Role of Fish Hepatic Aldehyde Oxidase in Oxidative in vitro Metabolism of Phenanthridine Heterocyclic Aromatic Compound

Khaled S. Al Salhen

Abstract—Aldehyde oxidase is molybdo-flavoenzyme involved in the oxidation of hundreds of endogenous and exogenous and N-heterocyclic compounds and environmental pollutants. Uncharged N-heterocyclic aromatic compounds such as phenanthridine are commonly distributed pollutants in soil, air, sediments, surface water and groundwater, and in animal and plant tissues. Phenanthridine as an uncharged N-heterocyclic aromatic compound was incubated with partially purified aldehyde oxidase from rainbow trout fish liver. Kinetic constant for the oxidation reactions were determined spectrophotometrically and showed that this substrate has a good affinity \( K_m = 78 \pm 7.6 \mu M \) for hepatic aldehyde oxidase, will be a significant pathway. This study confirms that partially purified aldehyde oxidase from fish liver is indeed the enzyme responsible for the in vitro production 6(5H)-phenanthridinone metabolite as it is a major metabolite by mammalian aldehyde oxidase, coupled with a relatively high oxidation rate \((0.77 \pm 0.03 \text{ nmol/min/mg protein})\). In addition, the kinetic parameters of hepatic fish aldehyde oxidase towards the phenanthridine substrate indicate that in vitro biotransformation by hepatic fish aldehyde oxidase will be a significant pathway. This study confirms that partially purified aldehyde oxidase from fish liver is indeed the enzyme responsible for the in vitro production 6(5H)-phenanthridinone metabolite as it is a major metabolite by mammalian aldehyde oxidase.

Keywords—Aldehyde oxidase, Fish, Phenanthridine, Specificity.

I. INTRODUCTION

ALDEHYDE oxidase (AO; EC 1.2.3.1) is molybdo-flavoenzyme involved in the oxidation of hundreds of endogenous and exogenous aldehydes and N-heterocyclic compounds many of which are drugs, vitamins and environmental pollutants[1]-[5]. AO is cytosolic enzyme belonging to a class of enzymes termed molybdenum hydroxylases [1]-[5]. Molybdenum hydroxylases are found in nearly every organism from humans to bacteria and have been determined to be present in a number of various tissues [1]-[5].

The examinations of AO from various different fish species have been studied [6], [7]. The roles of AO in fish have been examined in the metabolism of pollutants and the use of AO as biomarkers in response to pollution [8]-[10]. The AO in fish is responsible for metabolism of AO substrates such as endogenous vitamins [11].

Heterocyclic aromatic compounds are commonly distributed pollutants in soil, air, sediments, surface water and groundwater, and in animal and plant tissues [12]. The higher polarity and water solubility of the heterocyclic compounds is developed by the substitution of one carbon atom by nitrogen, sulfur or oxygen heteroatoms (NSO-HET)[13]. As well as a consequence, many heterocyclic compounds are mentioned to be included in the priority list of the European Water Framework Directive [14]. Heterocyclic aromatic compounds are known to show a large range of ecotoxic effects, e.g. acute toxicity, developmental and reproductive toxicity, cytotoxicity, photo-induced toxicity, mutagenicity, and carcinogenicity [12], [15]. Furthermore, some studies have shown that NSO-HET bioaccumulate in aquatic organisms, and acute toxicity has been reported for Daphnia, midge, and algae [16]-[18]. Almost all studies on heterocyclic compounds have been concentrated on heterocycles with an incorporated nitrogen atom that belong to the class of azarenes such phenanthridine[16], [17], [19]. Azarenes belonging to the new identified classes of anthropogenic pollutants. Anthropogenic reaction of azarenes is probably the main source of marine and lake sediments [20], [21]. Street dust is an important source of sedimentary azarenes [22]. Similar material can be washed from streets and roads by rainfall, carried by rivers and streams, and ultimately accumulated in the sediments. Outcome of atmospheric particulate matter containing azarenes from fossil fuel combustion is another source [22]. Bleeker et al. (2001) examined the metabolism of phenanthridine, as azarene, by cytochrome P450 in Carp (Cyprium carpio) into its metabolite 6(5H)-phenanthridinone (phenanthridine) [23]. The oxidative product, 6(5H)-phenanthridinone is a cyclic lactam substituted adjacent to a heterocyclic nitrogen atom (Fig. 1) and it is a major product of phenanthridine in vitro metabolism by aldehyde oxidase[1], [2], [24]. Because of there is only work on the biotransformation of phenanthridine remains that of Bleeker et al. examined the metabolism of phenanthridine by cytochrome P450 in Carp fish (Cyprium carpio) into its metabolite 6(5H)-phenanthridinone [23]. So, present study aims to identify the major metabolites of phenanthridine by partially purified aldehyde oxidase from rainbow trout fish liver. The specificity of liver rainbow trout fish (Oncorhynchus mykiss) aldehyde oxidase for this substrate was quantitatively explored by

