Changes in quaternary structure cause a kinetic asymmetry of glutamate racemase-catalyzed homocysteic acid racemization

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Glutamate racemases (GR) catalyze the racemization of D- and L-glutamate and are targets for the development of antibiotics. We demonstrate that GR from the periodontal pathogen Fusobacterium nucleatum (FnGR) catalyzes the racemization of D-homocysteic acid (D-HCA), while L-HCA is a poor substrate. This enantioselectivity arises because L-HCA perturbs FnGR’s monomer–dimer equilibrium toward inactive monomer. The inhibitory effect of L-HCA may be overcome by increasing the total FnGR concentration or by adding glutamate, but not by blocking access to the active site through site-directed mutagenesis, suggesting that L-HCA binds at an allosteric site. This phenomenon is also exhibited by GR from Bacillus subtilis, suggesting that enantiospecific, “substrate”-induced dissociation of oligomers to form inactive monomers may furnish a new inhibition strategy.

Keywords: Bacillus subtilis; enantioselective inhibition; Fusobacterium nucleatum; glutamate racemase; homocysteic acid; quaternary structure

Glutamate racemase (GR, EC 5.1.1.3) catalyzes the 1,1-proton transfer that reversibly interconverts the enantiomers of glutamate (Glu) via a two-base mechanism wherein two active-site cysteine residues located on opposite sides of the α-carbon stereocenter act as Brønsted acid-base catalysts depending on the reaction direction (Scheme 1) [1]. As such, the enzyme furnishes the supply of D-Glu required for the biosynthesis of peptidoglycan [2–4], which encapsulates the majority of bacterial cells, protecting them against osmotic lysis [5–8], and poly-γ-glutamate (in Bacillus spp.) [9–13]. Recently, a bifunctional enzyme from Chlamydia trachomatis capable of GR and diaminopimelate epimerase activities has also been described [14]. The genes encoding GRs have been shown to be essential in many bacterial species [15–19] and, consequently, a variety of compounds have been reported as GR inhibitors [20,21]. Primarily, two strategies for developing GR inhibitors have been described. First, a variety of Glu analogs have been successfully developed as inhibitors [22–31], and second, inhibitors that bind at allosteric sites have been discovered [4,26,27,32–45]. Because of the importance of GR as an antibacterial target, the development of alternative inhibition strategies remains an important goal.

Abbreviations
BN, blue native.; BsGR, Bacillus subtilis glutamate racemase; CD, circular dichroism; \( \bar{d}_H \), mean hydrodynamic diameter; DLS, dynamic light scattering; FnGR, Fusobacterium nucleatum glutamate racemase; GR, glutamate racemase; HCA, homocysteic acid; HPLC, high-performance liquid chromatography; IBLC, N-isobutyryl-L-cysteine; OPA, o-phthaldialdehyde; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reversed-phase HPLC; SDS, sodium dodecyl sulfate.
Previously, we characterized GR from *Fusobacterium nucleatum* (*FnGR*) [46]. This Gram-negative obligate anaerobe [47] promotes the onset of periodontal disease by facilitating the co-aggregation of different bacterial species in oral biofilms, leading to plaque formation, permanent establishment of pathogenic strains within the oral cavity, and subsequent periodontal pathology [48,49]. Unlike most other GRs, *FnGR* is a pseudosymmetric enzyme, catalyzing the racemization of Glu enantiomers with similar kinetic parameters and exists predominately in dimeric form [46]. While some GRs exhibit substrate-induced dimerization to form active enzyme [50–52], the position of the monomer–dimer equilibrium for *FnGR* is not substantially altered in the presence of Glu [46].

In the present study, we explored the ability of *FnGR*, as well as GR from *Bacillus subtilis* (*BsGR*), to utilize homocysteic acid (HCA) as an alternative substrate. Surprisingly, depending on the relative concentrations of substrate and enzyme employed in kinetic studies, we found that under certain conditions, only \( \delta \)-HCA was turned over, whereas under other conditions, both \( \delta \)- and \( \lambda \)-HCA were racemized by *FnGR*. Herein, we show that this paradoxical behavior arises because of enantiospecific, ligand-induced shifting of the monomer–dimer equilibrium to favor the inactive form of the enzyme, resulting in the loss of enzyme activity. Such enantioselective, “substrate”-induced shifting of the oligomerization equilibrium to favor the inactive form has not been observed previously with GRs and suggests a new inhibitor design strategy.

**Materials and methods**

**Reagents**

All reagents were from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada), unless otherwise specified. Continuous enzyme assays were conducted using a JASCO J-810 spectropolarimeter. For high-performance liquid chromatography (HPLC) analyses, a Waters 510 pump and 680 controller were used for solvent delivery. Injections were made using a Rheodyne 7725i sample injector fitted with a 50-\( \mu \)L injection loop. Analytes were detected using a Waters 474 scanning fluorescence detector.

