixture contained: 1 mM hydrogen peroxide, 100 μM sodium ferropicolinate, 100 μM DMPO in 50 mM phosphate buffer pH 7.8.

at the same time as a two-fold decrease in superoxide spin adduct formation occurs. The results obtained therefore clearly demonstrate the enhanced production of hydroxyl radicals by the xanthine/xanthine oxidase reaction in the presence of iron(III) picolinate. The reaction of superoxide radicals generated by pulse radiolysis with iron(III) picolinate was found to have a bimolecular rate constant $k_{cat} = 9.3 \times 10^4$ at pH 8.5. This compares with estimated rate constants for iron chelates [16, 17] and is several orders of magnitude slower than the enzymatic dismutation of superoxide. The iron(II) picolinate formed subsequently catalyses the disproportionation of hydrogen peroxide. This was confirmed by investigating the reaction between sodium ferropicolinate and hydrogen peroxide. The product of this reaction in the presence of DMPO is the hydroxyl spin adduct (Fig. 3).

The zinc(II) picolinate was found not to react with superoxide radicals generated by pulse radiolysis. The slight inhibition of free radical formation from the xanthine/xanthine oxidase reaction by this complex is probably due to the fact that the free ligand added to chelate adventitious metal is causing dismutation after chelation. The decrease in the hydroxyl adduct signal is due to the interaction of this radical with free α-picolinic acid.

Discussion

Copper(II) complexes with amino acids [18], oligopeptides [19] and various salicylates [20] have been shown to dismute superoxide. Their rates of activity measured by the xanthine/xanthine oxidase system are, on the whole, low compared to the activity of copper/zinc superoxide dismutase. Copper-(II) phenanthroline complex also shown to be a good quencher of superoxide radicals [21] has only slightly higher activity than the various complexes tested. The same argument applies to the various

copper and nickel polyamine complexes tested by Kimura *et al.* [22]. The most active among 30 chelates examined was a nickel(II) naphthyl methyl-dioxo-[16]ane-N_5 complex which had about 1/200th of the activity of copper/zinc superoxide dismutase.

Although the rate constants of the reaction between pulse radiolysis generated superoxide radicals and various copper(II) chelates of salicylic acid was found to be identical with that of copper/zinc superoxide dismutase [2.0×10^9 M^{-1} s^{-1}], these complexes and various others are unstable to EDTA. Recently Robertson and Fridovich [23] have demonstrated that the much reported [24, 25] superoxide dismutase activity of the complex [copper(I)$_8$copper(II)$_6$(D-penicillamine)$_{12}$Cl]$^{5-}$ is due to free copper which is itself known to catalyse the dismutation of superoxide radicals [26]. The copper penicillamine complex is not entirely stable and it slowly decomposes. The results of our investigation show that the copper(II) picolinate has a lower activity than copper/zinc superoxide dismutase and that this complex does not mimic the enzymic activity. It is, however, extremely stable although it would be expected to suffer photolytic degradation over a long period of time [27].

The 'slow' reaction observed between superoxide radicals and iron(III) picolinate appears to be more than sufficient to support the Fenton catalysis of the Haber–Weiss reaction. The Haber–Weiss reaction has been shown to occur in the presence of iron complexes [28]. Two iron containing proteins, namely transferrin [29] and lactoferrin [30] have been shown to catalyse a Haber–Weiss reaction. However, the site of action of transferrin, which is a plasma protein is restricted to the extracellular fluid, and the site of action of lactoferrin, which is secreted by neutrophils [31] is also restricted to the extracellular milieu [32]. Iron(III) picolinate, so long as it is formed intracellularly can be suggested as a Haber–Weiss catalyst with a wider sphere of action. This hypothesis deserves consideration under conditions of tissue damage (lipid peroxidation) by oxygen free radicals. Iron(II) picolinate may satisfy the requirements of the *general* Haber–Weiss catalyst needed to justify the superoxide theory of oxygen toxicity [33].

Acknowledgements

WHB and JVB thank the Wellcome Trust for Research Fellowships. PJT thanks the SERC for a Research Studentship and AJFS thanks the CRC for a Research Fellowship. We thank Dr. H. A. O. Hill for use of epr facilities purchased by a grant from the British Heart Foundation and Dr. R. L. Willson for use of pulse radiolysis facilities set up by a grant from the Cancer Research Campaign and the Medical Research Council. We also thank Dr. B. S. Wolfenden and Prof. R. J. P. Williams FRS for helpful advice.

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