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determining kinetic constants for phenanthridine as N-heterocyclic aromatic compound.

\[
\text{Phenanthridine} \rightarrow AO \rightarrow \text{6(5H)-Phenanthridone}
\]

**Fig. 1 Oxidation of phenanthridine to 6(5H)-phenanthridone by AO**

**Based on [1]**

**II. MATERIALS AND METHODS**

**A. Chemicals**

Phenanthridine, 6(5H)-phenanthridone, absolute ethanol and acetonitrile were purchased from Fisher Scientific and Sigma/Aldrich Chemical Company Ltd, Poole, UK. Solvents for chromatography were all of HPLC grade purity. Stock standard solutions of the phenanthridine and 6(5H)-phenanthridone (1mM) were made by dissolving in absolute ethanol. Solutions of lower concentrations were prepared by appropriate dilution of the stock solution with 50mM phosphate buffer saline (PBS) (pH 7.4).

**B. Preparation of Aldehyde Oxidase Fractions**

The liver rapidly excised from rainbow trout fish and weighted, chopped and placed immediately in 3-4 volumes of ice-cold isotonic potassium chloride solution (0.154M) containing 0.1mM EDTA and homogenized on ice in a homogenizer fitted with a Teflon pestle for 1-2 min at 4°C. The homogenate was then centrifuged at 4°C for 15 minutes at 10,000 xg, to pellet out the tissue and nuclear/mitochondrial fractions of the homogenate. The supernatant was then removed and centrifuged for a further 60 minutes at 4°C at 105,000 xg to obtain the cytosolic fraction. This fraction was then collected separated into 0.5ml aliquots and stored at -80°C. Partially purified aldehyde oxidase was prepared from liver supernatant by heat treatment and ammonium sulfate precipitation, as described by [25]. The fractions were restored in liquid nitrogen until used for spectrophotometric and HPLC analyses.

**C. Spectrophotometric Measurement of Enzyme Activity**

All spectrophotometric determinations were carried out at 37°C using Gen5™ software on a Windows XP PC connected to the microplate reader spectrophotometer (BioTek, UK). Enzyme activity of partially purified was monitored using phenanthridine [25]. The oxidation of phenanthridine was assayed by monitoring the increase in absorbance at 322nm (ε = 6,400 M⁻¹cm⁻¹) using molecular oxygen as the electron acceptor. All reactions were carried out in 50 mM PBS buffer pH 7.4. Phenanthridine substrate was tested for non-enzymatic interaction with PBS buffer prior to the addition of enzyme to wells (cuvettes).

