Biochemical Pharmacology 89 (2014) 141–147

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Mammalian flavin-containing monooxygenase (FMO) as a source of hydrogen peroxide

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ARTICLE INFO

Article history:
Received 18 December 2013
Accepted 11 February 2014
Available online 19 February 2014

Keywords:
Flavin-containing monooxygenase
Hydrogen peroxide
Pulmonary FMO2
Oxidative stress
Genetic polymorphism

ABSTRACT

Flavin-containing monooxygenase (FMO) oxygenates drugs/xenobiotics containing a soft nucleophile through a C4a hydroperoxy-FAD intermediate. Human FMOs 1, 2 and 3, expressed in Sf9 insect microsomes, released 30–50% of O_2 consumed as H_2O_2 upon addition of NADPH. Addition of substrate had little effect on H_2O_2 production. Two common FMO2 (the major isoform in the lung) genetic polymorphisms, S195L and N413K, were examined for generation of H_2O_2. FMO2 S195L exhibited higher "leakage", producing much greater amounts of H_2O_2, than ancestral FMO2 (FMO2.1) or the N413K variant. S195L was distinct in that H_2O_2 generation was much higher in the absence of substrate. Addition of superoxide dismutase did not impact H_2O_2 release. Catalase did not reduce levels of H_2O_2 with either FMO2.1 or FMO3 but inhibited H_2O_2 generated by FMO2 allelic variants N413K and S195L. These data are consistent with FMO molecular models. S195L resides in the GxGx**S**G/A $NADP^+$ binding motif, in which serine is highly conserved (76/89 known FMOs). We hypothesize that FMO, especially allelic variants such as FMO2 S195L, may enhance the toxicity of xenobiotics such as thioureas/thiocarbamides both by generation of sulfenic and sulfinic acid metabolites and enhanced release of reactive oxygen species (ROS) in the form of H_2O_2.

1. Introduction

Mammalian microsomal flavin-containing monooxygenase (FMO) is a superfamily of xenobiotic metabolizing enzymes with a single member in each family. Humans express five forms of FMO in a developmental- and tissue-specific manner (reviewed in [1,2]). FMO1 is the major form in fetal liver as well as adult kidney and intestine [3,4]. FMO2 is found primarily in the lung of most mammals including primates but humans have an interesting genetic polymorphism in expression such that all Caucasians and Asians sequenced to date carry a C to T transition mutation (*FMO2*2*) which results in a premature stop codon (TAG) and synthesis of a truncated and inactive enzyme (FMO2.2) [5]. Individuals of African (up to 49%) or Hispanic (2–7%) descent possess at least one allele (*FMO2*1*) of the ancestral gene coding for full length active enzyme (FMO2.1) [6–10]. FMO3 is present in adult liver; parturition provides some unknown signal that suppresses FMO1 expression and switches on the synthesis of FMO3 [11]. FMO3 is the enzyme responsible for metabolism of trimethylamine to trimethylamine N-oxide [12]. The genetic disease trimethylaminuria (TMAU, colloquially termed "fish odor syndrome") is due to a number of known mutations in the FMO3 gene [13–16]. An individual suffering from TMAU excretes large amounts of trimethylamine in urine and sweat resulting in an unpleasant body odor. TMAU patients also exhibit socio-psychological problems as well as altered metabolism of drugs [17–19]. The developmental expression of FMO3 following birth is sometimes delayed causing what is known as "transient TMAU" in infants [11].

FMO utilizes NADPH in the presence of O_2 to form a stable 4a-hydroperoxy-FAD (FAD-OOH) intermediate [20,21]. Any xenobiotic containing a soft-nucleophile that can gain access to this site reacts with the peroxy-flavin (Fig. 1). One atom of O_2 is incorporated into the substrate whereas the other atom forms H_2O (reviewed in [2,22,23]). Previous studies by other laboratories have observed "uncoupling" of this enzyme to yield either superoxide anion radical [24] or H_2O_2 [25]. Release of superoxide anion radical with purified pig liver FMO1 was a relatively minor percent of NADPH consumption [24]. Formation of H_2O_2 was observed with pulmonary FMO2 and reached 41% of NADPH oxidized by rabbit FMO2 upon addition of primary amines; but was not observed with hepatic FMO1 sources [25].

