Continuous Spectrophotometric Assays for Cytosolic Epoxide Hydrolase

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Two convenient and sensitive continuous spectrophotometric assays for cytosolic epoxide hydrolase are described. The assays are based on the differences in the ultraviolet spectra of the epoxide substrates and their diol products. The hydrolysis of 1,2-epoxy-1-(p-nitrophenyl)pentane (ENP) is accompanied by a decrease in absorbance at 302 nm, while the hydrolysis of 1,2-epoxy-1-(2-quinoxalinyl)pentane (EQUP) produces an increase in absorbance at 315.5 nm. The Km values for ENP and EQUP with purified mouse liver cytosolic epoxide hydrolase were 3.7 μM, 11,700 mmol/min/mg and 25 μM, 8300 mmol/min/mg, respectively. Both substrates are hydrolyzed significantly faster than trans-stilbene oxide, which is currently the most commonly used substrate for measuring cytosolic epoxide hydrolase activity. No spontaneous hydrolyses of the substrates is detectable under normal assay conditions. The assays are applicable to whole tissue homogenates as well as purified enzyme preparations. p-Nitrophenol oxide and p-quino-

xalinyl glyceryl ether were also examined and found to be very poor substrates for cytosolic epoxide hydrolase from mouse liver.

KEY WORDS: spectrophotometric assay, epoxide hydrolase.

Humans are exposed to a multitude of potentially mutagenic, carcinogenic, and cytotoxic epoxides present in natural products and industrial chemicals, as well as arising in vivo as biotransformation products and in the metabolism of olefinic and aromatic xenobiotics (1). Epoxide hydrolases (EC 3.3.2.5) catalyze the addition of water to epoxides to generate 1,2-diols (2,3). Several distinct forms of epoxide hydrolase have been identified in mammalian tissues, which differ in physical properties and substrate preferences. They include a "microsomal epoxide hydrolase" that hydrolyzes a wide range of epoxidized xenobiotics including arene oxides (2,4), a "cholesterol epoxide hydrolase" (also microsomai) that hydrolyzes cholesterol oxides (5,6), a "leukotriene A4 hydrolase" (7), and a "cyto-

somic epoxide hydrolase" that catalyzes the hydrolysis of a variety of epoxides not on cy-


1 This investigation was supported in part by National Institute of Environmental Health Sciences (NIHES) Grant ES00710-07 and by NIHES Training Grant 5-T32-ES07039 in Environmental Toxicology to R.N.W. B.D.H. is a Burroughs-Wellcome Toxicology Scholar.

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Fig. 1. Structures of spectrophotometric substrates for epoxide hydrolase.

MATERIALS AND METHODS

Synthesis of trans-1,2-epoxy-1-(2-quinolyl)pentane (EQUS) 6. The procedure of Still and Novack (12) for the direct epoxidation of carbonyl compounds was applied to the synthesis of 1,2-epoxy-1-(2-quinolyl)pentane. This method provides a trans stereoselective synthesis of epoxides via reaction of an stabilized arsonium ylide with an aldehyde. Quinoline 2-aldehyde (2-quinolinecarboxaldehyde) was available commercially (Chem Service, West Chester, PA) and the n-butyltriphenylarsionium fluoroaborate was prepared in three steps from n-butanol.

n-Butyl trflate was synthesized following the general procedure of Beard et al. (13) for preparation of alkyl triflates. A solution of 20.0 g (71 mmol) of trifluoroacetic anhydride (Aldrich Chemical Co., Milwaukee, WI) in 60 ml of methylene chloride at 0°C was added dropwise with stirring a solution of 4.60 g (63 mmol) of the ester and 4.90 g (0.062 mmol) of pyridine in 25 ml methylene chloride. After 20 min, the solution was washed with water, dried over sodium sulfate, and distilled under vacuum (77°C, ca. 80 mm Hg) to give 10.36 g (81% yield) of the product as a colorless oil.

For the preparation of n-butyltriphenylarsionium triflate (12), a solution of 10.3 g (50 mmol) n-butyl trflate and 18.4 g (60 mmol) triphepinearsinalon in 50 ml of methylene chloride was stirred at room temperature for 5 days. The solvent was removed by rotary evaporation to give a viscous oil which formed a white solid upon mashing with ether. The solid was filtered, washed with ether, and dried under vacuum, to give 23.6 g of the desired salt (92% yield).

Fluoroaborate exchange was accomplished by addition in a single portion of a solution of 10.2 g (20 mmol) n-butyltriphenylarsionium trflate in 110 ml methanol to a vigorously stirred solution of 220 g (2.0 mol) of sodium tetrafluoroaborate in 800 ml of water at room temperature (12). After stirring for 3 h, the reaction mixture was extracted four times with 400 ml of chloroform. The extracts were combined and dried over sodium sulfate, and the solvent was removed by rotary evaporation to give a light yellow oil, which produced 6.7 g (96% yield) of a white solid upon mashing with ether. This salt was used without further purification.