**Enzyme purification**

*Escherichia coli* BL21 (DE3) cells, transformed with either the pET-15b-*FnGR*, pET-15b-*BsGR*, or pET-15b-*FnGR* A151V variant-encoding plasmids, were used for protein overproduction, as described previously [46]. These plasmids encode their corresponding recombinant GRs (*BsGR* and *FnGR*) as fusion proteins with an N-terminal (His)\(_6\)-tag and an intervening thrombin recognition sequence. Protein overproduction was induced in mid-logarithmic-phase cultures grown in Luria Bertani medium at 37 °C by the addition of isopropyl-\( \beta \)-D-thiogalactopyranoside to the final concentration of 1 mM. The cells were lysed by sonication and the soluble (His)\(_6\)-tagged GRs purified using nickel ion affinity chromatography, as described previously [46]. The proteins were purified to >95% homogeneity as assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [53]. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Mississauga, ON, Canada) with bovine serum albumin (BSA) standards. The purified proteins were dialyzed into potassium phosphate buffer (assay buffer; 10 mM, pH 8.0) containing dithiothreitol (0.2 mM), aliquoted, and stored at −80 °C.

**Continuous enzyme assays**

*FnGR*-catalyzed racemization of \( \lambda \)-Glu and \( \delta \)-Glu was assayed directly using a circular dichroism (CD)-based assay [54], as described previously [46]. Reactions were conducted at 30 °C in assay buffer, and the racemization of both enantiomers of HCA (2.5–45.0 mM) was followed by monitoring the change in ellipticity at 204 nm. The values of [\( \theta \)]\(_{204}\) were determined to be 33.4 mdeg·mm\(^{-1}\)·cm\(^{-1}\) for \( \delta \)-HCA and 28.6 mdeg·mm\(^{-1}\)·cm\(^{-1}\) for \( \lambda \)-HCA. The ability of *BsGR* (6.25 \( \mu \)g·mL\(^{-1}\)) to catalyze the racemization of \( \delta \)-HCA (3.0–40 mM) was also monitored using the CD-based assay conducted at 30 °C.

The values of the Michaelis constant (*Km*) and maximal velocity (*Vmax*) were determined by fitting Eqn 1 to the initial velocity (\(v_i\)) data using nonlinear regression and the program *KaleidaGraph* v 4.02 (Synergy Software, Reading, PA). The values of *kcat* were calculated by dividing *Vmax* values by the total enzyme concentration ([E]\(_T\)) using *M*\(_T\) values (Da) of 32 046 and 32 162 for (His)\(_6\)-*FnGR* and (His)\(_6\)-*BsGR*.

![Scheme 1](image-url) GR-catalyzed racemization of Glu and HCA.
concentrations ranging from 18.75 to 125 μM. Following a 15-min preincubation with the appropriate amount of similarly pre-equilibrated solution of either D- or L-HCA in assay buffer. Aliquots (45 µL) were withdrawn at various times, the reaction was stopped by addition of HClO4 (3 M, 15 °C), and the supernatant was neutralized with KOH (6 M, 8°C, pre-equilibrated at 30 °C for 10 min, and the enzyme-catalyzed reaction was initiated by the addition of enzyme to the appropriate amount of similarly pre-equilibrated solution of either D- or L-HCA in assay buffer. Aliquots (90 µL) were withdrawn at various times, the reaction was stopped by addition of HClO4 (3 M, 15 µL), and the volume brought to 115 µL with the assay buffer. Following centrifugation (5 min, 12 000 g), the supernatant (100 µL) was neutralized with KOH (6 M, 8 µL), centrifuged (5 min, 12 000 g), and then frozen and stored at −20 °C. Immediately before derivatization with α-phthalaldehyde (OPA) and N-isobutyryl-l-cysteine (IBLC), the neutralized frozen supernatant solutions were thawed and diluted 200- to 400-fold in the reaction buffer. This solution (10 µL) was mixed with freshly prepared derivatization reagent (10 µL) obtained after combining 5 µL of OPA solution (2.2 mg OPA dissolved in 110 µL methanol, with the volume brought to 1 mL with borate buffer, 0.4 M, pH 9.3) and 5 µL IBLC solution (2.4 mg IBLC in 500 µL methanol). After a 2-min incubation at 25 °C, the reaction was stopped by the addition of sodium acetate buffer (175 µL; 25 mM, pH 6.2). An aliquot (45 µL) was immediately analyzed using RP-HPLC (50-µL injection volume) on a Restek Ultra IB column (5 μm; 100 Å; 150 × 4.6 mm; Chromatographic Specialties Inc., Brockville, ON, Canada). The isoindole derivatives were eluted under isocratic conditions at a flow rate of 1 mL-min⁻¹ with sodium acetate (25 mM, pH 6.2):methanol (80 : 20) and detected using fluorescence detection (λex = 340 nm, λem = 450 nm). The chromatograms were generated and integrated using PeakSimple software (Mandel Scientific, Guelph, ON, Canada).

