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Effect of soluble epoxide hydrolase polymorphism on substrate and inhibitor selectivity and dimer formation[S]

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Abstract **Epoxy FAs (EpFAs) are important lipid mediators that are mainly metabolized by soluble epoxide hydrolase (sEH). Thus, sEH inhibition is a promising therapeutic target to treat numerous ailments. Several sEH polymorphisms result in amino acid substitutions and alter enzyme activity. K55R and R287Q are associated with inflammatory, cardiovascular, and metabolic diseases. R287Q seems to affect sEH activity through reducing formation of a catalytically active dimer. Thus, understanding how these SNPs affect the selectivity of sEH for substrates and inhibitors is of potential clinical importance. We investigated the selectivity of four sEH SNPs toward a series of EpFAs and inhibitors. We found that the SNPs alter the catalytic activity of the enzyme but do not alter the relative substrate and inhibitor selectivity. We also determined their dimer/monomer constants ($K_{D/M}$). The WT sEH formed a very tight dimer, with a $K_{D/M}$ in the low picomolar range. Only R287Q resulted in a large change of the $K_{D/M}$. However, human tissue concentrations of sEH suggest that it is always in its dimer form independently of the SNP. These results suggest that the different biologies associated with K55R and R287Q are not explained by alteration in dimer formation or substrate selectivity.—**Morisseau, C., A. T. Wecksler, C. Deng, H. Dong, J. Yang, K. S. S. Lee, S. D. Kodani, and B. D. Hammock. **Effect of soluble epoxide hydrolase polymorphism on substrate and inhibitor selectivity and dimer formation.** ***J. Lipid Res.*** **2014.** 55: **1131–1138.**

Supplementary key words epoxy-fatty acids • inflammation • pain

In mammals, epoxy FAs (EpFAs), such as epoxyeicosatrienoic acids (EETs) derived from arachidonic acid, are produced by cytochrome P450s and are important lipid mediators that have key roles in the regulation of hypertension, inflammation, and angiogenesis, as well as in modulating both inflammatory and neuropathic pain (1, 2). These EpFAs are mainly metabolized endogenously by soluble epoxide hydrolase (sEH; EC 3.3.2.10) yielding 1,2-diols that are in general less biologically active than the original epoxides (3). In numerous animal models, pharmacological inhibition of sEH has been demonstrated to stabilize EpFA levels resulting in antihypertensive and cardioprotective effects, as well as reduction in inflammation and pain reduction mediated by the EpFAs (1–4). Thus, stabilization of EpFAs by sEH inhibition is a promising therapeutic target for the treatment of numerous ailments.

The sEH is expressed in multiple human tissues, where it is found mostly in the cytosol, but also in the peroxisomes of some organs (5, 6). At the molecular level, the sEH is a homodimer arranged in an antiparallel fashion, with each monomer having globular N- and C-terminal domains linked by a proline-rich bridge (7). While the C-terminal domain contains the EH activity, the smaller N terminal is a phosphatase (EC 3.1.3.76) (8). Several SNPs of sEH that result in amino acids substitutions have been identified (9, 10). Interestingly, several of these polymorphs of sEH (K55R, R103C, and R287Q) have been associated with various diseases ranging from increased cardiovascular risks to ischemia, kidney failure, and more recently, anorexia, underlying the potential clinical importance of sEH and of its natural mutants (11–14).

Hypotheses advanced to explain the role of the sEH mutants in these diseases involve change in activity and/or change in dimer formation (10, 15, 16). While not situated near any of the sEH active sites (7), several mutations affect activities, namely R287Q results in lower EH activity while K55R results in lower phosphatase activity (10, 15). However, the published results are in part from surrogate substrates and at high concentrations, which does not reflect actual cellular conditions. In addition, particularly for the phosphatase activity, some of the published results

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Abbreviations: EET, epoxyeicosatrienoic acid; EpFA, epoxy FA; [^{3}H]*t*-DPPO, [^{3}H]*trans*-1,3-diphenylpropene oxide; sEH, soluble epoxide hydrolase.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of Materials and Methods, three tables, and eight figures.

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are contradictory. A 2004 study suggests that the phosphatase activity of K55R, R103C, and R287Q was significantly lower than the WT sEH (15), while a 2006 study, by the same group using the same surrogate substrate, showed a marked increase in R103C and R287Q phosphatase activity (17).

Crystal structures of sEH show that Arg287 is localized on the dimer interface (7), and this residue has been hypothesized to form an intermonomer salt bridge with Glu254 of the other monomer to stabilize the dimer (10). Site-directed mutagenesis studies support such a role for these two residues (10, 15, 16). In addition, it was recently shown that only the sEH dimer is active (16). Put together, these data suggest that R287Q-associated biology may be functionally linked to its effects on dimerization. However, the effect of this mutation on dimer formation in vivo is not known.