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http://dx.doi.org/10.1016/j.bcp.2014.02.006
0006-2952/

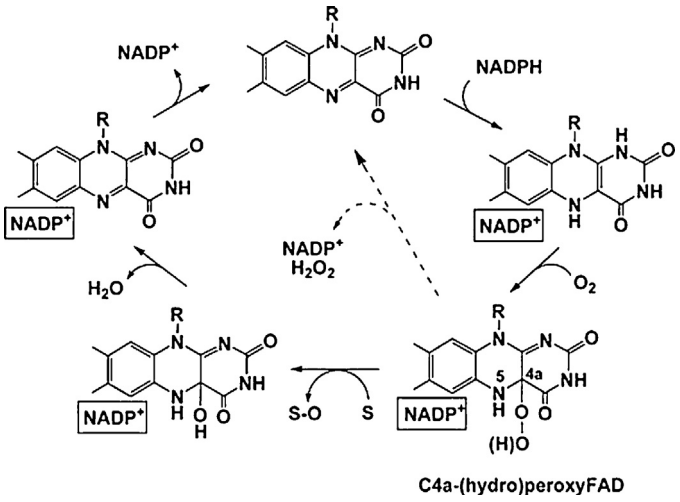

Fig. 1. Structure of FAD during the catalytic cycle of flavin-containing monooxygenases. Mammalian FMO and prokaryotic analogs have a catalytic cycle which first involves a rapid reduction by NADPH followed by binding of molecular oxygen and formation of the stable C4a-hydroperoxyflavin intermediate. This activated FAD was originally likened to a "cocked gun" by the late Dr. Henry Kamin, capable of reacting with any soft nucleophile (S) gaining proximity to this site. A nucleophilic attack by the substrate yielded an oxygenated product (S–O). The second atom of oxygen is then released as H_2O and the final step in the cycle is the release of $NADP^+$. The breakdown of the hydroxy-FAD pseudo base and release of $NADP^+$ are the slowest steps in the catalytic cycle and determine FMO turnover rate. Uncoupling (dashed line) with release of H_2O_2 is promoted if $NADP^+$ binding is compromised.
Source: This figure was taken from Alfieri et al. [37] with permission.

The relatively low K_ms for both NADPH and O_2 and the stability of the FAD-OOH raises an interesting question. If this is the major state of the enzyme present in the cell, in the absence of substrate, does the FAD-OOH release reactive oxygen species (ROS)? We, and others, have documented that there are some endogenous substrates for FMO (e.g., TMA, cysteamine, lipoic acid and methionine, reviewed in [2]) but characterization shows the K_ms (with the exception of TMA) tend to be high compared to concentrations in the cell. Our laboratory has expressed in Sf9 insect cells (baculovirus) the three major FMOs involved in drug metabolism, FMO1, FMO2.1 and FMO3 in addition to two common allelic variants of human FMO2 [26]. In order to assess the degree of ROS production by mammalian FMOs, the impact of xenobiotic substrates and potential relative differences among human isoforms, as well as some known human FMO2 allelic variants, we utilized a dual electrode system to simultaneously monitor O_2 consumption and H_2O_2 production with FMOs expressed in Sf9 insect microsomes. In addition, H_2O_2 production was assessed with Amplex Red [27] and the impact of substrate, catalase and superoxide dismutase examined.

As with any electron transport system, efficiency is rarely 100%. Within the cell, the mitochondrial electron transport chain is responsible for much of this ROS leakage but monooxygenases, such as cytochromes P450 (CYPs) [27] and now FMOs are known to contribute to the total ROS load within the cell. In addition to the potential toxicity from oxidative stress, the fact that H_2O_2 is becoming increasingly recognized as a signaling molecule, makes understanding of the cellular location and amplitude of H_2O_2 production important [28].