Native-PAGE analysis

Blue native (BN)-PAGE gels were prepared and discontinuous native-PAGE was conducted as described elsewhere [46,55,56]. The oligomeric profiles of FnGR in the presence or absence of Glu or HCA were determined in 10% BN-PAGE gels (30 : 0.8 total acrylamide ratio). When determining the effect of D- and L-Glu, and L-HCA on FnGR quaternary structure, the investigated compounds (45.0 mM) were incorporated in the gel matrix as well as in all the buffers. Following a 15-min preincubation with the appropriate substrate or substrate analog at 30 °C, the proteins (final concentrations ranging from 18.75 to 125 μg·mL⁻¹) were resolved on 10% BN-PAGE gels. The experiment was conducted in duplicate.

Reversed-phase HPLC (RP-HPLC)

The racemization of D- and L-HCA was also followed using a modified RP-HPLC-based assay [57]. Briefly, FnGR or BsGR, appropriately diluted with the assay buffer, was pre-equilibrated at 30 °C for 10 min, and the enzyme-catalyzed reaction was initiated by the addition of enzyme to the appropriate amount of similarly pre-equilibrated solution of either D- or L-HCA in assay buffer. Aliquots (90 µL) were withdrawn at various times, the reaction was stopped by addition of HClO4 (3 M, 15 µL), and the volume brought to 115 µL with the assay buffer. Following centrifugation (5 min, 12 000 g), the supernatant (100 µL) was neutralized with KOH (6 M, 8 µL), centrifuged (5 min, 12 000 g), and then frozen and stored at −20 °C. Immediately before derivatization with α-phthalaldehyde (OPA) and N-isobutyryl-l-cysteine (IBLC), the neutralized frozen supernatant solutions were thawed and diluted 200- to 400-fold in the reaction buffer. This solution (10 µL) was mixed with freshly prepared derivatization reagent (10 µL) obtained after combining 5 µL of OPA solution (2.2 mg OPA dissolved in 110 µL methanol, with the volume brought to 1 mL with borate buffer, 0.4 M, pH 9.3) and 5 µL IBLC solution (2.4 mg IBLC in 500 µL methanol). After a 2-min incubation at 25 °C, the reaction was stopped by the addition of sodium acetate buffer (175 µL; 25 mM, pH 6.2). An aliquot (45 µL) was immediately analyzed using RP-HPLC (50-µL injection volume) on a Restek Ultra IB column (5 μm; 100 Å; 150 × 4.6 mm; Chromatographic Specialties Inc., Brockville, ON, Canada). The isoindole derivatives were eluted under isocratic conditions at a flow rate of 1 mL-min⁻¹ with sodium acetate (25 mM, pH 6.2):methanol (80 : 20) and detected using fluorescence detection (λex = 340 nm, λem = 450 nm). The chromatograms were generated and integrated using PeakSimple software (Mandel Scientific, Guelph, ON, Canada).

Fluorescence experiments

Fluorescence spectra were recorded using a Cary Eclipse fluorescence spectrophotometer (Varian Canada Inc., Mississauga, ON, Canada) at 30 °C. Slit widths of 10 nm were used for both excitation and emission. The interaction of D,L-Glu with wild-type (1.63 mg·mL⁻¹) and A151V (0.83 mg·mL⁻¹) FnGRs was monitored by following the quenching of the intrinsic protein fluorescence (λex = 295 nm, λem = 310–400 nm). D,L-Glu was chosen to avoid concomitant enzymatic reactions. Spectra obtained in the presence of protein were recorded in triplicate and averaged. Likewise, spectra for the buffer were recorded in triplicate, averaged, and then subtracted from the averaged spectrum of the protein solution. The fluorescence quenching studies were conducted in assay buffer in the presence of different concentrations of D,L-Glu (2.5–40.0 mM). The apparent binding affinity (Kd) of wild-type FnGR for D,L-Glu was estimated by plotting the ratio of the decrease in protein fluorescence intensity at the emission maximum (329 nm) (F0 – F) to the total change in fluorescence (F0 – Fc) against the ligand concentration ([L]) according to Eqn 2 [58]: where F is the measured fluorescence, F0 is the starting fluorescence, Fc is the fluorescence of the ligand-FnGR variant complex, and Kd is the apparent dissociation constant for D,L-Glu.

\[
\frac{F_0 - F}{F_0 - F_c} = \frac{[L]}{K_d - [L]}
\]
(75 × 10 mm; Hellma Analytics, Plainview, NY) and DLS measurements were recorded at a fixed angle of 90° for 150 s. The temperature of the sample cell was maintained at 30 °C. To obtain the correlation function, a delay range of 5.0 μs was used for the first delay and 100.0 ms for the last delay, with 200 channels. Autocorrelation functions were generated by a TurboCorr Digital Correlator and the data were fitted using a nonnegative least squares algorithm (Brookhaven Instruments DLS software v. 5.89) to determine the intensity- and number-weighted distributions of GR monomers or dimers in the presence or absence of specific ligands. The refractive index and viscosity of the buffer (with and without GR) were determined using a Mettler Toledo Refracto 30GS refractometer (Mississauga, ON, Canada) and RheoSense µVISC viscometer (San Ramon, CA), respectively. The sample homogeneity was examined during each measurement by observing the polydispersity index.

