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Superoxide radical production by allopurinol and xanthine oxidase

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ROS, reactive oxygen species
SOD, superoxide dismutase
XO, xanthine oxidase
XOR, xanthine oxidoreductase
XDH, xanthine dehydrogenase
FAD, flavine–adenine dinucleotide

ABSTRACT

Oxypurinol, an inhibitor of xanthine oxidase (XO), is being studied to block XO-catalyzed superoxide radical formation and thereby treat and protect failing heart tissue. Allopurinol, a prodrug that is converted to oxypurinol by xanthine oxidase, is also being studied for similar purposes. Because allopurinol, itself, may be generating superoxide radicals, we currently studied the reaction of allopurinol with xanthine oxidase and confirmed that allopurinol does produce superoxide radicals during its conversion to oxypurinol.

At pH 6.8 and 25 °C in the presence of 0.02 U/ml of XO, 10 and 20 μM allopurinol both produced 10 μM oxypurinol and 2.8 μM superoxide radical (determined by cytochrome C reduction). The 10 μM allopurinol was completely converted to oxypurinol, while the 20 μM allopurinol required a second addition of xanthine oxidase to complete the conversion. Fourteen percent of the reducing equivalents donated from allopurinol or xanthine reacted with oxygen to form superoxide radicals. Superoxide dismutase prevented the reduction of cytochrome C by these substrates. At higher xanthine oxidase concentrations, or at lower temperatures, more of the 20 μM allopurinol was converted to oxypurinol during the initial reaction. At lower xanthine oxidase concentrations, or higher temperatures, less conversion occurred. At pH 7.8, the amount of superoxide radicals produced from allopurinol and xanthine was nearly doubled. These results indicate that allopurinol is a conventional substrate that generates superoxide radicals during its oxidation by xanthine oxidase. Oxypurinol did not produce superoxide radicals.

1. Introduction

Xanthine oxidoreductase (XOR) is implicated in generating reactive oxygen species (ROS) that contribute to the biology associated with ischemia and reperfusion injury as well as to the pathology of failing cardiac tissues [1,2], reviewed in [3–5]. XOR exists as either an oxidase (XO) that transfers reducing equivalents to oxygen, or as a dehydrogenase (XDH) that utilizes NAD or oxygen as the final electron acceptor [3,6–9]. Both forms contain an internal electron transport system that is capable of producing ROS [7,10,11]. The physiological substrates, xanthine and hypoxanthine, bind oxidized enzyme and donate two electrons into the molybdenum cofactor reducing it from Mo^{VI} to Mo^{IV}. Substrates are hydroxylated by H_2O at the molybdenum site as the electrons travel via two iron-sulfide residues to flavine–adenine dinu-

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cleotide (FAD). Reduced FAD can be divalently reoxidized by oxygen to produce hydrogen peroxide, or univalently reoxidized in two steps to generate two equivalents of superoxide radical (reviewed in [3,12–14]). Superoxide in particular has been identified as the probable reactive oxygen species that contributes to cardiac dysfunction in failing myocardium [1,2], reviewed [3–5].

Several studies in tissue culture, isolated hearts, laboratory animals and in cardiac patients have indicated that the XOR inhibitors, allopurinol and oxypurinol, are useful in preventing the formation of superoxide and improving cardiac function [1,2], reviewed in [3–5]. However, allopurinol by itself cannot prevent the generation of superoxide by XOR [15]. It is an efficient alternative substrate of XOR that must first be converted to oxypurinol, the actual inhibitor [16–22]. In contrast to allopurinol, which binds to the oxidized Mo^{VI}, oxypurinol binds to reduced Mo^{IV}. The reduced XOR–oxypurinol complex then slowly rearranges into a tightly bound inhibitory complex. Consequently, during the course of the reaction (with and without the physiological substrates) the rate of product formation prematurely decelerates as the enzyme becomes strongly inhibited by oxypurinol.

Although the inhibition appears to be irreversible, oxypurinol can slowly dissociate from XOR. Oxypurinol is referred to as a pseudo irreversible inhibitor that ''inactivates'' the enzyme.

In 1970 [18], allopurinol was shown to reduce cytochrome *C* while being oxidized by XO. A contemporary study demonstrated that the reduction of cytochrome *C* by xanthine and XO was mediated by superoxide [11]. Because allopurinol may be generating superoxide, we have studied the reaction of allopurinol with XO and confirmed that allopurinol does produce superoxide during its conversion to oxypurinol. We also examined the variables that determine the quantity of superoxide formed during the reaction.

