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Direct detection of stereospecific soman hydrolysis by wild-type human serum paraoxonase

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Human serum paraoxonase 1 (HuPON1; EC 3.1.8.1) is a calcium-dependent six-fold β-propeller enzyme that has been shown to hydrolyze an array of substrates, including organophosphorus (OP) chemical warfare nerve agents. Although recent efforts utilizing site-directed mutagenesis have demonstrated specific residues (such as Phe222 and His115) to be important in determining the specificity of OP substrate binding and hydrolysis, little effort has focused on the substrate stereoselectivity of the enzyme; different stereoisomers of OPs can differ in their toxicity by several orders of magnitude. For example, the C±P- isomers of the chemical warfare agent soman (GD) are known to be more toxic by three orders of magnitude. In this study, the catalytic activity of HuPON1 towards each of the four chiral isomers of GD was measured simultaneously via chiral GC/MS. The catalytic efficiency ($k_{cat}/K_m$) of the wild-type enzyme for the various stereoisomers was determined by a simultaneous solution of hydrolysis kinetics for each isomer. Derived $k_{cat}/K_m$ values ranged from 625 to 4130 mM$^{-1}$min$^{-1}$, with isomers being hydrolyzed in the order of preference C+P+ > C+P- > C-P+ > C-P-. The results indicate that HuPON1 hydrolysis of GD is stereoselective; substrate stereosepecificity should be considered in future efforts to enhance the OPase activity of this and other candidate bioscavenger enzymes.

Human serum paraoxonase 1 (HuPON1; EC 3.1.8.1) is a human plasma enzyme previously shown to hydrolyze insecticides and the highly toxic organophosphorus (OP) nerve agents sarin (GB), O-ethyl S-(2-diisopropylaminoethyl) methylphosphonothioate (VX), and soman (GD; pinacolyl methylphosphonofluoridate) in vitro and in vivo [1–3]. Although its catalytic efficacy against GB, VX, and GD is low, it is the capacity to hydrolyze these toxic nerve agents in vivo that makes HuPON1 attractive as a candidate bioscavenger of OP compounds. It has been theorized that a genetically engineered variant of HuPON1 with at least a 10-fold increase in activity would be highly protective in vivo against intoxication by OP compounds [4–7].

GD is a member of a class of highly toxic acetylcholinesterase inhibitors, all of which have their leaving groups attached to a chiral phosphorus atom [8–11]. GD contains a second chiral center at one of the alkyl side chain carbon atoms. Therefore, it exists as four stereoisomers C+P+, C+P-, C-P+, and C-P- (Fig. 1) [12–17]. Both of the P- isomers (C±P-) are much more toxic in vivo and more readily inhibit

Abbreviations
DFP, diisopropylfluorophosphate; GB, sarin; GD, soman; HuPON1, human serum paraoxonase 1; OP, organophosphorus; PON1, paraoxonase 1; VX, O-ethyl S-(2-diisopropylaminoethyl) methylphosphonothioate.
Results

Analysis of GD stereoisomer hydrolysis using GC/MS

The decrease in the concentration of each of the GD isomers in the presence of HuPON1 over time was followed using GC/MS analysis. All four stereoisomers and the internal standard disopropylfluorophosphate (DFP) were quantitatively separated (Fig. 2) using a Chiraldex γ-cyclodextrin trifluoroacetyl column [24]. The elution order of individual GD stereoisomers from a racemic sample was determined by examining the retention times of individual purified stereoisomers alone (data not shown).

The elution order detected was C−P−, C−P+, C+P−, and then C+P+ at approximately 12.0, 12.8, 13.2, and 13.6 min after injection, respectively (Fig. 2). Our elution order differs from those previously reported using different GC columns [9,16]. The DFP standard eluted after all four GD stereoisomers, at ≈ 17.3 min post injection. The clear separation of peaks in the elution profile allowed for the simultaneous determination of the fate of all four GD stereoisomers (Fig. 3) [9,12,25].