Finally, besides the EETs, epoxide of other FAs, especially n-3 FAs such as EPA (epoxyeicosatetraenoic acids) and DHA (epoxydocosapentaenoic acids: EpDPEs) are excellent substrates for the sEH (18). Like the EETs, the n-3 EpFAs are biologically active (1). Interestingly, while for inflammation and pain the EETs and n-3 EpFAs have similar effects (18, 19), EETs and EpDPEs have opposite effects on angiogenesis, with the former promoting it and the latter reducing it (20). Although the sEH SNPs were shown to change the enzyme specific activity (10), their effects on substrate selectivity, which can alter the biological role of sEH, have not been tested.

To answer these questions, we investigated the selectivity of four sEH SNPs (K55R, R103C, C154Y, and R287Q) toward a series of EpFAs and inhibitors, as well as determined their dimer/monomer constants ($K_{D/M}$) and the concentration of sEH in human tissue extract.

MATERIALS AND METHODS

Chemicals

All inhibitors and substrates tested were previously synthesized in our laboratory (4, 18, 21). Commercial reagents and solvents were from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and were used without further purification.

Overexpression and purification of human sEH polymorphs

Recombinant human sEH polymorphisms were expressed as previously published (10) and purified using affinity chromatography as previously reported (22). This method allows, in general, the recovery of 50% to 70% of the activity, with purification factors >20-fold. All enzymes displayed purities >95% as evaluated by SDS-PAGE analysis (see supplementary Fig. I). The purified proteins were aliquoted and stored at −80°C with 20% glycerol in sodium phosphate buffer (0.1 M, pH 7.4) until usage.

Kinetic assay conditions

Kinetic parameters for [^{3}H]*trans*-1,3-diphenylpropene oxide ([^{3}H]*t*-DPPO); 14,15-EET; attophos; and 1-myristoyl-2-hydroxy-3-glycerophosphate were determined under steady-state conditions as described (18, 21, 23, 24). Details are given in supporting information.

Substrate selectivity analysis

A mixture of 14 EpFAs from arachidonic acids, EPA, and DHA at 0.1 mM each in DMSO was prepared from purified regioisomers (18). Purified recombinant sEH proteins in 100 µL of sodium phosphate buffer (0.1 M, pH 7.4) containing 0.1 mg/ml BSA were each incubated with the mixture of EpFAs (each at a final concentration of 1 µM). After incubation at 30°C (see supplementary Table I for times), the reactions were stopped by the addition of 100 µL of methanol containing 400 nM of 12-(3-cyclohexylureido)dodecanoic acid as internal standard. Incubation times were optimized to ensure that the total turnover was <5% for the preferred substrate. The amount of each diol formed was quantified by LC/MS/MS as previously described (18). Results are average ± SD (n = 3).

IC_{50} determination

The IC_{50} was determined using racemic [^{3}H]*t*-DPPO as described (25). Details are given in the supplementary Materials and Methods.

Determination of the dimer-monomer dissociation constant ($K_{D/M}$)

Purified recombinant sEH proteins were each diluted in sodium phosphate buffer (0.1 M, pH 7.4) containing 0.1 mg/ml BSA to the desired concentration ($1 < [E]_{final} < 10{,}000$ pM). After 24 h incubation at 4°C, the remaining activity was measured using [^{3}H]*t*-DPPO as substrate ($[S]_{final}$ = 50 µM) (21). To ensure that, even at the lowest concentrations of enzyme, activity could be accurately measured, the diluted enzymes were incubated up to 5,760 min (96 h) at 30°C, before extraction and quantification of the diol formed. To avoid water evaporation, the glass tubes used for the assay were sealed and gently centrifuged twice a day to bring the water that condensed on the side of the tubes back to the bottom. Incubations of the substrate in buffer with the BSA alone were used to account for background chemical hydrolysis (supplementary Fig. II). Results obtained were corrected for activity lost during the incubation (supplementary Fig. III). Based on the recently reported observation that only the dimer is active (16), the dimer/monomer dissociation constant ($K_{D/M}$), the amount of enzyme for which 50% of the specific activity remained, was calculated by the nonlinear fitting of the following equation: $v = (A \cdot [S]^n)/(K^n + [S]^n)$ using the enzyme kinetic module of SigmaPlot version 9.01 (Systat Software Inc., Chicago, IL). Each datum point is the average ± SD of six replicates.

Western blot analysis

Standard protein and S9 samples were diluted in 4× sample buffer (Bio-Rad, Hercules, CA) to the appropriate concentration. Samples were loaded on Novex 4–20% Tris-Glycine Gels (Invitrogen, Grand Island, NY) and run at 125 V for 2 h. Membranes were blocked with 5% BSA and incubated with primary rabbit polyclonal anti-human sEH antibody (1:5,000) (26) and secondary goat anti-rabbit IgG (1:5,000) (Abcam, Cambridge, MA). Samples were developed with ECL detection reagent (GE Life Sciences) and imaged with a ChemiDoc MP (Bio-Rad). Images were processed and analyzed with Image Lab 5.0 (Bio-Rad).