For the synthesis of trans-1,2-epoxy-1-(2-quinolyl)pentane, 8.57 ml (12 mmol) of potassium hexamethyldisilazide (1.4 M solution in THF, Alfa Products, Danvers, MA) was added dropwise to a solution of 6.30 g (14 mmol) n-butyltriphenylarsionium fluoroaborate and 2 ml dry dimethylsulfoxidemide in 10 ml dry THF under nitrogen at −40°C. After it was stirred at −40°C for 15 min, the solution was cooled to −78°C, and 10 ml (10 mmol) of 2-quinolinecarboxaldehyde.

6 Abbreviations used: EQUS, trans-1,2-epoxy-1-(2-quinolyl)pentane; THF, tetrahydrofuran; ENPS, trans-1,2-epoxy-1-(p-nitrophenyl)pentane; PNSO, p-nitrophenylglycidyl ether; TSO, trans-stilbene oxide; ENPSO, 1,2-epoxy-3-(p-nitrophenyl)propene.
Cytosolic Epoxide Hydrolase Spectrophotometric Assays

The reaction mixture was stirred at 57°C for 5 min or until the aldehyde had disappeared, and the mixture was then evaporated to dryness. The residue was dissolved in ethanol and the optical density was measured at 540 nm. The NADPH NADP ratio was calculated from the optical density at 540 nm. The NADPH NADP ratio was then plotted against the concentration of NADPH. The resulting curve was used to determine the concentration of NADPH. The concentration of NADPH was then calculated from the equation:

\[
\text{NADPH (mM)} = \frac{\text{OD}_{540} - \text{OD}_{650}}{\text{NADPH standard}}
\]

The concentration of NADPH was then used to determine the activity of the enzyme. The activity of the enzyme was calculated from the equation:

\[
\text{Activity (U/mL)} = \frac{\text{OD}_{540}}{\text{time (min)}}
\]

The results were then plotted against the concentration of NADPH to determine the Michaelis-Menten constant (Km) and the maximum velocity (Vmax) of the enzyme. The Michaelis-Menten constant (Km) was calculated from the equation:

\[
\text{Km} = \frac{\text{Km} \cdot \text{Vmax}}{\text{Vmax} - \text{OD}_{540}}
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\]
<table>
<thead>
<tr>
<th>Substance/Product</th>
<th>m.p.</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>CO_2H</th>
<th>COO_2H</th>
<th>CH_3</th>
<th>CH_2</th>
<th>CH</th>
<th>CH_3</th>
<th>H_2N-CH-</th>
<th>H-NMR *</th>
<th>IR cm⁻¹</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESII (C_13H_20O_6)</td>
<td>108</td>
<td>0.95</td>
<td>6.74</td>
<td>6.49</td>
<td>6.91</td>
<td>7.50</td>
<td>7.80</td>
<td>6.50</td>
<td>9.50</td>
<td>9.20</td>
<td>9.70</td>
<td>9.00</td>
<td>9.20</td>
<td>9.40</td>
</tr>
<tr>
<td>DQUA (C_20H_24O_6)</td>
<td>106.1</td>
<td>0.25</td>
<td>7.15</td>
<td>7.35</td>
<td>7.15</td>
<td>7.35</td>
<td>7.50</td>
<td>6.50</td>
<td>9.50</td>
<td>9.20</td>
<td>9.70</td>
<td>9.00</td>
<td>9.20</td>
<td>9.40</td>
</tr>
<tr>
<td>TDO (C_4H_6O)</td>
<td>65-67</td>
<td>0.71</td>
<td>6.50</td>
<td>6.30</td>
<td>6.50</td>
<td>6.30</td>
<td>6.50</td>
<td>6.50</td>
<td>9.50</td>
<td>9.20</td>
<td>9.70</td>
<td>9.00</td>
<td>9.20</td>
<td>9.40</td>
</tr>
<tr>
<td>TPEI (C_22H_26O_6)</td>
<td>118-121</td>
<td>0.64</td>
<td>6.50</td>
<td>6.30</td>
<td>6.50</td>
<td>6.30</td>
<td>6.50</td>
<td>6.50</td>
<td>9.50</td>
<td>9.20</td>
<td>9.70</td>
<td>9.00</td>
<td>9.20</td>
<td>9.40</td>
</tr>
</tbody>
</table>

