A Long-Wavelength, Fluorogenic Probe for Epoxide Hydrolase: 7-(2-(Oxiran-2-yl)ethoxy) Resorufin

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Epoxide hydrolase plays an important role in the detoxification of genotoxic compounds and in the control of physiological signaling molecules. Altered levels of epoxide hydrolase activity are associated with many diseases, such as emphysema, lung cancer, ovarian cancer, and laryngeal carcinoma. We designed and synthesized a resorufin-based fluorogenic probe, 7-(2-(oxiran-2-yl)ethoxy) resorufin, which was hydrolyzed by microsomal epoxide hydrolase to form the corresponding diol, which upon further treatment with sodium periodate released the strongly fluorescent resorufin. The probe exhibits good biological compatibility and photophysical properties, such as long wavelength excitation (571 nm) and emission (585 nm) and a wide working pH range (from 6.0 to 10.0), and thus facilitates the determination of the activity of microsomal epoxide hydrolase.

Key words microsomal epoxide hydrolase; fluorogenic probe; long wavelength; resorufin

Epoxides are reactive electrophiles capable of modifying critical biological macromolecules, such as DNA and proteins, leading to potentially mutagenic, toxic, and carcinogenic effects.^{1,2)} The catalytic addition of water to epoxides or arene oxides by epoxide hydrolases (EH, EC 332.3) to yield the corresponding 1,2-diols is one of several benign ways that cells transform and thus detoxify epoxides.³⁾ The major form of epoxide hydrolase is microsomal epoxide hydrolase (mEH) which is membrane-bound and is present in the smooth endoplasmic reticulum.^{4,5)} Polymorphism studies have showed that altered levels of mEH activity are associated with emphysema,⁶⁾ several forms of cancer,^{7–9)} and Crohn's disease.¹⁰⁾ All of these have prompted interest in developing simple and sensitive approaches to determine its activity in biological systems.

Available assays for epoxide hydrolase activity include traditional analytical techniques, such as chromatography,^{11,12)} capillary electrophoresis,¹³⁾ and autoradiography.¹⁴⁾ Recently, fluorescence-based approaches have attracted much interest due to their intrinsically high sensitivity and operational simplicity. For example, Schmid and coworkers¹⁵⁾ developed a colorimetric mEH assay by taking advantage of the nucleophilic reaction of epoxide substrates with 4-(4-nitrobenzyl)pyridine. Although whole cells were used in the assay, the method exhibits reduced accuracy in cases where the overall conversion ratio is low. Benzo[α]pyrene-7,8-epoxide is a good substrate for mEH and could be converted to the corresponding fluorescent diol compound.¹⁶⁾ However, the low quantum yield and instability of the parent probe limited its application. A few years ago, Raymond and colleagues designed an umbelliferone-based fluorogenic probe to determine EH activity.^{17,18)} In this case, the short-wavelength photophysical parameters prevent its use in complex biological systems because of their overlap with endogenous fluorescence from proteins and cellular components. Moreover, it is unable to detect the activities of EH in acidic organelles, such as the nucleus, mitochondria, plasma membrane, and Golgi apparatus due to the sensitivity of the fluorescence signal to umbelliferone protonation, which occurs at physiologically relevant pH ($pK_a=7.8$).²⁾ Resorufin is a fluorophore

widely used in biological applications due to its stability and long-wavelength photophysical properties. Furthermore, it is known that *O*-alkylation of resorufin derivatives significantly reduces fluorescence, providing a paradigm for fluorescent “switch” development.¹⁹⁾ Moreover, the pH profile of resorufin implies that its fluorescent emission is stable throughout the physiologically relevant pH range. In this paper, we designed and synthesized a resorufin-based fluorogenic probe, 7-(2-(oxiran-2-yl)ethoxy) resorufin, which has favorable photophysical properties, a wide pH working range, and serves as a fluorogenic substrate for epoxide hydrolase. An assay based on this probe that allows the measurement of microsomal epoxide hydrolase activity across a wide pH range (from 6.0 to 10.0) as well as the determination of kinetic constants has been developed.

MATERIALS AND METHODS

Chemical Reagents and Apparatus 4-Bromo-1-butene, sodium hydride, hydrogen peroxide, sodium periodate, bovine serum albumin (BSA), (hydroxymethyl)aminomethane hydrochloride (Tris-HCl), acetophenone, and tetrabutylammonium bromide were purchased from Eastern China Chemical Co. (Hangzhou, China). Resorcinol and microsomal epoxide hydrolase (mEH) were purchased from Sigma-Aldrich.