RESULTS

Enzyme preparation

A histidine tag (His-tag) was attached to the recombinant sEH proteins to facilitate purification (10). While this kind of affinity purification is rapid, it yields both normal

and misfolded protein, which can result in lower apparent specific activity. To avoid this problem and lower the chance of artifacts in our experiments, the sEH WT and mutants were instead purified using an affinity gel that binds to the active site by mimicking the substrate (22). The targeted proteins were eluted with a slow-turnover substrate to yield only active enzymes (27). We observed that upon freezing and thawing, even when flash-freezing the samples and keeping them at −80°C, a significant amount of activity (>20%) was lost, especially for the R287Q mutant. Before freezing, the addition of 20% glycerol was sufficient to avert the loss of activity during storage.

Kinetic constant analysis

For each mutant, we determined the kinetic constant for a surrogate and natural substrates for both the EH and phosphatase activities (**Table 1**). The results fit well with the Michaelis-Menten equation ($r^2 > 0.96$; **Fig. 1**). The results obtained for the current WT enzyme were very similar to the results obtained previously with the recombinant human sEH expressed without a His-tag (18, 23), suggesting that this tag has little effect on the activity of the enzyme and that the purification and storage methods did not alter the enzyme significantly. Across the SNPs, the results obtained for the EH activity ([^{3}H]*t*-DPPO and 14,15-EET) follow the pattern published previously (10). However, the results obtained for the phosphatase activity (attophos and 1-myristoyl-2-hydroxy-3-glycerophosphate) are quite different than the pattern previously published with *para*-nitrophenyl-phosphate, a poor surrogate substrate of sEH phosphatase activity (15). K55R, R103C, and R287Q were previously shown to have a significantly lower phosphatase activity than WT (15), while we found that K55R and C1545Y have higher activity and that R103C and R287Q have lower activity than WT when assayed with a natural substrate. Interestingly, measurement of activity with *para*-nitrophenyl-phosphate (supplementary Table II) yields values that are globally similar to the ones previously published (15), suggesting that the change of pattern observed here is mostly due to substrate differences.

Selectivity for EpFAs

To test the substrate selectivity of sEH, we previously determined the kinetic constants for a series of EpFAs (18). Here, we took the complementary and faster approach of the direct competition between the substrates to determine the selectivity of the enzymes. This technique was developed originally for lipases (28) and then applied to other enzymes. However, to our knowledge, this is the first time it has been applied to an EH. The results obtained are summarized in supplementary Fig. IV. The substrate preference obtained for the WT is similar to the one calculated from the kinetic constants (18), underlining the accuracy of the method used. It also indicates that there are probably no substrate/product allosteric interactions as seen with lipoxygenase (29). While the total amount of diol formed by each SNP was different, the relative amounts of diol among the different EpFA substrates were similar (supplementary Fig. IV), indicating that the mutations do not affect the substrate selectivity of the sEH.

Selectivity of urea-based inhibitors

To understand if genetic variants of sEH display differential susceptibility against known WT sEH inhibitors, we screened each polymorphism against a series of structurally diverse, urea-containing compounds (4). As shown in supplementary Fig. V, globally the potency profile is similar for all the SNPs, suggesting that the mutations do not dramatically affect the binding of the urea-based inhibitors. Nevertheless, there is one significant difference in potency. Triclocarban (TCC), a high-volume antimicrobial additive found in personal care products, is 10-fold less potent against the K55R variant than for the other enzymes (supplementary Table III).

Dimer/monomer dissociation

Over the years, we have tried several methods (e.g., analytical ultracentrifugation, gel filtration, surface plasmon resonance, and microcalorimetry) to determine the dissociation constant of the sEH dimer. However, each time our results indicated a dissociation constant well below the