*H-NMR data not provided in the table.
ence of epoxide hydrolases. Styrene oxide is a good substrate for the microsomal epoxide hydrolase and a poor to moderate good substrate for the cytosolic enzyme (2,4,19,20). trans-β-Alklystyrone oxides, in contrast, such as trans-β-ethylstyrone oxide and trans-β-propylstyrone oxide, are very good substrates hydrated almost exclusively by the cytosolic enzyme (19-21). A very useful spectrophotometric assay for the microsomal epoxide hydrolase utilizing p-nitrosoxy oxide was developed by Westwayer and Hanslik (10). The above results suggested that the addition of a trans-δ-alyl group to p-nitrosoxy oxide might produce a useful spectrophotometric substrate for the cytosolic enzyme. The presence of the nitro group in the substrate was also expected to increase its solubility in water and decrease the volatility problems encountered with the trans-β-alklystyrone oxides. This was the basis for the synthesis of ENP5 which has proved to be an excellent substrate for the cytosolic enzyme whose hydrolase may be monitored by a decrease in absorbance at 302 nm. Another factor considered in the design of the new substrates was the desirability of a product appearance assay in contrast to the currently available substrate disappearance assays. A necessary requirement for such a substrate would be a significant uv spectral shift on the hydrolysis of the epoxide diol. It was thought that hydrolyzing the possibility of intramolecular hydrogen bonding in the product diol such a spectral shift might be obtained. This was the basis for the synthesis of EQU5 in which the ring nitrogen and δ-hydroxyl group of the corresponding diol are in a favorable position for hydrogen bonding. The quinoline moiety was also expected to provide a good uv chromophore and impart desirable water solubility characteristics, while the trans-δ-alyl group would accommodate the enzyme's substrate preferences. A significant spectral shift was observed (Fig. 2) and served as the basis for a convenient product appearance assay at 315 nm. Alternatively, the disappearance of EQU5 may be monitored at 320

<table>
<thead>
<tr>
<th>Compounds</th>
<th>λ (nm)</th>
<th>ɛ L (M⁻¹ cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Styrene oxide</td>
<td>280</td>
<td>44,100</td>
</tr>
<tr>
<td>trans-β-ethylstyrone oxide</td>
<td>290</td>
<td>44,100</td>
</tr>
<tr>
<td>trans-β-propylstyrone oxide</td>
<td>290</td>
<td>44,100</td>
</tr>
<tr>
<td>p-nitrosoxy oxide</td>
<td>280</td>
<td>44,100</td>
</tr>
</tbody>
</table>

* All data were determined by ultraviolet absorption in water at pH 7.4, and were run in triplicate.

** Spectra were recorded in 95% ethanol at pH 7.4 using a Varian Cary 30 spectrophotometer.
nm. Two other logical candidates for spectrophotometric substrates were synthesized \([\textit{trans-p}-\textit{nitrilotriethane oxide and trans-1,2-epoxy-1-(2-pyridyl)pentane}\] but are not included in this study owing to undesirable UV spectral characteristics \((\lambda_{\text{max}} \approx 271 \text{ nm})\) in the first case and a propensity to form micelles at concentrations below 0.05 mM with the latter.

\textbf{Assay validity, linearity, and kinetics.} The diol was shown by several methods to be the only metabolite produced from EQU5 and ENPS5 by either crude cytosol or the homogenate enzyme. The UV spectrum of the assay solution following incubation of the epoxide with enzyme matched exactly that of the authentic diol. Extraction of the assay solution with ethyl ether followed by TLC analysis resulted in a single spot on TLC of higher \(R_f\). The EQU5 and ENPS5 spectrophotometric assays were demonstrated to be linear with time and protein concentration \((\text{EQU5: 0-12 min, 0-1 \mu g purified enzyme; ENPS5: 0-5 min, 0-0.5 \mu g purified enzyme})\) (25). For EQU5 an apparent \(K_m\) of 25 \(\mu M\) and a \(V_{\text{max}}\) of 8300 nmol/min/mg were determined at 25°C using purified cytosolic epoxide hydrolase from livers of clofibrate-treated mice (Fig. 3A). An apparent \(K_m\) of 1.7

\[\text{pM and a } V_{\text{max}} \text{ of 11,700 nmol/min/mg were determined for ENPS5 (Fig. 3B). These results compare with values of 5 \mu M and 3300 nmol/min/mg measured at 37°C for TSO with the purified mouse liver enzyme (20). Effect of pH on EQU5 hydration. The pH optimum for EQU5 hydrolase by purified cytosolic epoxide hydrolase from mouse liver is shown in Fig. 4A. Maximal activity was observed near pH 7, which coincides with the pH optimum determined for TSO (20,22). Molar extinction coefficients for the difference spectrum of EQU5 and its diol were determined at each pH value (Fig. 4B) and were found to increase with decreasing pH, which is attributable to the strong hyperchromic effect that results from protonation of a quinolone nitrogen (23).