Calculation of hydrodynamic diameter (d_H)

The BI-200SM software records the intensity of the scattered light along a scattering vector (q) as a function of time. The resulting autocorrelation function for fluctuations in intensity of scattered light with respect to time was fitted by Eqn 3 to determine the decay times (τ) for the particles in the solution [59].

\[ G(τ) = \exp\left( -\frac{τ}{τ} \right) \]

This decay time was subsequently converted into a decay or relaxation rate (Γ) using Eqn 4.

\[ Γ = \frac{1}{τ} \]

The decay rate (Γ) is directly related to the translational, diffusive motion of the center of mass of the scatterer and is expressed as the translational diffusion coefficient (D_T) as given in Eqn 5.

\[ D_T = Γ \frac{q^2}{C} \]

As shown in Eqn 6, the magnitude of the scattering vector (q) is proportional to the refractive index of solvent (R_I), which was 1.332.

\[ q = \frac{4πR_I}{λ} \sin \left( \frac{θ}{2} \right) \]

The scattering angle (θ) was 90° and the wavelength (λ) of incident laser was fixed at 637 nm. For small spherical scatterers, the hydrodynamic diameter (d_H) may be calculated from the diffusion coefficient using the Stokes–Einstein equation (Eqn 7).

\[ d_H = \frac{k_BT}{3πηD_T} \]

where \( k_B \) is the Boltzmann constant; \( T \) is the absolute temperature; and \( η \) is the viscosity of the bulk solvent. The latter was 0.811 mPa·s (at the assay temperature of 303 K) and did not change under any of the experimental conditions.

Results and discussion

In general, GRs exhibit restricted substrate specificity with respect to the charge distribution and side chain length of the potential substrate [50,51,54]. These observations are in accord with the incommodious nature of the active site and specific electrostatic interactions observed in crystal structures of GRs with bound substrate or inhibitors [4,20,29,51,60–63]. Since L-homocysteine sulfinate was shown to be a substrate for GRs from B. subtilis [64] and Pediococcus pentosaceus [65], we explored the ability of FnGR to utilize the Glu analog HCA as a substrate and compared the results with those obtained for BsGR.

HCA as a substrate

Under the assay conditions we employed routinely for the CD-based assay of FnGR activity with Glu as the substrate (i.e., \([E]_T = 6.25 \ μg·mL^{-1}\)), only D-HCA appeared to be racemized by the enzyme but not the L-enantiomer (Table 1). In fact, D-HCA was a poor substrate since FnGR catalyzed the racemization of D-HCA with an efficiency (0.8 mm⁻¹·s⁻¹) that was 19-fold lower than the efficiency for the racemization of D-Glu (15 mm⁻¹·s⁻¹). This lower efficiency arose primarily from the weaker binding of D-HCA (K_m = 23 mM) by FnGR, relative to D-Glu (K_m = 1.7 mM) (assuming K_m approximates K_S). Only when the concentration of FnGR was increased to 25.0–50.0 μg·mL⁻¹ were we able to observe turnover of L-HCA to D-HCA, but only at low concentrations of L-HCA (i.e., 2.5–8.0 mM; Table 1).

Because CD-based assays are not particularly well suited for measurements of relatively small changes in ellipticity of solutions containing high concentrations of substrate, we turned to a more sensitive RP-HPLC-based assay to assess whether FnGR could utilize L-HCA as a substrate at higher concentrations (i.e., 20.0–30.0 mM). We followed the racemization of both enantiomers of HCA over time by measuring the fluorescence of the OPA-IBLC derivatives of both the substrate and the reaction product. Using the same enzyme concentration (6.25 μg·mL⁻¹) and buffer
Table 1. Kinetic parameters for FnGR and BsGR.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>[E]_{T}a</th>
<th>K_m (mM)</th>
<th>k_cat (s^{-1})</th>
<th>k_cat/K_m (mM^{-1}.s^{-1})</th>
<th>Substrate range (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FnGR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Glu</td>
<td>1x</td>
<td>1.7 ± 0.1</td>
<td>26 ± 1</td>
<td>15 ± 1</td>
<td>0.15-5.00</td>
</tr>
<tr>
<td>D-Glu</td>
<td>1x</td>
<td>1.04 ± 0.07</td>
<td>17.4 ± 0.8</td>
<td>17 ± 1</td>
<td>0.15-5.00</td>
</tr>
<tr>
<td>L-HCA</td>
<td>1x</td>
<td>23 ± 6</td>
<td>17 ± 2</td>
<td>0.8 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>D-HCA</td>
<td>4x</td>
<td>28 ± 10</td>
<td>20 ± 6</td>
<td>0.7 ± 0.3</td>
<td>2.5-8.0</td>
</tr>
<tr>
<td>L-HCA</td>
<td>4x</td>
<td>10 ± 3</td>
<td>9 ± 2</td>
<td>0.8 ± 0.3</td>
<td>2.5-8.0</td>
</tr>
<tr>
<td>D-HCA</td>
<td>6x</td>
<td>29 ± 9</td>
<td>19 ± 5</td>
<td>0.7 ± 0.3</td>
<td>2.5-8.0</td>
</tr>
<tr>
<td>L-HCA</td>
<td>6x</td>
<td>33 ± 22</td>
<td>22 ± 6</td>
<td>0.7 ± 0.5</td>
<td>2.5-8.0</td>
</tr>
<tr>
<td>D-HCA</td>
<td>8x</td>
<td>26 ± 7</td>
<td>16 ± 4</td>
<td>0.6 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>BsGR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Glu</td>
<td>1x</td>
<td>1.24 ± 0.08</td>
<td>4.72 ± 0.09</td>
<td>3.8 ± 0.3</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>D-Glu</td>
<td>1x</td>
<td>14 ± 1</td>
<td>42 ± 2</td>
<td>3.0 ± 0.2</td>
<td>1.0-4.0</td>
</tr>
<tr>
<td>L-HCA</td>
<td>1x</td>
<td>15 ± 2</td>
<td>26 ± 2</td>
<td>0.56 ± 0.07</td>
<td>-</td>
</tr>
<tr>
<td>D-HCA</td>
<td>1x</td>
<td>NAd</td>
<td>NAd</td>
<td>NAd</td>
<td>3.0-4.0</td>
</tr>
</tbody>
</table>