Spontaneous hydrolysis of GD stereoisomers

Hydrolytic assays were carried out in the absence of HuPON1 enzyme to define any effects of spontaneous hydrolysis at pH 7.4 at room temperature. The ratios of the areas under the curve for each stereoisomer were determined at 0.5, 1.0, 3.0, 5.0, 15.0, and 240 min following incubation of 2.0 mM racemic GD in supernatant from cells transfected with empty plasmid vector. The ratios of C−P−/C−P+ and C−P−/C+P− were identified relative to the DFP internal standard and were 23.4/26.7/26.6/23.2%, respectively, in good agreement with previous reports [26,27]. The absolute amount of GD and the relative percentages of each stereoisomer were consistent across all sampling times, differing by no more than 0.2% (data not shown), indicating negligible spontaneous hydrolysis.

Effects of GD stereoisomer racemization

Spontaneous racemization of GD stereoisomers is known to occur at the phosphorus atom in the presence of excess fluoride ion [26,27]. To determine if such racemization was occurring in our experimental system, studies were performed at room temperature in 50 mM glycine buffer (pH 7.4) with supernatant from cells transfected with empty plasmid vector. The extent
Fig. 2. Gas chromatographic separation of GD stereoisomers. Shown is a reconstructed ion chromatogram (m/z 126) of a 2.0 mM racemic sample of GD (no enzyme) analyzed by GC/MS after separation using a Chiralpak γ-cyclodextrin trifluoroacetyl column at 80 °C isothermal, with labels identifying peaks corresponding to the individual stereoisomers. The internal standard DFP eluted at ≈17.3 min (not shown).

Fig. 3. Overlay of reconstructed ion chromatograms (m/z 126) of GD hydrolysis by HuPON1. Typical ion chromatograms indicating the relative abundance of the four GD stereoisomers (0.75 mM racemic GD) after different incubation periods (i.e. 0, 5, 15, and 120 min, as indicated) with wild-type HuPON1 enzyme. The various GD stereoisomers were eluted in the same order as shown in Fig. 2.

of racemization was studied in reactions containing semipurified 0.30 mM C-P-/C-P+ or C+P-/C+P+ mixtures of GD isomers in the presence of excess fluoride ions (which varied from 0 to 2.0 mM NaF). In addition, we incubated 1.0 mM racemic GD with 2.0 mM NaF under the same experimental conditions to determine the extent of racemization under those conditions. The results obtained from both sets of experiments indicated that under the conditions used, the presence of excess fluoride ions caused no appreciable racemization of either the C±P− or the C±P+ isomers. Furthermore, we did not observe any alteration in the GC/MS isomer elution profile after incubating 1.0 mM racemic GD with excess (2.0 mM NaF) fluoride ions.

Characterization of wild-type HuPON1 activity

Initial rates of enzymatic hydrolysis of the individual GD stereoisomers were estimated by plotting GD concentration (for the individual stereoisomers) as a function of time (Fig. 4). The concentration of each
specific stereoisomer was derived from a previously determined GD standard curve and the area under the curve for each stereoisomer was then normalized against the DFP internal standard. The kinetic parameter $K_m$ of HuPON1 for each of the four stereoisomers of GD was determined from the derived kinetic model (Fig. 5, Table 1) as detailed in the Experimental procedures, and ranged from 0.27 to 0.91 mM in the following order: C-P- > C+P- > C-P+ > C+P+. The $k_{cat}$ values for the hydrolysis of each stereoisomer were also determined from the derived model (Table 1); the values range from 501 to 1030 min$^{-1}$, where C+P+ > C-P+ > C+P- > C-P−. The bimolecular rate constants derived from the model ranged from 4130 to 625 mM$^{-1}$min$^{-1}$ for C+P+ > C-P+ > C+P- > C-P−, respectively. The average $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values for all four GD stereoisomers in aggregate are 0.62 mM, 669 min$^{-1}$, and 1739 mM$^{-1}$min$^{-1}$, which is in reasonable agreement with previously reported values obtained using a racemic mixture of GD and plasma derived HuPON1 in a different assay of enzymatic activity [1]. Finally, the kinetics of HuPON1-mediated GD hydrolysis (2 mM) determined in the presence of added NaF (1 mM) were indistinguishable from those measured in the absence of NaF; these results indicate that under the experimental conditions used, liberated fluoride ions do not enhance racemization of GD or influence the stereospecificity of HuPON1-mediated GD hydrolysis.