\textbf{Comparison with other epoxide hydrolase spectrophotometric substrates.} The rates of enzymatic hydrolase for the five spectrophotometric substrates examined in this study are listed in Table 3. ENPS5 and EQU5 are hydrolized by the cytosolic epoxide hydrolase at rates approximately 15-fold and 5-fold higher.

\[\text{FIG. 2.} \text{ Ultraviolet spectra of EQU5, its corresponding diol, and their difference spectrum. All spectra were measured at a concentration of 0.005 mM in pH 7.4 sodium/potassium phosphate buffer (7 = 0.2). The presence of bovine serum albumin at 100 \mu g/ml had no effect on the absorbance spectra.}\]
than that of TSO. No detectable hydrolysis of ENPS or EQU5 was observed in crude microsomal preparations from mouse liver at protein concentrations up to 800 μg/mL, suggesting that the new substrates exhibit the same selectivity for the cysteolic enzyme as observed for the trans-β-alkylstyrrene oxides. PNSO and ENPO3 have been suggested previously as possible spectrophotometric substrates for the cysteolic epoxide hydrolase (3,9). PNSO is currently used to monitor purified microsomal epoxide hydrolase activity spectrophotometrically at 310 nm (10), while ENPO3 is a commercially available substrate for glutathione S-transferase whose conjugate can be monitored by an increase in absorbance at 360 nm (11). Both PNSO and ENPO3, however, appear to be poor substrates for the cysteolic enzyme from mouse liver (Table 3). This indicates that PNSO might be very useful in conjunction with EQU5 or ENPS for monitoring both epoxide hydrolases.

### Table 3: Hydrolysis of Spectrophotometric Substrates by Epoxide Hydrolase from Mouse Liver

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cytosol</th>
<th>Purified cysteolic epoxide hydrolase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENPS</td>
<td>129 ± 1</td>
<td>10,900 ± 50</td>
</tr>
<tr>
<td>EQU5</td>
<td>45.5 ± 0.6</td>
<td>3,240 ± 10</td>
</tr>
<tr>
<td>TSO</td>
<td>8.80 ± 0.05</td>
<td>677 ± 13</td>
</tr>
<tr>
<td>PNSO</td>
<td>1.45 ± 0.10</td>
<td>16.3 ± 0.1</td>
</tr>
<tr>
<td>ENPO3</td>
<td>0.13 ± 0.01</td>
<td>6.34 ± 0.02</td>
</tr>
</tbody>
</table>

*Initial rates of hydrolysis (mmol/min/mg ± SEM) at 25°C and 0.050 mM substrate concentration in buffer A.*
hydrolyses important in the hydration of xenobiotics. Similarly, ENP3 could prove useful for monitoring some of the glutathione S-transferase isozymes even in the presence of the cysteic epoxide hydrolase.

Advantages and limitations of the spectrophotometric assays for cysteic epoxide hydrolase. The TSO, ENP5, and EQU5 assays all offer the advantages of sensitivity and stability, with ENP5 being the most sensitive. TSO is commercially available, while ENP5 and EQU5 require a four-step synthesis for their preparation. The low aqueous solubility (0.1 mM) of TSO is a limitation that is less of a problem with the more water-soluble ENP5 and EQU5 substrates. TSO hydration is monitored at a wavelength of 229 nm, where most aromatic compounds absorb strongly, whereas the hydration of ENP5 and EQU5 are monitored above 300 nm, which allows convenient evaluation of most aromatic inhibitors and activators. The utility of EQU5 in examining the inhibition of cysteic epoxide hydrolase by a chalcone oxide is demonstrated in Fig. 5. Such an experiment would prove very difficult with TSO due to the high extinction coefficient of the inhibitor. EQU5 was also used to examine the effect of T-2 toxin, a 12,13-epoxytrichothecone on purified cysteic epoxide hydrolase activity. At concentrations up to 0.5 mM, no inhibition or activation of enzyme activity was observed, as expected from the lack of hydration of radioactive T-2 toxin by the enzyme. The EQU5 spectrophotometric substrate has the additional advantage of providing a product appearance assay.

Further applications. The EQU5 and ENP5 spectrophotometric assays could offer dramatic time savings if adapted for use in kinetic microtitre plate readers likely to be available in the near future that are modified to operate at wavelengths down to 300 nm (24). These substrates should also be suitable for the development of assays that simultaneously monitor cysteic epoxide hydrolase and glutathione S-transferase activities following separation of the products by partition-, TLC-, or HPLC-based methods.

REFERENCES


FIG. 5. Inhibition of EQU5 hydration by a chalcone oxide. The 4-fluorochrome oxide (FCHOs) inhibitor was added in 10 μl of ethanol to 980 μl of purified mouse liver cysteic epoxide hydrolase (0.093 mg/ml) and pre-incubated with the enzyme for 10 min at 25°C prior to the addition of EQU5. Less than 5% variation in rates was observed on repetition of the experiment.