Direct calorimetric measurement of substrate turnover by human FMO3

(Supplementary Information)

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Chemicals and materials

Chemicals including Luria Bertani (LB), tryptone, agar, ampicillin, Isopropyl-beta-D-thiogalactopyranoside (IPTG), phenylmethanesulfonylfluoride (PMSF), salts and agarose were purchased from Sigma Aldrich. Benzydamine, Ethionamide, Methimazole and Trimethylamine were purchased from Sigma Aldrich.

Protein expression and purification

Human FMO3 gene containing a C-terminal poly-histidine tag was heterologously expressed in *E. coli* cells using the pJL2 expression vector. LB liquid cultures (1% w/v tryptone, 0.5% w/v yeast extract and 1% w/v sodium chloride and supplemented with 100 µg/ml ampicillin) of the transformed *E. coli* JM109 with pJL2-FMO3 were incubated overnight at 37°C in a Gallenkamp shaker, 200 rpm. Two liters flasks containing 500 mL of Terrific Broth (1.2% w/v tryptone, 2.4% w/v yeast extract and 2.5% v/v glycerol) were inoculated with 5 mL overnight cultures, supplemented with 100 µg/ml ampicillin and 50 mL KPi salts and grown at 37°C in the Gallenkamp shaker, 200 rpm. At OD600 values between 0.5 and 0.8, FMO3 production was induced upon addition of IPTG at a final concentration of 1 mM, 50 µM riboflavin (used as FAD precursor) and 100 µg/ml ampicillin; overnight incubation at 26 °C in the Gallenkamp shaker, 180 rpm. Cells were harvested by centrifugation at 4,000 rpm for 20 minutes at 4 °C. After discarding the supernatant, the cells were resuspended in 50 mM KPi pH 7.4 and centrifuged again in order to remove all growth medium and stored at -20°C until use. Human FMO3 pellet was resuspended in 50 mM KPi pH 7.4, 20 % glycerol, 5 mM β-mercaptoethanol, 0.5 mM PMSF. Then 2 mg/ml of lysozyme was added to the cell suspension. The suspension was transferred to a glass beaker kept on ice and after stirring for 1 hour at 4°C, subjected to sonication on ice 7 x 30 seconds with 1 minute intervals to cool the cells. The resulting lysed cell preparation (lysate) was subjected to centrifugation at 247,000 g at 4 °C for 1 hr. The pellet was resuspended in 50 mM KPi pH 7.4, 20% glycerol, 5 mM ß-mercaptoethanol, 0.5 mM PMSF, 1% IGEPAL CA-630 detergent using dounce homogenizer. The suspension was stirred for 2 hours at 4°C in order to solubilize the membrane fraction and then subjected to centrifugation at 247,000 g at 4°C for 1 hr. The supernatant was loaded onto a DEAE-Sepharose Fast Flow column (volume =150 mL) connected to a Nickel-chelating Sepharose Fast Flow column (volume =20 mL), both previously equilibrated with 50 mM KPi pH 7.4, 20% glycerol (v/v), 5 mM ß-mercaptoethanol, 0.1% IGEPAL CA-630 detergent. After the loading the two columns were washed using the above-mentioned buffer and then disconnected. The Nickel column, which binds the His-tagged FMO proteins, was then washed with two column volumes of 50 mM KPi pH 7.4, 20% glycerol, 5 mM ß-mercaptoethanol, 0.1% IGEPAL, 50 mM KCl, 100 µM FAD, followed by two column volumes of same buffer plus 50 mM Glycine, 2 column volumes of the same buffer plus 1 mM Histidine and finally 1 column volume of same buffer plus 5 mM Histidine. FMO3 was eluted with 50 mM KPi pH 7.4, 20% glycerol, 5 mM ß-mercaptoethanol, 0.1% IGEPAL CA-630 detergent, 50 mM KCl, 100 µM FAD and 40 mM Histidine. The protein was stored in 50 mM KPi pH 7.4, 20% glycerol, 1 mM EDTA.

Isothermal titration calorimetry experiments

ITC experiments were performed using the ITC200 calorimeter at 25 °C. For general single injection experiments 2 μl of 30 μM substrate solution were injected into the sample cell containing 280 μl of reduced protein (1.2 mM NADPH, 4.8 μM hFMO3) using feedback "none" mode, 120 seconds of initial delay, a reference power of 3.84, stirring at 350 rpm. For competition experiments 2 μl of 30 μM substrate solution were injected into the sample cell containing 280 μl of reduced protein (1.2 mM NADPH, 4.8 μM hFMO3, 0-128 μM Methimazole) using feedback "low" mode, 120 seconds of initial delay, a reference power of 12, stirring at 350 rpm. For general multiple injection experiments 2 μl of 30 μM substrate solution were injected into the sample cell containing 280 μl of reduced protein (1.2 mM NADPH, 4.8 μM hFMO3) using feedback "low" mode, 120 seconds of initial delay, spacing 120 seconds, a reference power of 12, stirring at 350 rpm.