Discussion

It has recently been reported that a gene-shuffled, bacterially expressed variant of PON1 exhibits in vitro stereospecificity for the less toxic isomers of both GD and cyclosarin [22]. In that study, enzymatic hydrolysis was determined by simultaneously measuring the amount of OP and the inhibitory capacity of the same OP after incubation with the hybrid PON1 enzyme for different time intervals [22]. Although that approach suggested preferential degradation of the less toxic isomers, the results could not distinguish between the C+ and C− isomers. Attempts to obtain $K_m$ and $k_{cat}$ values for the degradation of specific stereoisomers using this approach were unsuccessful [22].

In this study, we have demonstrated that recombinant wild-type HuPON1 exhibits modest, but distinct, stereoselectivity in its catalytic hydrolysis of the four GD stereoisomers. Whereas the C+P+ isomer was preferentially hydrolyzed by HuPON1 (Figs 3, 4; Table 1), the $k_{cat}$ value for each of the C±P− isomers was similar to that for C-P+ and was only half that for the C+P+ isomer. Kinetic constants were determined directly for each stereoisomer after measuring the individual stereoisomer concentrations as a function of time. A critical assumption in the analytical model we developed to determine the kinetic constants of each stereoisomer is that each isomer behaves as an independent but competitive substrate in the reaction (see Supplementary material for a more detailed description of the model used).

Although our chromatographic technique obtained distinct baseline peak separation among the four GD stereoisomers (Fig. 2), it must be appreciated that the liberation of fluoride ions during hydrolysis has the potential to racemize the phosphorus chiral center of the unhydrolyzed GD in solution. Under conditions of excess fluoride ions, neither the enantiomeric nor racemic GD mixtures displayed observable differences in peak magnitude or elution order for the individual stereoisomers. Furthermore, the presence of added fluoride ions had no detectable effect on the stereoselectivity of HuPON1-mediated hydrolysis of GD, suggesting that fluoride-induced racemization at the phosphorus atom of GD does not contribute to the decrease in concentration of any particular stereoisomer. Rather, the results support the premise that each stereoisomer is behaving as an independent substrate competing for the same active site, as stipulated by our analytical model (Fig. 5). In addition, because HuPON1 was not purified in our experimental approach, the possibility also existed that other enzymes in the
Fig. 5. Reaction schematic of the racemic GD/HuPON1 system. A–D, various GD stereoisomers; E, PON1 enzyme; E<sub>A</sub>–E<sub>D</sub>, PON1–GD stereoisomer complexes; P–S, hydrolyzed products; k9, association/dissociation constants.

Table 1. Kinetic parameters for the enzymatic hydrolysis of the various GD stereoisomers by recombinant wild-type HuPON1. HuPON1 catalyzed GD hydrolysis was assayed in the presence of at least 1.0 mM CaCl<sub>2</sub> as described in Experimental procedures. Kinetic results presented for each isomer were determined from at least eight independent kinetic experiments (n = 8).