a The standard total concentration ([E]_{T}) of FnGR bearing an N-terminal (His)_6-tag in the assays was 6.25 μg·mL^{-1} (denoted as 1x). Higher enzyme concentrations are denoted as multiples of 1x. All assays were conducted at 30 °C.

b K_{eq} values were calculated using the Haldane relation: K_{eq} = (k_{cat}/K_m)^{D/L}[(k_{cat}/K_m)^{D/L}].

c Data from reference [46].

d No enzymatic activity observed.

e The k_{cat} and k_{cat}/K_m values are apparent values since the full kinetic mechanism incorporating inhibition arising from the effect of L-HCA on the monomer–dimer equilibrium has not been established.

Indeed, such deviations have been reported for variants of GR from *Lactobacillus fermenti* [66,67] and wild-type GRS from other organisms [4,50,51]. These deviations from unity may arise due to the enzymes operating via an iso mechanism, in which the rate of interconversion of two free enzyme forms differing in protonation state is kinetically significant [68]. To the best of our knowledge, however, asymmetrical substrate enantioselectivity of the magnitude exhibited by HCA has not been observed for any GRs studied to date, with one notable exception. The L-Asp/Glu-specific racemase from *E. coli* exhibits the unidirectional conversion of either L-Asp or L-Glu to their corresponding enantiomers [61].
However, it has been proposed that this enantiospecificity arises because of an “unbalanced” pair of catalytic residues at the active site. The L- and D-specific Bronsted bases are Cys 197 and Thr 83, respectively, as opposed to most GRs, which have two catalytic Cys residues at their active sites. Indeed, conversion of Thr 83 to a Cys using site-directed mutagenesis yielded a GR variant capable of turning over D-Glu [61]. Intriguingly, both FnGR and BsGR exhibit enantioselectively with HCA as a substrate, despite having a “balanced” active site.

Effect of enzyme concentration

Notably, upon increasing the concentration of FnGR in the reaction mixture from 25.0 μg·mL⁻¹ to 50.0 μg·mL⁻¹, we were able to observe slow conversion of l-HCA to d-HCA (Fig. 1C), indicating that the t-enantiomer could indeed serve as a substrate of FnGR. When higher concentrations of l-HCA were assayed, higher concentrations of FnGR were required in order for racemization to be detected within 30 min after the initiation of reaction (i.e., 12.5 μg·mL⁻¹ FnGR for 5.0 mm l-HCA, 25.0 μg·mL⁻¹ FnGR for 10.0 mm l-HCA, and 50.0 μg·mL⁻¹ FnGR for 20.0 mm l-HCA; data not shown). Thus, increasing the concentration of FnGR permitted the enzyme to overcome an apparent inactivity or inhibition effect so that racemization of l-HCA could proceed. It is conceivable that the observed turnover of l-HCA accompanying the increase in the concentration of FnGR was due to a contaminant protein. However, the preparation of FnGR was >98% pure, as judged using SDS-PAGE analysis, and the RP-HPLC analysis revealed that l-HCA was indeed converted to d-HCA, consistent with the activity of a racemase. That a contaminating racemase would be responsible for this activity seemed highly unlikely.