Data analysis

Data analysis was performed using the Origin software suite embedded in the Malvern instrument package using the "enzyme assay" mode. The data analysis performs calculation and yields automatically the final Michaelis-Menten parameters. In order for the software to proceed with the calculations two different experiments need to be carried out: one in single-injection mode and one in multiple-injections mode. The first experiment is used to calculate the ΔH of the reaction that is obtained upon complete conversion of substrate to product. The second experiment is used to determine the heat flow at different substrate concentrations (dQ/dt) that will be correlated to the reaction kinetics.

Below the steps that are needed to determine Michaelis-Menten parameters for each substrate:

STEP 1) Single injection mode is used to determine the enthalpy of the reaction according to equation 1:

$$\Delta H = \frac{\int_{t=0}^{\infty} \frac{dQ}{dt} dt}{V[S]_{t=0}}$$ Equation 1

where H is enthalpy, Q is the heat variation, t is time, V is volume of the reaction and [S] is substrate concentration.

Equation 1 can be re-elaborated into equation 2:

$$Q = n \cdot \Delta H = V \cdot \Delta H \cdot [P]$$ Equation 2

Where n are the moles of product and [P] is the product concentration.

Equation 2 can be re-elaborated into equation 3:

$$v = \frac{dP}{dt} = \frac{1}{V \cdot \Delta H} \cdot \frac{dQ}{dt}$$

Equation 3

Step 2) The dQ/dt measured by the multiple-injections mode is obtained by calculating the difference between the original baseline and the new baseline obtained after each injection was used to convert the experimental data to reaction rate using the ΔH previously calculated

Step 3) Data were fitted using the Michaelis-Menten equation 4:

$$v = \frac{k_{cat} \cdot [E] \cdot [S]}{K_M + [S]}$$

Equation 4

Fitting the plot of the competitive inhibition of TMA oxidation with Methimazole

The data points are the result of the enthalpic contributions for each Methimazole concentration normalized to the highest activity in the absence of Methimazole. For the determination of the IC50 concentration the enthalpy values obtained in the presence of different concentrations of inhibitor were plotted as a function of the concentration of the inhibitor and fit to a sigmoidal curve
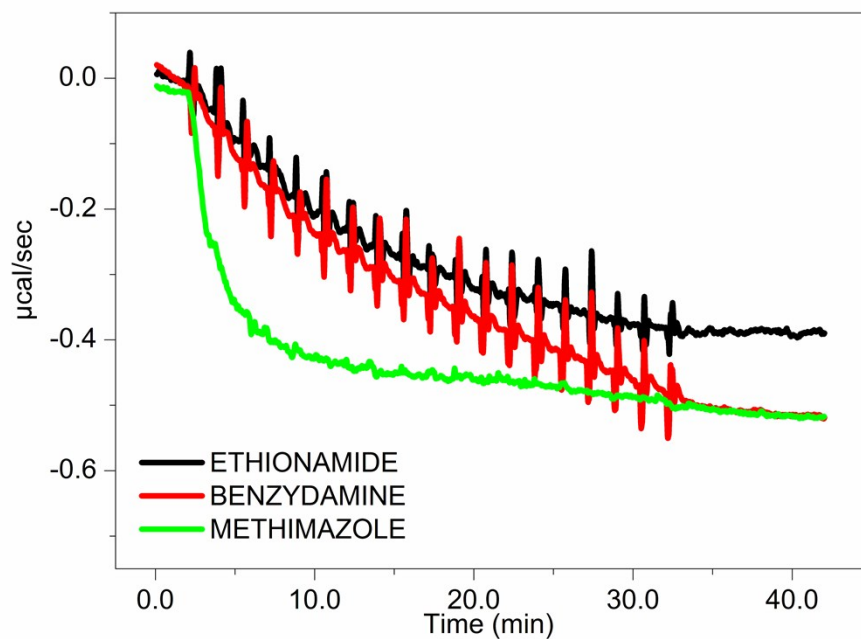
Supporting figures