<table>
<thead>
<tr>
<th>GD isomer</th>
<th>k&lt;sub&gt;m&lt;/sub&gt; (mM)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt; (min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;/k&lt;sub&gt;m&lt;/sub&gt; (mM&lt;sup&gt;-1&lt;/sup&gt;min&lt;sup&gt;-1&lt;/sup&gt;)</th>
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<tbody>
<tr>
<td>C–P&lt;sup&gt;-&lt;/sup&gt;</td>
<td>0.91 ± 0.34</td>
<td>501 ± 45</td>
<td>625 ± 241</td>
</tr>
<tr>
<td>C–P&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.58 ± 0.23</td>
<td>593 ± 54</td>
<td>1180 ± 469</td>
</tr>
<tr>
<td>C&lt;sup&gt;+&lt;/sup&gt;P&lt;sup&gt;-&lt;/sup&gt;</td>
<td>0.71 ± 0.49</td>
<td>553 ± 163</td>
<td>1040 ± 465</td>
</tr>
<tr>
<td>C&lt;sup&gt;+&lt;/sup&gt;P&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.27 ± 0.08</td>
<td>1030 ± 94</td>
<td>4130 ± 1090</td>
</tr>
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supernatant might be partially responsible for the observed hydrolysis of GD. However, supernatant collected from cells transfected with empty vector plasmids showed negligible GD hydrolysis, thus demonstrating that the observed hydrolysis of GD was mediated by only the HuPON1 enzyme.

The stereospecificity of several different enzymes for OP acetylcholinesterase inhibitors such as GD has been studied for several decades. To date, the enzymes examined have almost universally exhibited considerable stereospecific preference for the less toxic isomers of GD, including the recent results of Amitai et al. with a recombinant gene-shuffled version of PON1 [22,28]. Initial studies by Benschop et al. [12,25] showed that acetylcholinesterase was selectively inhibited by the C ± P<sup>-</sup> GD stereoisomers by three orders of magnitude more rapidly than by the C ± P<sup>+</sup> isomers. Likewise, a bacterial phosphotriesterase [29] was found to hydrolyze the P<sup>+</sup> GD analog diastereomers 1000-fold faster than the more toxic P<sup>-</sup> isomers. Benschop et al. [25] and De Jong et al. [9] reported that for plasma and liver homogenates from guinea pigs, mice and marmosets, binding and/or hydrolysis of the C ± P<sup>+</sup> stereoisomers was preferred. The only previous report of a lack of stereospecificity in the enzyme-catalyzed hydrolysis of GD was a study by Little et al. [18] who reported that an enzyme with a molecular mass of 40 kDa, isolated as a single peak by HPLC from a rat liver homogenate, hydrolyzed all four GD stereoisomers at identical rates. The fact that PON1 is a liver-expressed serum enzyme with a molecular mass of 42 kDa and only modest stereoselectivity for GD suggests that PON1 may have been responsible for the majority of the enzymatic activity in that study. In this study, the detection of stereoselectivity against GD by HuPON1 may be the result of different sources of the enzyme (recombinant human versus rat plasma-derived) and/or improved instrumental resolution.

Akin to many OP hydrolases, HuPON1 has broad substrate specificity [3,7,19,20,22,30–34]. The recent publication of the crystal structure of a gene-shuffled, primarily rabbit PON1 variant [20] (the enzyme used in the report of Amitai et al. [22]) and of a DFPase-based HuPON1 homology model [5,7] have provided a framework to support the efforts currently underway to enhance PON1's enzymatic activity against OP substrates using rational design. This study demonstrates that the catalytic efficiency (k<sub>cat</sub>/k<sub>m</sub>) for hydrolysis of each of the GD stereoisomers by wild-type HuPON1 differs by less than one order of magnitude (Table 1). The k<sub>cat</sub> values of the individual isomers are quite similar, with the turnover of the C ± P<sup>+</sup> isomer being...
only twice that for the other three stereoisomers. The $K_m$ values for the individual stereoisomers with wild-type HuPONI show a wider (almost fourfold) variation, with the $P-$ isomers exhibiting the highest values. This suggests that either the $P-$ isomers of GD have a lower affinity for HuPONI than the $P+$ isomers, or that the $P-$ isomers form more stable enzyme-substrate complexes. Given the lack of information about the rate of enzyme/substrate to enzyme/product transitions in this system, it is not currently possible to distinguish between these nonmutually exclusive possibilities [35].