TABLE 1. Kinetic parameters for WT and four sEH polymorphs

Substrate	WT	K55R	R103C	C154Y	R287Q
[^{3}H]*t*-DPPO					
K_M (μM)	3.3 ± 0.1	5.8 ± 0.5	5.9 ± 0.8	4.0 ± 0.1	10.1 ± 0.9
k_{cat} (s^{-1})	0.99 ± 0.04	1.72 ± 0.06	0.76 ± 0.09	2.55 ± 0.09	0.076 ± 0.009
k_{cat}/K_M (s^{-1} μM^{-1})	0.30 ± 0.02	0.30 ± 0.04	0.13 ± 0.03	0.64 ± 0.04	0.008 ± 0.001
14,15-EET					
K_M (μM)	7 ± 1	7.4 ± 0.8	10 ± 1	9.2 ± 0.9	9 ± 1
k_{cat} (s^{-1})	5.0 ± 0.3	7.5 ± 0.3	2.1 ± 0.1	15.0 ± 0.5	0.44 ± 0.01
k_{cat}/K_M (s^{-1} μM^{-1})	0.71 ± 0.05	1.0 ± 0.1	0.21 ± 0.03	1.6 ± 0.2	0.05 ± 0.01
Attophos					
K_M (μM)	9.7 ± 0.3	7.4 ± 0.4	14 ± 3	7.4 ± 0.3	17 ± 3
k_{cat} (10^{-3} s^{-1})	13.1 ± 0.1	18.2 ± 0.4	5.0 ± 0.6	27.0 ± 1.2	1.0 ± 0.2
k_{cat}/K_M (10^{-3}·s^{-1}·μM^{-1})	1.35 ± 0.05	2.5 ± 0.1	0.36 ± 0.04	3.63 ± 0.02	0.06 ± 0.01
1-Myristoyl-2-hydroxy-3-glycerophosphate					
K_M (μM)	11 ± 2	19 ± 3	7 ± 1	6 ± 1	12 ± 2
k_{cat} (10^{-3} s^{-1})	150 ± 9	420 ± 30	68 ± 4	320 ± 20	4.6 ± 0.3
k_{cat}/K_M (10^{-3}·s^{-1}·μM^{-1})	14 ± 2	22 ± 5	10 ± 2	52 ± 5	0.4 ± 0.1

Enzyme assays were performed in $NaPO_4^{3-}$ buffer (100 mM, pH 7.4) containing 0.1 mg/ml of BSA at 30°C. Results are average ± SD (n = 3).

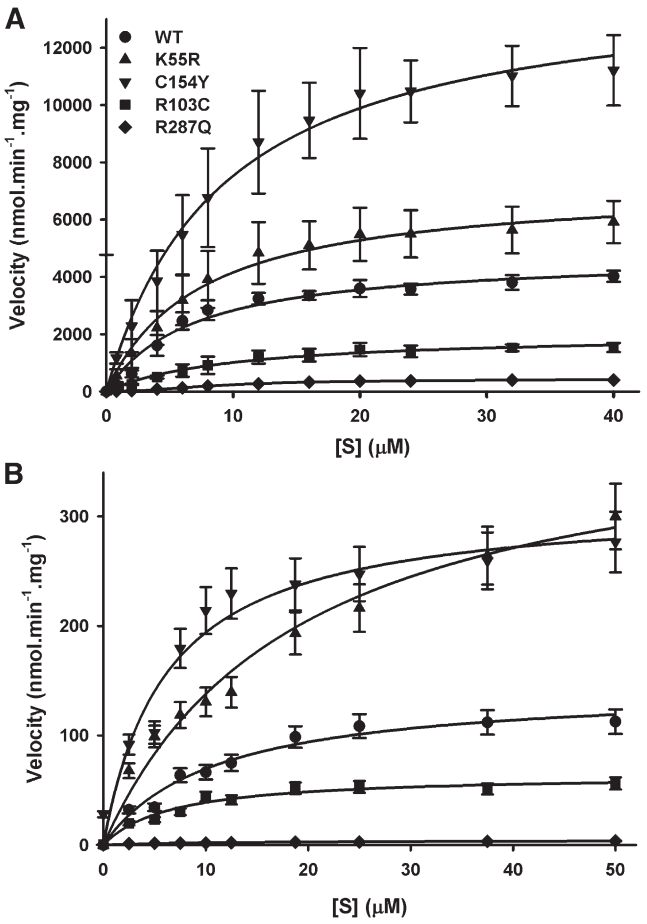

Fig. 1. Determination of the kinetic constants for 14,15-EET (A) and 1-myristoyl-2-hydroxy-3-glycerophosphate (B) with the human sEH ($[E]_{final} \approx 3$ nM) in Bis-Tris HCl buffer (25 mM, pH 7.0) containing 0.1 mg/ml of lipid-free BSA at 30°C. The kinetic constants (K_M and V_m) were calculated by nonlinear fitting of the Michaelis equation using the enzyme kinetic module of SigmaPlot version 9.01 (Systat Software Inc.). Results are average ± SD (n = 3).