Stock solution of the probe (1 mM) was prepared in 1:1 dimethyl sulfoxide (DMSO)–water, and 2-phenyl-2-(trichloromethyl)oxirane (PTCO) was dissolved in acetonitrile (10 mM). mEH was prepared as solutions (around 1 mg/ml) in distilled water. All of the above materials were stored at -78°C . Fluorescent data were recorded on a Spectrum M2 (Molecular Device Company), and fluorescence spectra were not corrected. $^1\text{H-NMR}$ spectra were obtained using a Bruker 300 MHz NMR spectrometer.

Preparation of Fluorogenic Probe: 7-(2-(Oxiran-2-yl)ethoxy) Resorufin A mixture of resorufin sodium salt (20 mg, 0.079 mmol), 4-bromo-1-butene (35 μl , 0.32 mmol), and potassium carbonate (17 mg, 0.12 mmol) in 5 ml of dimethylformamide (DMF) was heated to 80°C for 60 h. After

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cooling to room temperature, the reaction mixture was diluted with 100 ml of ethyl acetate, washed with 1 M sodium hydroxide (10×4 ml) and brine (15×2 ml), and dried over anhydrous magnesium sulfate. After evaporation, the residue was purified by flash chromatography on silica gel (eluent: ethyl acetate/petroleum ether=3/1) to give the intermediate 7-(but-3-enyloxy)-resorufin. ¹H-NMR (CDCl₃) δ: 7.63 (1H, d, *J*=5.8 Hz), 7.35 (1H, d, *J*=5.8 Hz), 6.87 (1H, m), 6.76 (2H, m), 6.27 (1H, s), 5.83 (1H, m), 5.12 (2H, m), 4.06 (2H, t, *J*=3.9 Hz), 2.53 (2H, m). Electrospray ionization (ESI)-MS *m/z*: 268.2 (*M*⁺+1).

The intermediate (0.115 g, 0.5 mmol) was dissolved in anhydrous dichloromethane (5 ml) and hydrogen peroxide (2.49 mmol) was added. The mixture was then stirred for 3 d at 0 °C, and the solution was extracted with ethyl acetate, washed with brine, and dried over Na₂SO₄ to give crude product. Further purification was performed by flash chromatography (silica gel, ethyl acetate/hexane=3:1) to afford Probe R as a white solid 85 mg (70%). The HPLC purity was above 95%.

Probe R: ¹H-NMR (CDCl₃) δ: 7.65 (1H, d, *J*=5.4 Hz), 7.36 (1H, d, *J*=3.0 Hz), 6.89 (1H, m), 6.78 (2H, m), 6.28 (1H, m), 4.16 (2H, m), 3.11 (1H, m), 2.80 (1H, m), 2.54 (1H, m), 2.17 (1H, m), 1.88 (1H, m). ESI-MS *m/z*: 284.2 (*M*⁺+1). *Anal.* Calcd for C₁₆H₁₃NO₄: C, 67.84; H, 4.63; N, 4.94. Found: C, 67.81; H, 4.66; N, 4.90.

Preparation of 2-Phenyl-2-(trichloromethyl)oxirane (PTCO) Acetophenone (1 mmol, 120 mg), 50% NaOH (1 ml), and tetrabutylammonium bromide (10 mg) were added to CCl₄ (5 ml), and the mixture was stirred at room temperature for 8 h. The solution was extracted with ethyl acetate, washed with brine, and dried over Na₂SO₄ to give crude product. Further purification was performed by flash chromatography to afford product (42 mg, 18%). ¹H-NMR (CDCl₃) δ: 7.40 (5H, m), 3.57 (1H, d, *J*=6.1 Hz), 2.85 (1H, d, *J*=6.1 Hz). ESI-MS *m/z*: 237.2 (*M*⁺+1).

Enzyme Assay by Fluorescent Probe One microliter of the probe (10 μM), 2 μl of enzyme (20 μg/ml) and 1 μl sodium periodate (1 mM) were added to 95 μl of 50 mM Tris buffer (pH 8.4), or citric buffer (pH 6.0), or glycine buffer (pH 10.0) at 37 °C. The fluorescent intensities were monitored at λ_{em} of 585 nm with λ_{ex} of 540 nm in Spectrum M2 96-well black flat-bottom plates.

For determination of kinetic constants, the probe concentrations were varied from 0.5–5 mM for mEH. The initial rates of the reactions were calculated by the following equation:

$$\text{initial rate} = \frac{\left(\frac{n_{\text{st}} \times (F_t - F_0)}{F_{\text{st}}} \right)}{\text{time}}$$

Where *F_t* and *F₀* represent the fluorescence at time *t* and 0, *n_{st}* is the corresponding moles of the product standard, and *F_{st}* is the fluorescence resulting from *n_{st}* of product.