Data from HuPONI presented in Table 1 suggest that the observed variations in catalytic efficiency for GD can be attributed largely to differences in the $K_m$ values of the enzyme for the various stereoisomers. Although the stereochemistry of the substrates may be important for binding, the results suggest that once bound, the catalytic machinery is not overly sensitive to the chirality of the groups around the phosphorus atom. Therefore, small changes (via site-directed mutagenesis) that reduce the $K_m$ for the more toxic isomers might be singularly sufficient to make the enzyme a viable bioscavenger for detoxification of OP anticholinesterase poisons in vivo. For example, a reduction in $K_m$ by 10-fold with no change in the $V_{max}$ value, would enhance catalytic turnover of the more toxic stereoisomers of GD such that they would be preferentially hydrolyzed by several fold [4,5,7]. Such a mutant would have considerable potential as a bioscavenger capable of providing protection against nerve agent poisoning.

**Experimental procedures**

**Production of HuPONI**

Wild-type recombinant HuPONI enzymes were produced as described previously [7]. Briefly, a pcDNA3 plasmid (Invitrogen, Carlsbad, CA) encoding recombinant wild-type HuPONI was transiently transfected into human 293T embryonic kidney cells, grown in DMEM (Cambrex Bioscience, Walkersville, MD) supplemented with 5% fetal bovine serum and 2% L-glutamine) at 70-90% confluency. Secreted HuPONI protein in cultured supernatant was harvested seven days after transfection. HuPONI expression was detected by immunoblotting with mouse anti-HuPONI mAb (kindly provided by R. James, University Hospital of Geneva, Switzerland), probed with an alkaline-phosphatase conjugated rabbit anti-mouse serum, and quantitated by densitometry analysis (Un-Scan-It version 5.1, Silk Scientific Corp., Orem, UT) with a PONI standard of known concentration (Randox Laboratories Ltd, Antrim, UK), and verified by enzymatic assays for phenyl acetate and paraoxon hydrolysis [36-38].

**Determination of GD hydrolysis**

Racemic GD (2.0 mg·mL$^{-1}$ in saline), containing 2.5% diisopropyl carbodiimide added as a stabilizer, was obtained from the Research Development and Engineering Command (Aberdeen Proving Ground, MD). Analysis using nuclear magnetic resonance spectroscopy showed it to be 96.7% pure. The pure individual GD stereoisomers were previously prepared in ethyl acetate by the TNO Prins Maurits Laboratory (Rijswijk, the Netherlands) [12].

Somacnic activity was determined at room temperature as detailed in Broomfield et al. [8] with minor variations. Specifically, GD hydrolysis experiments were carried out using 1.50 mL of supernatant from cells transfected with either the wild-type HuPONI gene or empty vector. Supernatants were incubated with the indicated concentrations of GD in 50 mM glycine–NaOH buffer, pH 7.4 with 10 mM CaCl$_2$. Total reaction volume was 3.0 mL. At selected time intervals, 400 μL aliquots were removed and inactivated through extraction with an equal volume of GC-grade ethyl acetate (EM Science, Cherry Hill, NJ) previously dried over a type 4A/grade 514 molecular sieve (Fisher Scientific, Fairlawn, NJ). The organic layer (containing unhydrolyzed GD) was then removed and dried over molecular sieve again. A 50-μL sample of this dried sample was collected and spiked with DFP (Sigma-Aldrich, St Louis, MO) to a final concentration of 50 μM as the internal standard before injection into the gas chromatograph [12]. The quantity of GD in each sample was determined by comparison with both the DFP internal standard present in each sample and a standard GD calibration curve. Calibration curves were obtained by using GD at five different concentrations also spiked with a final concentration of 50 μM DFP in ethyl acetate as the internal standard. Kinetic parameters of GD hydrolysis were determined using at least eight different initial substrate concentrations that ranged from 0.2 to 3.0 mM.