Consequently, we could reliably monitor l-HCA racemization using the CD-based assay only when the enzyme concentration was increased by at least fourfold (i.e., from 6.25 μg·mL⁻¹ to 25.0 μg·mL⁻¹) relative to the concentration routinely used to follow the racemization of d-HCA under the standard assay.
that curvature with respect to \([E]_T\) suggested the possibility of a dependence on \([E]_T\) (Fig. 3C). Consequently, the initial velocities exhibited a linear dependence on \([E]_T\) (Table 1). Accurate determination of the kinetic parameters for \(FnGR\)-catalyzed racemization of \(L\)-HCA was hampered by the fact that pronounced inhibition was observed at saturating concentrations of the substrate. Hence, even though assay conditions were chosen to facilitate the measurement of the initial rates, that is, relatively high \([E]_T\) (25.0–50.0 \(\mu\)g\cdot mL\(^{-1}\)) and low substrate concentrations (2.5–8.0 mM), considerable error accompanied the determination of both \(k_{cat}\) and \(K_m\) (see Table 1). Despite this experimental obstacle, the estimated kinetic parameters for \(L\)-HCA were similar, within error, to those for \(D\)-HCA when the initial velocity data were obtained under the same assay conditions (i.e., higher \([E]_T\)). In addition, the values of \(K_{eq}\) calculated using the Haldane relation [69] were approximately unity (Table 1). Thus, when the apparent inhibitory effect was minimized, the racemization of \(L\)-HCA was catalyzed by \(FnGR\) with a similar efficiency to \(D\)-HCA, underscoring the pseudosymmetrical character of the enzyme observed when Glu is the substrate (Table 1) [46].

**Effect of HCA on \(FnGR\) quaternary structure — BN-PAGE**

The effect of enzyme concentration on the ability of \(FnGR\) to utilize \(L\)-HCA as a substrate led us to consider the effect of HCA on the oligomeric state of the enzyme. To determine if an association–dissociation phenomenon such as ligand-induced shifting of the monomer–dimer equilibrium to favor the monomeric (inactive) form of the enzyme could be giving rise to the apparent asymmetry of the \(FnGR\)-catalyzed racemization of HCA, we analyzed the effect of a high concentration of \(L\)-HCA (45.0 mM) on \(FnGR\) quaternary structure, as a function of enzyme concentration using both BN-PAGE (Fig. 4) and DLS (*vide infra*). While neither \(D\)- nor \(L\)-Glu (45.0 mM) appreciably affected the apparent ratio of dimeric to monomeric species (Figs. 4A–C), a clear increase in the relative concentration of the monomeric species was observed in the presence of \(L\)-HCA (Fig. 4D). As might be expected, this effect was most pronounced at lower enzyme concentrations, that is, at greater \([L\text{-HCA}]\) to \([E]_T\) ratios [e.g., see gel lanes corresponding to \([E]_T\) = 18.75 \(\mu\)g\cdot mL\(^{-1}\) (3 \(\times\)) to 62.50 \(\mu\)g\cdot mL\(^{-1}\) (10 \(\times\))], in agreement with the kinetic data obtained from the CD-based assays. It is important to note that BN-PAGE is a nonequilibrium technique, in
which the gel acts as a molecular sieve to separate the various oligomeric states of the protein, which are bound to Coomassie dye [55]. As such, one cannot conclude that the rate of establishing the equilibrium between the $F_{nGR}$ monomers and dimers is slow based on the observation of two distinct bands corresponding to each of these species. In fact, the DLS experiments (vide infra) and initial rate studies suggested that equilibration between the oligomeric forms of $F_{nGR}$ is rapid.

Interestingly, the $l$-HCA-induced monomerization was partially reversed when $F_{nGR}$ was simultaneously incubated with $l$-HCA and $L$-Glu (Fig. 4E). This effect was particularly noticeable when $[E]_T \geq 37.5 \, \mu g\cdot mL^{-1}$ (6×). Because the presence of $L$-Glu itself does not appear to affect $F_{nGR}$ quaternary structure [46], this suggested that to alleviate the inhibitory effect, $L$-Glu must be capable of either displacing $l$-HCA from its binding site or inducing a conformational change in the protein that, to some degree, overrides the inhibitory effect of $l$-HCA, with or without a concomitant displacement of $l$-HCA. Indeed, it has been shown that GRs may undergo considerable structural transition upon substrate binding [4].

To distinguish whether $l$-HCA binds at the active site or at an allosteric site to exert its inhibitory effect, we examined the effect of the A151V substitution on the apparent monomer–dimer ratio. In all X-ray crystal structures of GRs solved to-date, residue 151 ($F_{nGR}$ numbering) resides at the entrance to the active site. Previously, we showed that increasing the steric bulk of this residue by substitution with Val abolished the ability of $F_{nGR}$ to catalyze the racemization of D- and L-Glu [46]. In the present study, we used fluorescence quenching experiments to show that A151V $F_{nGR}$ does not bind D,L-Glu