Fig. S1

Multiple injections ITC measurements of substrate in a hFMO3 solution. Conditions are 1.2 mM NADPH, 30 µM substrate in 50 mM Kpi buffer pH 7.4, 25°C, 300 rpm stirring, mid-feedback mode, 120 seconds spacing, 2 µl of titration volume, 1.2-9.6 µM hFMO3
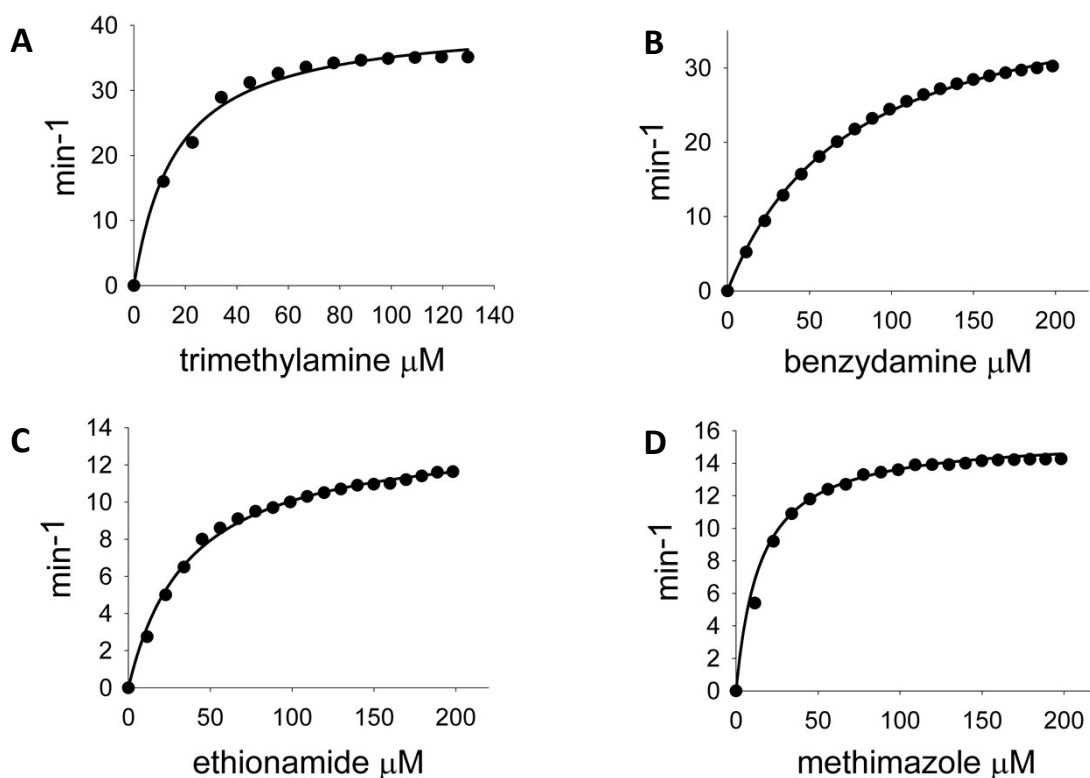
Fig S2 Michaelis-Menten plots obtained after data analysis by the origin Suite for trimethylamine (A), benzydamine (B), ethionamide (C) and methimazole (D)
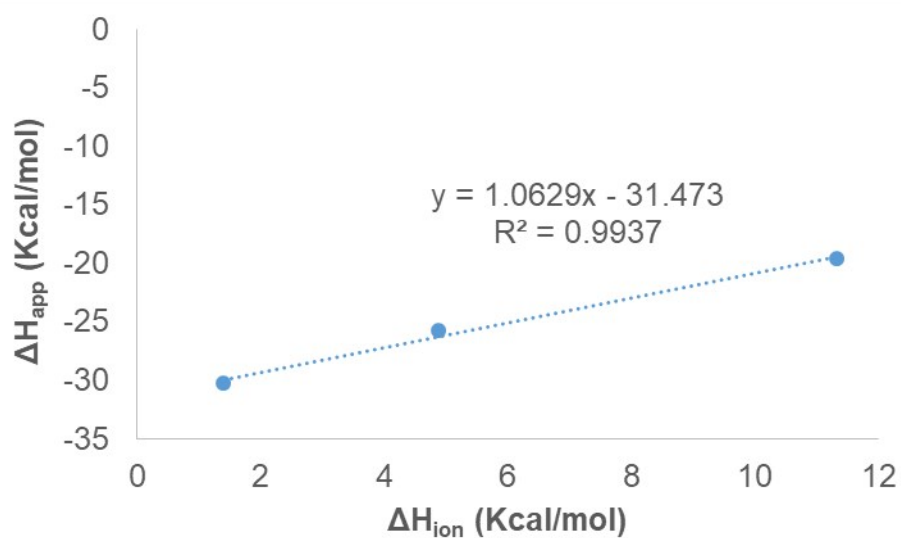

$$y = 1.0629x - 31.473$$
$$R^2 = 0.9937$$

Fig S3 Calculation of ΔH_{int} for the TMA oxidation reaction. Measurements of ΔH_{app} were carried out in 3 different buffers for which ΔH_{ion} are known:20 mM HEPES, 20 mM TrisHCl and 20 mM Phosphate.

ΔH_{int} of the reaction is obtained after applying linear regression analysis. The resulting equation is exploited also to determine the number of protons exchanged during TMA oxidation (the "a" coefficient for x = 1.06)

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