2. Materials and methods

2.1. Chemicals

Ethylene thiourea (ETU) was from Lancaster Synthesis (Pelham, NJ). Ethionamide, methyl-*p*-tolyl sulfide, NADPH, $NADP^+$, glucose-6-phosphate dehydrogenase, glucose-6-phosphate, potassium phosphate, sodium phosphate, glycerol, EDTA, cytochrome *c*, superoxide dismutase, and catalase were purchased from Sigma Chemical (St. Louis, MO). Protease Inhibitor Cocktail Set III was from Calbiochem (Billerica, MA). Coomassie Plus reagent was purchased from Thermo Fisher Scientific Corp. (Rockford, IL). The Amplex Red assay kit and all reagents used in expression of FMO proteins were obtained from Invitrogen Life Technologies Corp. (Grand Island, NY). Rat CYP3A2 supersomes were purchased from BD Biosciences (Franklin Lakes, NJ).

2.2. Expression of human FMO1, ancestral FMO2.1, FMO3 and FMO2 allelic variants (S195L and N413K).

A baculovirus system was utilized to express hFMO1, ancestral hFMO2.1, hFMO3 and the S195L and N413K allelic variants of hFMO2. The generation of these expressed enzymes has been described previously [26,29]. The Sf9 insect cells were harvested 96 h post-infection and microsomes isolated by ultra-centrifugation [30]. Microsomal protein was resuspended in buffer (10 mM KPO_4, pH 7.25, 20% glycerol, 1 mM EDTA, 1 μl/ml protease inhibitor cocktail). Protein concentration was quantitated by the Bradford [31] based Coomassie Plus Reagent. Flavin content was determined by an HPLC assay of FAD [32]. The specific content of the expressed flavoproteins varied from 0.99 to 1.58 nmol (average = 1.18 ± 0.24) FAD/mg microsomal protein, corresponding to 0.91–1.5 nmol FMO/mg microsomal protein (corrected by subtraction of constitutive FAD specific content in Sf9 microsomes).

2.3. H_2O_2 production and O_2 consumption

An Apollo 4000 Free Radical Analyzer (World Precision Instruments, Sarasota, FL) was equipped with an ISO-HPO-2 hydrogen peroxide electrode (World Precision Instruments) and an MI-730 oxygen microelectrode (Microelectrodes, Inc., Bedford, NH) to simultaneously measure H_2O_2 production and O_2 consumption in a closed 3 ml incubation chamber connected to a circulating water bath, to maintain 37 °C. The H_2O_2 electrode was calibrated using fresh H_2O_2 standard solutions ranging from 0 to 32 μM while the O_2 electrode was calibrated by saturating buffer with N_2 gas (0% O_2), air (21% O_2), and O_2 gas (100%) all at 37 °C. The formula for conversion of $\%O_2$ to molarity was $S = (a/22.414)(760 - p/760)(r\%/100)$, where S = solubility of gas in molar concentration, a denotes the absorption coefficient of gas at 37 °C, p is the vapor pressure of water at 37 °C, and $r\%$ represents analyzer output in percent O_2. Calibrations were done immediately before and after incubations to verify electrode stability. Expressed FMO protein (100 pmol FAD) was added to 100 mM PBS, pH 7.4, 1 mM EDTA, 1 mM NADPH or a regenerating system of 1 mM $NADP^+$, 10 U glucose-6-phosphate dehydrogenase, 2.5 mM glucose-6-phosphate) and incubated 3–5 min open to the atmosphere at 37 °C in a 3 ml sample port connected to a recirculating water bath (World Precision Instruments). Assays were performed in the presence of known FMO substrates (50 or 100 μM ETU, 50 μM trifluoperazine, 100 μM methyl-*p*-tolyl sulfide or 75 μM ethionamide) or in the absence of substrate, during which time O_2 consumption and H_2O_2 were measured for up to 60 min. Assays were performed at pH 7.4 to demonstrate biological relevance (mammalian FMOs typically have pH optima from 8.5 to 9.5). We have expressed the truncated FMO2 Q472X (FMO 2*2 A) protein in Sf9 insect cells. FAD concentration in these microsomes average 0.14 ± 0.06 nmol/mg protein and is similar to FAD in the Sf9 expressed FMO2 d337G variant which also lacks FAD binding resulting in an estimated 0.15 ± 0.05 nmol FAD/mg protein. Thus we assumed the Q472X variant would not produce appreciable amounts of H_2O_2 when incubated with NADPH and/or substrates and