![Fig. 3. $F_{nGR}$-catalyzed racemization of D- and L-HCA monitored by CD-based assays. Initial rates for the conversion of either D- or L-HCA were measured in potassium phosphate buffer (10 mM, pH 8.0) at 30 °C, as described in the Materials and Methods. The substrate concentrations used were 2.5 mM (●), 8.0 mM (■), 16.0 mM (▲), and 24.0 mM (▼). When D-HCA was used as a substrate, the $F_{nGR}$ concentration was varied from 1.78 $\mu g\cdot mL^{-1}$ (0.056 µM) to 21.88 $\mu g\cdot mL^{-1}$ (0.683 µM). When L-HCA was used as a substrate, the enzyme concentration was varied from 6.25 $\mu g\cdot mL^{-1}$ (0.195 µM) to 50 $\mu g\cdot mL^{-1}$ (1.56 µM). (A) Dependence of the initial velocities of racemization at low concentrations of $l$-HCA on $F_{nGR}$ concentration. The approximately parabolic increase in initial velocity with increasing $[F_{nGR}]$ shown is typically observed at concentrations of $l$-HCA >16 mM. (B) Dependence of the initial velocities of racemization at a higher concentration of L-HCA (16.0 mM) on $F_{nGR}$ concentration. (C) Dependence of the initial velocities of racemization at low (2.5 and 8.0 mM) and high (16.0 and 24.0 mM) concentrations of D-HCA on $F_{nGR}$ concentration. Note that the dependencies are linear at high concentrations of D-HCA, while the corresponding concentrations of L-HCA typically gave parabolic dependencies.}
(Fig. S2). d,L-Glu quenched the native tryptophan fluorescence of wild-type FnGR, yielding an apparent $K_d$ value of 3.4 ± 0.6 mM, but had minimal effect on the fluorescence of A151V FnGR. Hence, substitution of Ala 151 by Val prevents binding of Glu at the active site.

BN-PAGE analysis of A151V FnGR revealed that, similar to wild-type FnGR, the variant enzyme exists in a monomer–dimer equilibrium, regardless of the presence of either d- or l-Glu (Figs. 5A–C). Intriguingly, a shift in the equilibrium to favor the
monomeric species was also observed in the presence of L-HCA (45.0 mM) (Fig. 5D), suggesting that L-HCA perturbs the quaternary structure of FnGR by binding at an allosteric site and not the active site. In the presence of L-Glu (45.0 mM), the L-HCA-induced monomerization of A151V FnGR (Fig. 5E) was not reversed, suggesting that L-Glu probably exerts its “rescue” effect on wild-type FnGR by binding at the active site (not possible with A151V FnGR) and inducing a conformational change that overcomes the inhibitory effect of L-HCA.

Effect of HCA on FnGR quaternary structure — DLS

We also employed DLS to examine the oligomerization states of FnGR and BsGR in the presence and absence of D- and L-Glu, and D- and L-HCA under solution conditions using a concentration of enzyme that showed the most pronounced effects on the solution conditions using a concentration of enzyme A151V FnGR or wild-type BsGR (64.0 μg·mL⁻¹, i.e., ca. 10×) were mixed separately with each substrate or potential substrate (45.0 mM), and the value of the mean hydrodynamic diameter (d_H) determined at 30 °C (Figs. S3–S8). A single population of wild-type FnGR was detected in the absence of ligands with a mean hydrodynamic diameter of 6.9 nm (Table 2). Upon addition of L-Glu, d-Glu, d,L-Glu, or d-HCA, the value of d_H remained between 7.0 and 7.7 nm, consistent with FnGR existing as a dimer in the presence of these ligands. In the presence of L-HCA, however, the value of d_H dropped to 4.8 nm, consistent with a shift of the oligomeric equilibrium to favor the monomeric species of FnGR. For A151V FnGR in the absence of ligands, the dimeric species was observed with a mean hydrodynamic diameter of 7 nm (Table 2). Upon addition of L-Glu, d-Glu, d,L-Glu, or d-HCA, the value of d_H remained between 5.6 and 7.0 nm, consistent with A151V FnGR existing as a dimer in the presence of these ligands. In the presence of L-HCA, however, the value of d_H dropped to 4.5 nm, consistent with a shift of the oligomeric equilibrium to favor the monomeric species of A151V FnGR. This observation agrees with

<table>
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<th>Parameter</th>
<th>No ligand</th>
<th>L-Glu a</th>
<th>d-Glu a</th>
<th>d,L-Glu a</th>
<th>L-HCA b</th>
<th>d-HCA b</th>
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<tr>
<td>τ (μs)</td>
<td>36.2 ± 0.3</td>
<td>39.4 ± 0.3</td>
<td>37.3 ± 0.3</td>
<td>37.2 ± 0.3</td>
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<td>Γ (×10⁵ s⁻¹)</td>
<td>27.5 ± 0.2</td>
<td>25.3 ± 0.2</td>
<td>26.7 ± 0.2</td>
<td>26.8 ± 0.2</td>
<td>39.8 ± 0.4</td>
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<tr>
<td>D (×10⁻⁶ m²·nm⁻²)</td>
<td>79.7 ± 0.7</td>
<td>73.2 ± 0.5</td>
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<td>115 ± 1</td>
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<td>d_H (nm)</td>
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<td>7.5 ± 0.1</td>
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<td>7.0 ± 0.1</td>
<td>4.8 ± 0.1</td>
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<td>Dimer</td>
<td>Dimer</td>
<td>Dimer</td>
<td>Monomer</td>
<td>Dimer</td>
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<th>d,L-Glu a</th>
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<th>d-HCA b</th>
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<td>30 ± 3</td>
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<td>Γ (×10⁵ s⁻¹)</td>
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<td>31 ± 4</td>
<td>29 ± 4</td>
<td>34 ± 4</td>
<td>42 ± 4</td>
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<tr>
<td>D (×10⁻⁶ m²·nm⁻²)</td>
<td>80 ± 15</td>
<td>91 ± 10</td>
<td>84 ± 11</td>
<td>98 ± 10</td>
<td>121 ± 10</td>
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<td>d_H (nm)</td>
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<td>d_H (nm)</td>
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<td>4.1 ± 0.5</td>
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<td>Dimer</td>
<td>Dimer</td>
<td>Dimer</td>
<td>Monomer</td>
<td>Monomer</td>
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</table>