The *V_{max}* and *K_m* were calculated by the following equation:

$$\frac{1}{V} = \frac{K_m + [S]}{V_{\text{max}}[S]} = \frac{K_m}{V_{\text{max}}[S]} + \frac{1}{V_{\text{max}}}$$

Microsome Isolation from Beef Liver Beef liver (500 g) was coarsely minced with a knife on a cold glass plate and homogenized thoroughly in cold solution containing 0.25 M sucrose, 0.01 M potassium phosphate, and 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0 (4 ml/g liver). The mixture was centrifuged for 15 min at 600 *g*, and then 20 min at 12000 *g*. The solution was decanted to yield a post-mitochondrial supernatant. The resulting pellet was suspended in 0.8 M CaCl₂ solution, stirred slowly on ice for 15 min, and then centrifuged at 10000 *g* for 20 min. The pellet (yield 100–130 mg/g liver) was resuspended in cold 0.25 M sucrose (3 ml/g) and further diluted with 100 ml of cold 0.2 M Tris buffer (pH 8.7). The resulting solution contained protein at a concentration of 6.8 mg/ml as determined by the Lowry method and was used directly for the assay.

Inhibition of mEH with PTCO Ninety-four microliters of 50 mM Tris (pH 8.4), 2 μl of enzyme (20 μg/ml), and 2 μl of PTCO (0.2 mM) were incubated in small sample vials at 37 °C for 30 min. Next, 1 μl of the probe (10 μM) and 1 μl of sodium periodate (1 mM) were added to the incubation solution and incubated for another 3 h. The emission spectra were collected at an excitation wavelength of 540 nm in Spectrum M2 96-well black flat-bottom plates.

RESULTS AND DISCUSSION

Principle of the Screening Strategy Probe R is prepared *via* a two step sequence. First, resorufin sodium salt, 4-bromo-1-butene, and potassium carbonate are dissolved in anhydrous DMF. The mixture is stirred for 60 h at 80 °C to obtain a white solid, which is oxidized by hydrogen peroxide at 0 °C for 3 d to afford Probe R in moderate yield. Spectroscopic characterization of Probe R was carried out under assay conditions (Tris, 50 mM, pH 8.4). The main absorption peak was found at 479 nm (ε=18900 M⁻¹ cm⁻¹). Compared with resorufin (Φ=0.23) the probe is non-fluorescent (Φ<0.01).

The principle of the epoxide hydrolase assay using Probe R is illustrated in Fig. 1. With the treatment of EH, the epoxide group in Probe R is hydrolyzed to form a diol product, which is subsequently oxidized to form the aldehyde in the presence of sodium periodate. Spontaneous β-elimination releases resorufin, which exhibits a strong fluorescent emission. Thus, the EH activity could be measured in terms of the fluorescence increase.

Enzymatic Assay for EH and Microsomes The activities of commercial mEH in different pH-adjusted buffers (50 mM citrate, pH 6.0; 50 mM Tris, pH 8.4; or 50 mM

Fig. 1. Epoxide Hydrolase Assay with Probe R

Fig. 2. (a) Continuous Fluorescent Analysis of mEH Under Different Conditions

The probe was diluted into buffered solution to give a final concentration of $10\ \mu\text{M}$, and then mEH ($20\ \mu\text{g/ml}$) and sodium periodate ($1\ \text{mM}$) were added.

(b) Continuous Fluorescent Analysis of Microsomes from Beef Liver Under Different Condition.

Fluorescence excitation was provided at $540\ \text{nm}$ and emission intensity was collected at $585\ \text{nm}$ after $3\ \text{h}$. Each value corresponds to the mean of three experiments (standard errors were less than 6% of the mean in all cases).

glycine, pH 10.0) were examined. The enzyme was deactivated by heating at 100°C for $30\ \text{min}$ as a negative control. In a typical experiment, Probe R was diluted into buffer to give a final concentration of $10\ \mu\text{M}$, and then mEH ($20\ \mu\text{g/ml}$), BSA ($10\ \mu\text{g/ml}$), and sodium periodate ($1\ \text{mM}$) were added. Fluorescence excitation was provided at $540\ \text{nm}$ and emission intensity was collected at $585\ \text{nm}$ after $3\ \text{h}$. The results revealed that the fluorescence enhancement in different pH buffers was 56 (pH 6.0), 80 (pH 8.4), and 55 (pH 10.0) fold, respectively. The distinct “turn-on” response for mEH proves Probe R is an excellent reporter for mEH assay.