limit of detection (1–10 nM) of these methods. Recently, a split firefly luciferase protein fragment-assisted complementation system was used to demonstrate that only the dimer form of sEH is active (16). However, because this system increases the size of the studied protein significantly (chimeric proteins were 30% to 100% larger than sEH), one can speculate that the method may change the way the sEH behaves, especially with regard to dimer formation. Based on this later study's observation that only the dimer is active, one should be able to titrate down the sEH enzyme concentration until it is only in the monomeric form, and thus the specific activity should accordingly disappear. In this way, one should be able to determine the apparent dimer/monomer dissociation constant corresponding to the loss of half of the activity. To reduce the chance that some loss of activity resulted from nonspecific binding of the sEH proteins to the tube, 0.1 mg/ml of BSA was added to the buffer prior to the dilution. In the end-point setup of the assay, one needed to proportionally increase the incubation time to still be able to significantly measure activity above background as the enzyme is diluted. For routine assays, an sEH concentration of around 1–10 nM is normally used, and incubation for 5–10 min is usual (30). In this report, as low picomolar concentrations of enzymes were reached, incubation times of roughly 5,000 min (≈ 96 h) were needed to significantly measure activity. To test our ability to measure activity over these long incubations, we first looked at the substrate stability in absence of enzyme under the assay conditions (supplementary Fig. II). While only 20% of the [^{3}H]*t*-DPPO is chemically hydrolyzed after 4 days, the attophos is completely hydrolyzed in less than 24 h. In addition, after 1 day, the fluorescence resulting from the attophos product decreased steadily, suggesting that this product is not stable. Thus, attophos is unsuitable as a substrate for this experiment. We then looked at the stability of the diluted sEH mutants over time under the assay conditions (supplementary Fig. III). Globally, the SNPs gave similar activity-time curves with a loss of around 50% of the activity after 3–4 days. These data were used to correct the activity measured with the WT and mutants. Using the split firefly luciferase protein fragment-assisted complementation system, it took several minutes to a few hours for the dimer to form (16). Thus, to allow significant time for the dissociation to occur, after dilution the enzymes were incubated for 24 h at 4°C before the substrate was added, and the remaining activity was measured at 30°C. For all the sEH SNPs, we observed that their specific activity for [^{3}H]*t*-DPPO dropped toward zero as their concentration decreased (**Fig. 2**). This loss of activity was not due to loss in the assay sensitivity, as the activity measured for the lowest enzyme concentrations was significantly higher than the background, but it was also 10- to 20-fold lower than the theoretical values calculated based on the dimer activity. Thus, our results support the hypothesis that only the sEH dimer is active (16). Curve fitting with the Hill equation allowed us to determine the apparent dimer/monomer dissociation constant ($K_{D/Mapp}$; **Table 2**). The values from the Hill equation are very low and suggest that sEH forms a very tight dimer. The R103C and R287Q mutants yield dimerization constants 2- and 20-fold higher than WT, respectively, while the K55R and C154Y SNPs gave dimerization constants similar to the WT.

In the second experiment, we tested the time dependence for the WT and R287Q enzymes to reach dimer/monomer equilibrium. We diluted the enzymes to their $K_{D/Mapp}$ values (Table 2) and stored them at 4°C until aliquots were taken at different time points to measure the remaining activity. For both enzymes, we obtained a biphasic curve (**Fig. 3**). In the first phase, a rapid loss of the activity over the first few hours approaches 50% of the initial specific activity, presumably corresponding to the dissociation of half of the dimeric enzymes as expected. While this phase took 5–6 hours for the WT enzyme, the plateau was reached in less than an hour for the R287Q. The faster dissociation is consistent with previous findings (10) and supports the hypothesis that the R287Q forms a weaker dimer, resulting in a higher $K_{D/Mapp}$. In a second phase, over the next few days, there is a further slow loss of the specific activities. The relative speed of activity loss was similar (9 ± 0.5%/day) for both enzymes. Interestingly, at

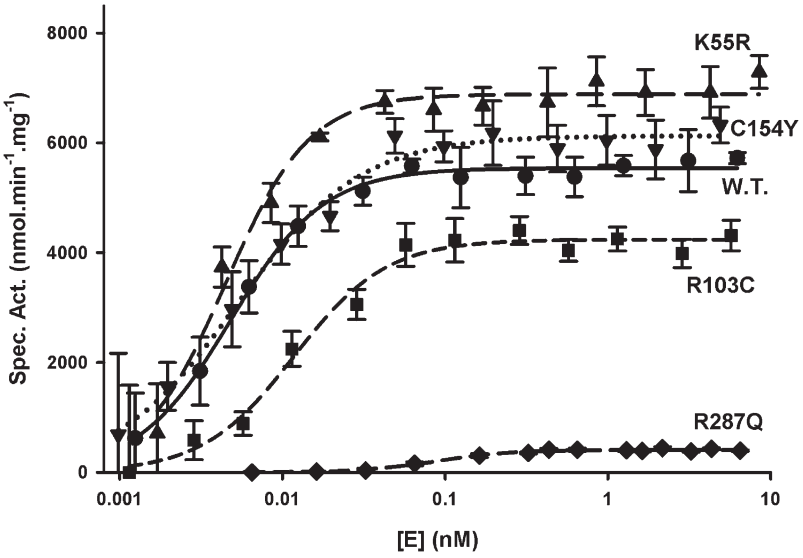

Fig. 2. Effect of sEH polymorphism on monomer/dimer formation. Purified recombinant sEH proteins were serially diluted in sodium phosphate buffer (0.1 M, pH 7.4) containing 0.1 mg/ml BSA. After 24 h incubation at 4°C, the remaining activity was measured using [^{3}H]*t*-DPPO as substrate ($[S]_{final}$ = 50 μM). Based on the recently reported observation that only the dimer is active, the dimer/monomer dissociation constant ($K_{D/M}$), the amount of enzyme for which 50% of the specific activity remained, was calculated by the nonlinear fitting of the following equation: $v = (A \cdot [S]^n)/(K^n + [S]^n)$.