**D. Determination of Kinetic Constants for Oxidation of Phenanthridine by Aldehyde Oxidase**

Aldehyde oxidase activity was determined spectrophotometrically in PBS buffer pH 7.4 at 37°C, using phenanthridine as the substrate. Six concentrations of phenanthridine, ranging from 25 to 100µM, were prepared in 30µl PBS buffer and the reaction started by adding 50µl of the enzyme fraction. All assays were carried out in triplicate in 100µl reaction volumes. All samples were frozen and thawed only once, and the spectrophotometric data were collected at 5-second intervals for 1 to 3 minutes using the microplate reader spectrophotometer (BioTek, UK). The activity of the aldehyde oxidase enzyme was tested by monitoring the oxidation of phenanthridine by an increase in absorbance at 322nm (ε = 6,400 M⁻¹cm⁻¹) [25]. These values were used for calculating initial velocities and then Ka and Vmax values from an Eadie Hofstee plot for the phenanthridine oxidation catalyzed by hepatic fish aldehyde oxidase. The line of the best fit through the points on the plot was calculated using linear regression by the least squares method using Microsoft Excel (Microsoft Office).

**E. Protein Determination**

Protein concentrations of partially purified enzyme fractions were determined using a Pierce Bicinchoninic acid (BCA) Protein assay kit with bovine serum albumin as the protein standard [26].

**F. Incubations with Partially Purified Aldehyde Oxidase Fractions**

Phenanthridine (50µM) were incubated with 200µl partially purified fish liver fractions at 37°C in a total volume of 1000µl of 700µl 50mM phosphate buffer saline, pH 7.4. Incubations were performed in 1000µl-closedvials, which were placed in a DriBlock heater (DB-2D, Techne, UK). Aliquots (200µl) were removed at 5 and 10min and added to either 100µl 20% trichloroacetic acid to terminate the reaction. Samples were centrifuged in a Microfuge 16 Centrifuge (Beckman Coulter, UK) at 10,000rpm for 2min and the supernatants were subsequently analysed by HPLC. In all in vitro assays no spontaneous oxidation of any substrate was observed when control incubations were carried out without enzyme fraction.

**G. HPLC Analysis of Phenanthridine Oxidation**

The reversed-phase high performance liquid chromatography (HPLC) system used was the Beckman system gold™ 127 solvent modules (dual pump) and a programmable UV detector (module 166). The system was fitted with a 20µl injection loop and the column used was a stainless steel Kromasil (25cm X 4.6mm, 5µm; RP-C18) with guard column (5µm) to avoid contamination of the column with any biological materials at ambient temperature. At the beginning of each working day the system was started and left to run for approximately 30 minutes. Oxidized metabolites were identified by comparison of their HPLC retention times and UV spectra with those of authentic standards. The method used for the phenanthridine study by HPLC was a gradient...
phenanthridone metabolites were detected at 254nm. The buffer for 20min [24]. The gradient system programmed with product formation of 6(5H)-phenanthridone at 322nm. The pollutant, which is a prototypical uncharged xenobiotic purified liver fish aldehyde oxidase. The result phenanthridine was calculated to reflect the relative the activity is shown as nmol substrate hydroxylated/min/mg protein and are ± S.D., n = 3. Phenanthridine concentration = 50 µM.

III. RESULTS AND DISCUSSION

Phenanthridine is a good example of an exogenous toxic pollutant, which is a prototypical uncharged xenobiotic substrate of AO. The oxidative product, 6(5H)-phenanthridine is a cyclic lactam substituted adjacent to a heterocyclic nitrogen atom (Fig. 1) [1], [2], [24]. Once the assays were optimized, AO activities were measured in the partially purified liver fish aldehyde oxidase. The result phenanthridine oxidase assay is summarized in the following Table I. The result demonstrates phenanthridine oxidase activity, which is present in liver fish (Table I). The oxidation activity of phenanthridine was calculated to reflect the relative the aldehyde oxidase activity in the fish species (Table I).

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>HEPATIC ALDEHYDE OXIDASE ACTIVITY IN LIVER FISH</th>
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<tbody>
<tr>
<td>Species</td>
<td>Enzyme activity towards phenanthridine (nmol/min/mg protein)</td>
</tr>
<tr>
<td>Rainbow trout fish</td>
<td>Phenanthridine(50 µM) 0.77 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Activity was determined as described in materials and methods. The activity is shown as nmol substrate hydroxylated/min/mg protein and are expressed as means ± S.D., n = 3.</td>
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</tbody>
</table>

TABLE II

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>SUBSTRATE</th>
<th>K_M (µM)</th>
<th>V_MAX (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainbow trout fish</td>
<td>Phenanthridine</td>
<td>78 ± 7.6</td>
<td>1.58 ± 0.32</td>
</tr>
</tbody>
</table>

The oxidation rate of phenanthridine was measured spectrophotometrically at 37°C with molecular oxygen as an acceptor. The value shown are the mean ± S.D., n = 3. Phenanthridine concentration = 50 µM.