To determine the elution/retention time profile of the four GD stereoisomers, samples of individual stereoisomers were run under the same conditions as those used to determine the calibration curve.

**Excess fluoride/racemization control experiments**

To determine whether racemization occurs in our experimental system, three independent control experiments were performed under the same conditions as those used to determine the calibration curve. First, 1.0 mM of racemic GD was incubated with culture medium from cells transfected with empty plasmid vector control in the presence of excess fluoride ions (2.0 mM NaF). Second, semipurified individual stereoisomers were also incubated with excessive...
fluoride ions. Finally, wild-type HuPON1 was reacted with 2 mM GD as described above, but in the presence of 1 mM NaF.

**GC/MS analysis**

GC separation of the GD stereoisomers was performed using a modification of a previously developed method [24]. An Agilent 6890 gas chromatograph (Palo Alto, CA) was fitted with a 20 m x 0.25 mm inside diameter Chiraldex γ-cyclodextrin trifluoroacetyl column, 0.125 μm film thickness (Advanced Separation Technologies, Inc., Whippany, NJ). A 2.5 m x 0.25 mm inside diameter cyano/phenyl/methyl deactivated fused silica retention gap (Chrompack, Inc., Raritan, NJ) was installed at the injection end of the GC and connected to the analytical column using a Chrompack deactivated Quick-Seal glass connector. Helium was used as carrier gas at a linear velocity of 45 cm·s⁻¹. The oven temperature was held initially at 80 °C for 14 min, programmed from 80 to 90 °C at 5 °C·min⁻¹, and held at 90 °C for 3 min. Split injections of 1 μL volume were made using an Agilent 7683 autosampler. The injection port temperature was 210 °C and the split ratio was 1 : 100. The GC was interfaced to an Agilent 5973 mass spectrometer (MS) with an electron impact ion source. The MS operating conditions were as follows: ion source pressure ≈ 1.0 x 10⁻⁶ torr; source temperature, 230 °C; electron energy, 70 eV; electron multiplier voltage + 200 V relative to the autotune setting; and transfer line temperature, 230 °C. The MS was operated using selected ion monitoring (SIM). Four ions (m/z 69, 82, 99 and 126) were monitored for GD and DFP, respectively. A dwell time of 50 m's⁻¹ for each ion resulted in a scan rate of 3.77 cycles's⁻¹ [39]. Three ions (m/z 69, 101 and 127) were monitored for DFP [40]. A dwell time of 50 m's⁻¹ for each ion resulted in a scan rate of 5 cycles's⁻¹. The m/z 126 and 127 ions were used for quantitation of GD and DFP, respectively.

**Calculation of kinetic constants**

In the presence of a racemic mixture of GD, the catalyzed reaction is analogous to simultaneously deriving the kinetic constants for the hydrolysis of four competitive substrates. To do this, we used the model of GD–HuPON1 interaction shown in Fig. 5 and described in detail in the supplementary material. The first-order rate equations of the enzyme–substrate intermediates were set equal to zero (the enzyme ‘steady-state’ assumption). The resulting set of equations was solved to express the steady state enzyme–substrate intermediate levels as functions of the substrate concentrations and the kinetic parameters. A conservation of enzyme assumption was employed to obtain the free enzyme level in terms of the four enzyme–substrate intermediates. These relationships, each substrate rate equation was cast in terms of a single substrate and integrated with respect to time to arrive at the solutions. The derived solution for all four of the substrates is shown below:

$$T_A = (A_0/V_{maxA})(1 - (A/A_0)(K_{mA}/K_{mA})(V_{maxA}/V_{maxA})) + (B_0/V_{maxB})(1 - (B/A_0)(K_{mB}/K_{mB})(V_{maxB}/V_{maxB})) + (C_0/V_{maxC})(1 - (C/A_0)(K_{mC}/K_{mC})(V_{maxC}/V_{maxC})) + (D_0/V_{maxD})(1 - (D/A_0)(K_{mD}/K_{mD})(V_{maxD}/V_{maxD})) + (K_{mA}/V_{maxA}) \log (A/A_0)$$