concentrations where WT and R287Q are only in their dimer form ([E] = 1.2 and 13.0 nM, respectively), no significant loss of activity was observed after 1 week of storage at 4°C, agreeing with previous observations (31). This suggests that the second slow phase may correspond to the slow denaturation of the monomeric proteins, which displaces the monomer/dimer equilibrium toward more monomer and thus less activity.

To test if the dissociation of the dimer to monomer was reversible, the WT enzyme was diluted to a concentration where it is mainly in the monomer form (1.2 pM) based on the results in Fig. 2. The diluted enzyme was then concentrated around 1,000-fold by ultrafiltration at 4°C. This took 3 days, after which the remaining activity was measured (supplementary Fig. VIA). As expected, the dilution resulted in the apparent loss of >90% of the activity of the enzyme. However, concentration to the point at which it should be a dimer did not restore the activity. Interestingly, when diluting the WT enzyme to a concentration where it is mainly in the dimer form (12 pM), no significant loss of activity was observed during the concentration step (supplementary Fig. VIB). In light of the previous experiment, it suggests that in solution the monomeric sEH is not stable over a long period of time and thus cannot reform the active dimer, leading to the loss of activity observed in the first dilution/concentration experiment (supplementary Fig. VIA).

TABLE 2. Apparent dimer/monomer dissociation constants calculated from the results displayed in Fig. 2

	$K_{D/Mapp}$ (pM)	r^2
WT	5 ± 1	0.98
K55R	4 ± 1	0.98
R103C	12 ± 1	0.98
C154Y	5 ± 1	0.98
R287Q	90 ± 8	0.99

Results are values ± standard error (n = 1).

Finally, to test the strength of the dimer, we examined whether the R287Q sEH can swap monomers with the WT enzyme, leading to a restored high activity level for the resulting chimera dimer. However, in our hands, we were not able to see such enhancement of activity when mixing the WT and R287Q proteins for 24 h at 4°C (supplementary Fig. VII), suggesting that the sEH dimer is too tight to allow significant interdimer swapping on the timescale of the experiment. Our results agree with previous findings (16).

Human sEH concentration in tissue extracts

In some tissues, the sEH is localized in both the cytosol and peroxisomes (5). The presence of free sEH monomers in the cytosol has been proposed to enhance peroxisomal targeting (6). To test whether in tissue sEH is in monomer or dimer form, the concentration of sEH in human tissues was determined using [^{3}H]*t*-DPPO specific activity and quantitative Western blot (**Table 3**; supplementary Fig. VIII). Similar variations in sEH concentrations were observed with both methods, with the Western blot yielding values 20–50 nM higher than with [^{3}H]*t*-DPPO. We observed up to 140-fold variation in sEH concentrations among tissues, from 3 nM in the lungs to >400 nM in the liver, which is 30–1,000 times higher than the $K_{D/Mapp}$ found for the sEH mutants (Table 2). As an aside, we did not observe any difference in sEH concentration between the lungs of nonsmokers and smokers.

DISCUSSION

Several human genetic studies have associated the polymorphs of sEH with a range of diseases (11–14). Previous work demonstrated there are significant differences in the specific activity of the SNPs using surrogate substrates (10, 15, 17). Here we performed full kinetic analysis for both sEH activities with natural substrates (Table 1). For all the substrates studied, the mutations