2. Materials and methods

2.1. Materials

Xanthine, allopurinol, uric acid, 8-methyl xanthine, EDTA disodium salt and horse heart Cytochrome *C* type III were purchased from Sigma (St. Louis, MO). Oxypurinol was obtained from Sigma (St. Louis, MO) and from DSM Pharmaceuticals (Greenville, NC). Analytical grade phosphate buffer, acetonitrile, tetrabutylammonium hydrogen sulfate and trichloroacetic acid were purchased from Fisher. Amicon ultrafree centrifugal filter devices 5000 NMWL were from Millipore Corporation (Bedford, MA). Catalase and superoxide dismutase were purchased from Calbiochem (a brand of EMD Biosciences Inc., La Jolla, CA). XO was purified from unpasteurized cow's milk (obtained from the experimental dairy herd at the Ohio State University) using the modified [23] method of Massey et al. [24] and was kindly provided by Dr. Russ Hille of The Ohio State University, Department of Molecular and Cellular Biology, Columbus OH. A Cary 100 spectrophotometer equipped with a Peltier temperature control system and CaryWin software, was used for the spectrophotometric measurements (Varian, Quebec Canada). HPLC analyses used a Gilson system coupled to an Agilent PDA detector.

2.2. Methods

The XO-catalyzed conversion of xanthine to uric acid was monitored spectrophotometrically at 295 nm, $\Delta\varepsilon_{295} = 9.7\ mM^{-1}\ cm^{-1}$ [25]. Reactions were carried out in Buffer A (50 mM potassium phosphate buffer, pH 6.8, containing 0.1 mM EDTA and 200 U/ml catalase) unless otherwise indicated.

The production of superoxide was monitored by following the superoxide-dependent reduction of cytochrome *C* spectrophotometrically at 550 nm, $\Delta\varepsilon_{550} = 21\ mM^{-1}\ cm^{-1}$ [26]. The reactions were initiated with the addition of xanthine oxidase after the desired temperature was reached.

The conversion of allopurinol to oxypurinol was quantified by HPLC analysis. The concentrations of XO were varied and the initial allopurinol concentration was 10 or 20 μM. The reactions were quenched after product formation had reached a plateau using a 10% TCA solution that contained 12.5 μM 8-methyl xanthine as an internal standard. Protein was removed by centrifugation through the Amicon filters with a 5000 NMWL molecular weight cut-off. Samples were injected (25 μl) onto a Zorbax C8 column (150 × 4.5 mm) with a C8 guard and isocratically eluted with 2% acetonitrile in 50 mM potassium phosphate buffer (pH 5.0) with 0.5 mM tetrabutylammonium-hydrogensulfate, at a flow rate of 1 ml/min at 23 °C. Analytes were quantified by their UV absorbance at 260 nm. Standards and quality control samples contained 2.5–20 μM allopurinol and oxypurinol in Buffer A (without XO). The calibration curves for both analytes were linear (correlation coefficients = $R^2 > 0.99$). The precision of the method was within 10% CV and the accuracy ranged from −8 to +11% of nominal for oxypurinol and from −6 to +4% of nominal for allopurinol.

3. Results

3.1. Superoxide production by xanthine and allopurinol

The production of superoxide from XO was monitored spectrophotometrically by following the reduction of cytochrome *C* in reactions at pH 6.8 and 37 °C. The data in Fig. 1 shows that during the complete conversion of 20 μM xanthine to uric acid (confirmed by the direct spectral assay), approximately 5 μM superoxide was formed. This represents approximately 13% of the total electron flux.[1] At the end of the reaction with xanthine, the addition of 20 μM allopurinol resulted in a similar production of superoxide. However, as previously seen for the oxidation of allopurinol [16,19], the rate

[1] Each mole of substrate donates 2 mol of reducing equivalents. Thus, 20 μM xanthine represents 40 μM reducing equivalents, and 5 μM superoxide (univalent reduction of oxygen) is 13% of this total. Presumably, the remaining 35 μM reducing equivalents (87%) produced 17.5 μM H_2O_2 (divalent reduction of oxygen) superoxide.