$$T_B = (A_0/V_{maxA})(1 - (B/B_0)(K_{mB}/K_{mB})(V_{maxA}/V_{maxB})) + (B_0/V_{maxB})(1 - (B/B_0)(K_{mB}/K_{mB})(V_{maxB}/V_{maxB})) + (C_0/V_{maxC})(1 - (C/B_0)(K_{mC}/K_{mC})(V_{maxC}/V_{maxC})) + (D_0/V_{maxD})(1 - (D/B_0)(K_{mD}/K_{mD})(V_{maxD}/V_{maxB})) + (K_{mB}/V_{maxB}) \log (B/B_0)$$

$$T_C = (A_0/V_{maxA})(1 - (C/C_0)(K_{mC}/K_{mC})(V_{maxC}/V_{maxC})) + (B_0/V_{maxB})(1 - (C/C_0)(K_{mC}/K_{mC})(V_{maxB}/V_{maxC})) + (C_0/V_{maxC})(1 - (C/C_0)(K_{mC}/K_{mC})(V_{maxC}/V_{maxC})) + (D_0/V_{maxD})(1 - (C/C_0)(K_{mC}/K_{mC})(V_{maxD}/V_{maxC})) + (K_{mC}/V_{maxC}) \log (C/C_0)$$

$$T_D = (A_0/V_{maxA})(1 - (D/D_0)(K_{mD}/K_{mD})(V_{maxA}/V_{maxD})) + (B_0/V_{maxB})(1 - (D/D_0)(K_{mD}/K_{mD})(V_{maxB}/V_{maxD})) + (C_0/V_{maxC})(1 - (D/D_0)(K_{mD}/K_{mD})(V_{maxD}/V_{maxC})) + (D_0/V_{maxD})(1 - (D/D_0)(K_{mD}/K_{mD})(V_{maxD}/V_{maxD})) + (K_{mD}/V_{maxD}) \log (D/D_0).$$

Where $K_{mA}$, $K_{mB}$, $K_{mC}$, and $K_{mD}$ are the Michaelis–Menten constants for the four stereoisomers of GD; $V_{maxA}$, $V_{maxB}$, $V_{maxC}$, and $V_{maxD}$ are the corresponding maximum velocities; and $A_0$, $B_0$, $C_0$, and $D_0$ are the initial concentrations of each stereoisomer.

Although complex, the solutions give the time it would take for each substrate (normalized to its initial level) to fall to a particular level. As such, they were used to graph curves of the substrate levels as functions of time. By adjusting the kinetic parameters we were able to use a Microsoft EXCEL 2003 spreadsheet to fit these model curves to the experimentally derived data (see Supplementary material). The bimolecular rate constants ($k_{cat}/K_m$) shown in Table 1 are the average of eight independent experiments' ± standard deviation (n = 8).

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References


**Supplementary material**

The following supplementary material is available online:

Fig. S1. Reaction schematic of the racemic GD HuPON1 system.

Fig. S2. Hydrolysis of 0.37 mM racemic GD by HuPON1.

Fig. S3. Comparison of theoretical and numerical solutions.

Fig. S4. Comparison of assumed enzyme ‘steady-state’ levels and actual (numerically integrated) levels.

Fig. S5. Lineweaver–Burke plot of theoretical solutions and measured data for hydrolysis of 1.67 mM racemic GD by HuPON1.

Fig. S6. Hanes–Woold plot of theoretical solutions and measured data for hydrolysis of 1.67 mM racemic GD by HuPON1.

Fig. S7. Eadie–Hofstee plot of theoretical solutions and measured data for hydrolysis of 1.67 mM racemic GD by HuPON1.

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