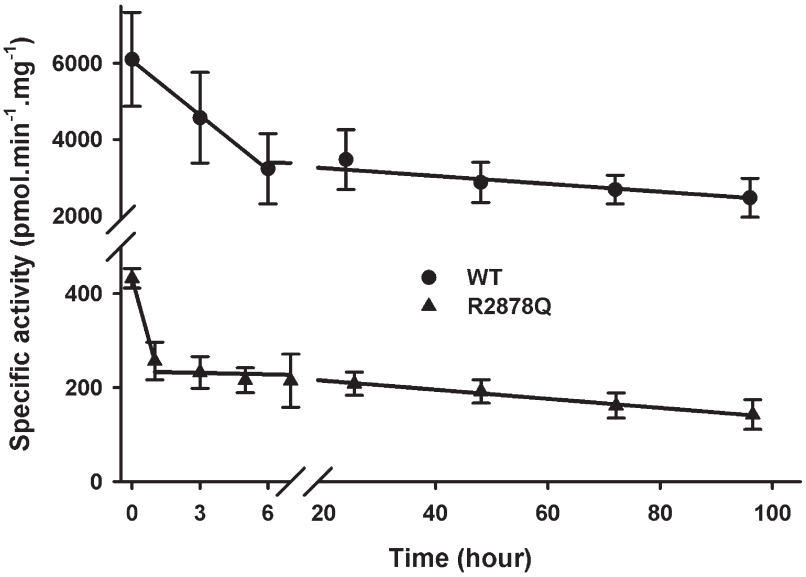

Fig. 3. Time dependence of dimer dissociation. The purified WT and R287Q enzymes were diluted in chilled sodium phosphate buffer (0.1 M, pH 7.4) containing 0.1 mg/ml BSA to concentrations closed to their $K_{D/M}$ ($[E]_{final}$ = 5 and 93 pM, respectively). The diluted enzymes were kept at 4°C until use. At different time points, aliquots were taken and activity was measured using [^{3}H]*t*-DPPO as substrate ($[S]_{final}$= 50 μM). Results are average ± SD (n = 3).

affect mainly the k_{cat} values, while the K_M values are similar for each substrate. Overall, the results for the EH activity are similar to published findings (10), whereas the results obtained for the phosphatase activity are quite different from previous results (15). However, published data for the phosphatase activity were obtained with a poor surrogate substrate, yielding results that are probably not representative of this activity. With natural substrates, our results do not support the claim that K55R and R287Q have opposite and inverse effects on the EH and phosphatase activities (15). The two SNPs with mutation near the phosphatase catalytic site, K55R and C154Y, were the most active mutants compared with WT, 1.5- to 3-fold higher k_{cat} values, respectively. On the other hand, the SNPs with mutation near the dimer interface, R103C and R287Q, show loss in overall catalytic function. R103C displays between 50% and 80% of the activity of WT, and R287Q is 30- to 300-fold less active. The simplest explanation for these results is that each of the enzyme preparations contains some inactive protein, with a higher content for the enzyme with the lower activity, thus affecting the measurement of k_{cat}. To avoid such artifacts, an sEH selective affinity purification method that yields only active enzyme was used (22). In addition, the results obtained for the EH activity were similar to those obtained with a different purification method (10), suggesting that the presence of inactive protein in the enzyme preparation is probably not the main cause of the observed k_{cat} variation among SNPs.

Alternatively, the effects of the mutations on the activities of sEH could be through changes in its structure that disturb the catalytic mechanism. Because the mutations do not alter the selectivity of multiple inhibitors and substrates for the sEH (supplementary Figs. IV and V) or the K_M values (Table 1), the results suggest that the mutations did not adversely affect the overall structure of the active site. The EH activity is the best studied of the two activities (32). The EH has a two-step mechanism involving the formation and hydrolysis of a covalent intermediate (equation 1) (32).

TABLE 3. The concentration of sEH in the S9 fraction of pooled (4–50 persons) human tissues (Xenotech LLC, Lenexa, KS)

Tissue	[Protein] (mg ml^{-1})	[^{3}H]*t*-DPPO Specific Activity (nmol·min^{-1}·mg^{-1})	[sEH] (nM)	
			Estimated from Activity	Estimated from Western Blot
Liver	16.98	8.50 ± 1.80	420 ± 89	500 ± 150
Kidney	4.15	3.67 ± 0.29	44 ± 3	80 ± 15
Lung (N.S.)	4.92	0.21 ± 0.02	3.0 ± 0.3	22 ± 6
Lung (S.)	4.49	0.22 ± 0.02	2.8 ± 0.3	21 ± 4
Intestine	4.23	6.53 ± 0.71	80 ± 9	110 ± 10

N.S., nonsmoker; S., smoker. Concentrations estimated from the specific activity using [^{3}H]*t*-DPPO as substrate ($[S]_{final}$= 50 μM) and by Western blot using recombinant purified human sEH as a standard. Results are average ± SD (n = 3).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\,S \xrightarrow{k_2} E\text{-}P \xrightarrow[+H_2O]{k_3} E + P \qquad (Eq.\ 1)$$