a [enzyme] = 64.0 μg·mL⁻¹ (i.e., at 10×).

b [ligand] = 45.0 mM.
the BN-PAGE results and suggests that l-HCA exerts its effect by binding at an allosteric site. Also in agreement with the BN-PAGE results, the $d_H$ value of A151V FnGR remained at 4.5 nm in the presence of both l-HCA and l-Glu, indicating that l-Glu does not reverse the effect of l-HCA unless it is able to bind at the active site.

For BsGR in the absence of ligands, the mean hydrodynamic diameter was 4.1 nm, indicating that unlike FnGR, BsGR is monomeric in solution (Table 2). The addition of either l-Glu, d-Glu, or D,l-l-Glu yielded a population of dimers with $d_H$ values ranging between 6.3 and 7.0 nm. These DLS measurements are in agreement with the results obtained from gel-filtration experiments [46,52] that suggest that the quaternary structure of BsGR is monomeric for the free enzyme and dimeric in the presence of either l- or d-Glu. Interestingly, in the presence of either l- or D-HCA, the observed mean hydrodynamic diameters were 4.1 and 4.8 nm, respectively, indicating that, unlike d- and l-Glu, neither l- nor d-HCA was capable of inducing dimerization of BsGR (Table 2). This observation may account for why d-HCA is a poorer substrate for BsGR than for FnGR.

Concluding remarks

Our initial observation that d-HCA was a substrate for FnGR, while l-HCA was not, was highly unusual considering the pseudosymmetry of FnGR observed when Glu is the substrate (i.e., $k_{\text{cat}}^\text{l-Glu} \approx k_{\text{cat}}^\text{d-Glu}$ and $K_m^\text{l-Glu} \approx K_m^\text{d-Glu}$) [46]. However, assays conducted with increased enzyme concentrations revealed that the racemization of l-HCA was catalyzed by FnGR, but that the ability of the enzyme to use this enantiomer as a substrate was masked by an l-HCA-induced shift of the monomer–dimer equilibrium toward the monomeric (inactive) form of FnGR. Our combined kinetic, BN-PAGE, and DLS data support the hypothesis that l-HCA destabilizes the quaternary structure of the enzyme, most likely through binding at an allosteric site.

Perturbation of the quaternary structure of proteins by the binding of small effector molecules is a common mechanism of enzyme regulation (e.g., see references [70–72]). However, while such regulatory mechanisms usually involve substrate-induced oligomerization to generate a higher order species that is catalytically active (or more active), substrate-induced dissociation of oligomers appears to be a rare phenomenon (e.g., [73–82]). Our observation that l-HCA can disrupt the active dimeric forms of FnGR and BsGR suggests an alternative strategy for developing inhibitors of GRs, that is, designing ligands that promote the formation of inactive, monomeric forms of GR (cf. references [83–86]). Interestingly, complexes of 1,3,5-triazapentadienate with Zn$^{2+}$ and Mn$^{2+}$, identified as GR inhibitors through screening studies, were shown to function by the converse mechanism, that is, binding to the GR-substrate complex and promoting formation of an inactive dimeric form of the enzyme [43]. Although D,l-HCA (>1 mm) has been shown to be toxic to Glu-utilizing E. coli K12 cells [87], whether this toxicity arises because of l-HCA-dependent inhibition of the GR activity remains to be elucidated.

Acknowledgements

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Author contributions

JM and SLB conceived the study and, along with HK, designed the experiments; JM and HK performed the experiments; JM, HK, and SLB analyzed the data; JM and SLB wrote the manuscript; all authors reviewed and approved the manuscript before submission.

References


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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Racemization of d-HCA catalyzed by BsGR.

Fig. S2. Quenching of the intrinsic protein fluorescence of wild-type and A151V FnGRs by D,L-Glu.

Fig. S3. Effect of Glu on the oligomeric state of FnGR determined using DLS.

Fig. S4. Effect of Glu on the oligomeric state of BsGR determined using DLS.

Fig. S5. Effect of HCA on the oligomeric state of FnGR determined using DLS.

Fig. S6. Effect of HCA on the oligomeric state of BsGR determined using DLS.

Fig. S7. Effect of Glu on the oligomeric state of A151V FnGR determined using DLS.

Fig. S8. Effect of HCA on the oligomeric state of A151V FnGR determined using DLS.