Phenanthridine oxidase was monitored by following product formation of 6(5H)-phenanthridone at 322nm. The assay showed linear reaction kinetic under the conditions used (Fig. 2). Kinetic constants for phenanthridine with partially purified liver fish aldehyde oxidase are shown in Table II. K_m and V_max values were calculated from linear oxidation rates using Eadie-Hofstee plot (Fig. 3). The K_m and V_max values for oxidation of phenanthridine with partially purified fish liver aldehyde oxidase was also determined spectrophotometrically as described in Methods. The kinetic parameters obtained was K_m = 78 ± 7.6µM and V_max = 1.58 ± 0.32nmol/min/mg protein (Mean ± SD, N = 3) for hepatic fish aldehyde oxidase. The values of K_m and V_max of AO were determined using Eadie Hofstee (Fig. 3), due to this plot being superior to other methods of plotting data [27]. Eadie Hofstee plots of the data obtained gave good correlation coefficients (r²) > 0.97 (Fig. 3).

In order to determine that there was definitely activity present in liver fish for phenanthridine as substrates a sensitive HPLC assay was performed with this substrate and was used to confirm the product formation. This is shown in Fig. 4, where product was detected even after extended incubation periods. One metabolite was observed in chromatograms arising from the incubation of phenanthridine with partially purified fish liver aldehyde oxidase (Fig. 4). In the present study, biotransformation of phenanthridine to 6(5H)-phenanthridone has been found, which is agreement with previous studies [3], [24], [29], [33], [36], [37]. 6(5H)-phenanthridine (Phenanthridone) has been shown to be a major metabolite in mammalian studies [1], [3], [24], [28]-[31], [38].
Fig. 2 Spectrophotometric assay of aldehyde oxidase activity using partially purified liver fish aldehyde oxidase at 37˚C with 50µM phenanthridine substrate.

Fig. 3 Eadie-Hofstee plot for oxidation phenanthridine by partially purified rainbow trout fish liver aldehyde oxidase at 37˚C with molecular oxygen as an acceptor. The typical results are from three independent experiments.

Fig. 4 HPLC analysis of the *in vitro* biotransformation of phenanthridine by partially purified rainbow trout fish liver aldehyde oxidase.

HPLC chromatograms are offset on the vertical axis to allow comparison between extended incubation periods. The Lower HPLC chromatogram is *in vitro* oxidation of phenanthridine to phenanthridone at zero time. The middle chromatogram is *in vitro* oxidation of phenanthridine to phenanthridone after 5 minutes incubation time at 37˚C. The upper chromatogram shows oxidation of phenanthridine to phenanthridone after 10 minutes incubation time at 37˚C. Analytes were injected onto a RP-18 column (Kromasil 5µm) and eluted with triethylamine: acetonitrile gradient system as described in method as mobile phase. The wavelength of the detection was 254nm.
In conclusion, initial study using spectrophotometric and HPLC assays indicated that meldonio-flavonenzyme aldehyde oxidase in liver fish were able to catalyse the biotransformation of phenanthidine substrate of the well-characterised mammalian AO. This identification of major metabolite of phenanthrene by partially purified liver fish aldehyde oxidase has already brought novel information about aldehyde oxidase activity in fish species and it is expected that fish are better capable of transforming phenanthrene into its metabolite, due to their activity of aldehyde oxidase.

REFERENCES
[3] C. Beedham, “Xanthine Oxidoreductase as Aldehyde Oxidase,” (Second ed. Chapter 4.10: Comprehensive Toxicology. Copyright © 2010 Elsevier Ltd. All rights reserved.