In previous studies,^{17,18)} BSA was found to be essential for ether bond cleavage, which was the crucial step in the assay. However, for Probe R, fluorescence could be generated without the aid of BSA after the initial hydrolysis. As shown in Fig. 2a, the fluorescence was unchanged regardless of the presence of BSA. The reason for this is unclear, but we hypothesize that the ether bond of the probe is less stable than that of the umbelliferone motif used in previous reports. Therefore, the following experiments were carried out in the absence of BSA.

Meanwhile, to approximate the *in vivo* environment, microsomes known to contain mEH were isolated from beef liver and the activity was determined. As shown in Fig. 2b, microsomes also gave strong fluorescence responses across a range of pH values.

The intrinsic stability of Probe R was also evaluated. The probe was incubated in Tris buffer (pH 8.4) in the absence of mEH for $3\ \text{h}$. The results show that Probe R is very stable under the assay conditions (Fig. 3). To further assess the selectivity of the fluorescence response, we incubated mEH from beef liver with $0.2\ \text{mM}$ PTCO, a known mEH inhibitor, for $1\ \text{h}$ prior to the assay.¹⁶⁾ Although incomplete inhibition was observed, relative fluorescent intensity was decreased nearly ten fold, from 0.98 to 0.11 (Fig. 3). This result suggests Probe R is not activated when mEH is inhibited, whereas efficient hydrolysis is observed with active mEH. Taken together, the results validate the activity-based design strategy of the probe.

Kinetic Assay of mEH In order to calculate the conversion for each reaction, a standard curve to correlate fluorescence intensity with the mEH concentration was examined

Fig. 3. Comparison of Fluorescence Intensity of Active and Deactivated mEH

mEH was incubated with PTCO, a known mEH inhibitor at $0.2\ \text{mM}$. After $1\ \text{h}$, Probe R was added and the fluorescent intensity was monitored at $585\ \text{nm}$.

Fig. 4. Fluorescence Emission Spectrum ($540\ \text{nm}$ Excitation) Obtained from mEH Treated with Probe R ($10\ \mu\text{M}$) measured at $1, 2, 3, 4,$ and $5\ \text{h}$

The insert in this figure shows the change in fluorescence intensity at $585\ \text{nm}$ as a function of time.

and good linearity was observed in the range from 1 — $100\ \mu\text{g/ml}$ (data not shown). Next, the time-dependent fluorescent intensity was examined. Probe R ($10\ \mu\text{M}$) was incubated with $20\ \mu\text{g/ml}$ mEH and sodium periodate ($1\ \text{mM}$) at 37°C in $50\ \text{mM}$ Tris buffer (pH 8.4). As shown in Fig. 4,

Fig. 5. Lineweaver-Burk Plots Generated from Continuous Kinetic Assay Method

The assay was performed by adding mEH to five concentrations in 50 mM Tris buffer, pH 8.4. Fluorescence was recorded ($\lambda_{\text{ex}} = 540$ nm, $\lambda_{\text{em}} = 585$ nm) at 1 h time intervals for 5 h. Data were converted to concentration of resorufin product produced using a standard curve created using known resorufin in Tris buffer (pH 8.4). Each point corresponds to the mean of three times (standard errors were less than 7% of the mean in all cases).

fluorescence intensity increased with time and the observed emission maximum was consistent with the emission spectrum (around 585 nm) of resorufin. The increase in fluorescence emission at a single wavelength, shown in the insert for 585 nm, was linear over the course of a 5 h experiment.

Since the fluorescence increase correlated linearly with time, the kinetic parameters could be directly derived from the fluorometric data. The initial rates were calculated according to previously published procedures.²⁰⁾ mEH kinetics plots are given in Fig. 5. These experiments afforded the Michaelis-Menten constant values $K_m = 3.31 \mu\text{M}$, and $V_{\text{max}} = 0.07 \mu\text{mol/mg/min}$, which shows Probe R has better affinity than some known substrates, such as styrene-7,8-oxide ($K_m = 0.98 \text{ mM}$)²¹⁾ and benzo(a)pyrene ($K_m = 10 \mu\text{M}$).²²⁾

CONCLUSION

We have successfully designed and synthesized an activity-based probe for microsomal epoxide hydrolase. The enzymatic conversion of Probe R to resorufin is further validated by the fluorescence reduction when mEH is incubated with inhibitor. It is also interesting to note that the assay works even in the absence of BSA, which was necessary in the previous approach. In addition, the long excitation and emission wavelengths of the probe reduce the impact of biosample photodamage and interfering autofluorescence. Furthermore, different from previously reported EH probes, Probe R exhibits a relatively constant fluorescent response

across a wide range of pH values from 6.0 to 10.0. Michaelis-Menten parameters were readily assessed and the results show Probe R is a good substrate for mEH.

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