K_M in this case is not a measure of the affinity of the substrate for the enzyme. Rather, K_M reflects the concentration of substrate for which the velocity is half maximal. The second step of the sEH reaction mechanism (k_3) is at least an order of magnitude slower than the first step (k_2). Thus, k_{cat} values represent largely the rate of hydrolysis (k_3) of the covalent intermediate (32). In light of these facts, the kinetic data suggest that the natural mutations of sEH do not change how the substrates bind to it or the formation of the covalent intermediate but strongly influence the hydrolysis step. This latter step is strongly dependent on the optimal positioning of a water molecule in the active site and on its activation by a histidine (His523 for the human sEH) and an aspartic acid (Asp495 for the human sEH) charge relay (32). Mutation studies showed that alteration of these residues greatly changes the activity (32–34). It is possible that the SNPs induce small changes in the sEH structure that can result in the positioning of the His523-Asp495 pair, thus influencing the activation of the water molecule and the resulting k_{cat}. R287Q, the closest mutation to the active site, has the greatest effect on k_{cat} (Table 1). Interestingly, K55R, R103C, and C154Y, mutations in the N-terminal phosphatase domain, are relatively far from the C-terminal EH catalytic cavity, but they still have some limited effects on k_{cat}. These small structural changes probably also slightly alter the binding of some inhibitors, especially TCC, which is very hydrophobic, and establish hydrogens bonds only between its urea function and the catalytic tyrosines and Asp333 (4). More polar compounds, such as *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid, that form additional bonds with the enzyme are less affected. Unfortunately, the relative low resolution (≈ 3 Å) of the available crystal structure of the human sEH (7, 35, 36) does not allow the effective modeling of such small changes.

Based on X-ray crystal structure, the R287Q mutation was suggested to alter the dimer formation (10) and, because only the dimer is active (16), to alter the activity of this mutant. To test this hypothesis, we determined the monomer/dimer dissociation constants (Table 2). Effectively, this mutation results in a 20-fold weaker dimer interaction, while the other mutations had none or little effect on this interaction. In addition, the R287Q dimer dissociates to the monomer faster than the WT dimer (Fig. 3). When measuring kinetic constants (Table 1) and selectivity of series of substrates and inhibitors (supplementary Figs. IV and V), [E] values far above (>100-fold) the $K_{D/M}$ were used, indicating that all the enzymes were in their dimer form during in vitro measurement. However, one could ask if, in vivo, some of the sEH is in its monomer form, thus influencing activity.

To answer this question, the concentration of sEH was determined in human tissue extracts (Table 3). Pooled samples were purposely used and came mostly from the WT genotype, which represents 60%–70% of the population (10). We observed concentrations of sEH between 3 nM in lung extract and 400 nM in liver extract. While sEH is homogeneously distributed across the liver, in other tissues, especially in the lungs, sEH in concentrated in particular cells types (5, 26, 37). In addition, during preparation, the tissues were diluted in buffer. Thus, the actual sEH concentration in cells is much higher in the sEH-containing cells than the ones measured in the diluted tissue extract solutions (Table 3).

Nevertheless, the sEH concentrations measured are 30- to 1,000-fold higher than the dimer/monomer dissociation constant obtained for the SNPs (Table 2), indicating that if the expression of the SNPs is similar, it is highly likely that the sEH is always in its dimer form in vivo, even for R287Q, contrary to what was previously proposed (10). Interestingly, when the WT was diluted to the monomer state, we were not able to recover any activity after reconcentration. Similarly, when WT and R287Q were diluted to a 50:50 monomer-dimer mixture, we observed a slow loss of activity (Fig. 3). Put together, these data suggest that in its monomeric form the sEH is not stable. The presence of stable sEH monomer was previously proposed to be essential for the import of sEH into the peroxisomes (6). Our data do not support the existence of such sEH monomers inside cells. However, it is possible that particular cellular conditions or components could stabilize sEH monomers.

Interestingly, the concentrations of EpFAs in tissues are usually in the low nanomolar range (1, 18, 19), suggesting that in tissues the amount of sEH activity is not the limiting factor for the conversion of EpFAs, but likely the accessibility to these substrates, like for most hydrolases. Thus, SNPs that slightly (<3-fold) increase (K55R and C154Y) or decrease (R103C) the sEH activity probably do not effectively change the apparent metabolism of EpFAs in tissues. Only R287Q, which is >10-fold less active than WT, might possibly have a direct effect on EpFA metabolism in tissues. Higher plasma FA epoxide-to-diol ratios were observed in R287Q patients, supporting a lower in vivo activity (12). Lower plasma FA epoxide-to-diol ratios were observed in K55R Caucasians but not African Americans (38), suggesting that other factors besides sEH catalytic activity play a role in the pathologies associated with this SNP. There is no indication in the literature that the pathologies associated with the other SNPs resulted from altered EpFA metabolism (11–14). Separately, the observation that in some tissues the concentration of sEH (Table 3) is higher than its natural substrates (1, 18, 19) suggests that >90% inhibition of the enzyme is needed to significantly alter EpFA metabolism is some tissues, such as the liver and kidneys.

In conclusion, our data suggest that natural mutants of sEH affect the activities of the enzyme through slight tweaking of the structure, but not through major structural changes, such as dimer formation, because the SNPs do not alter the selectivity for a series of substrates and inhibitors. Extrapolating to the in vivo situation, our results suggest that there is no reason to forecast any special susceptibility to sEH inhibitors in the population due to sEH SNPs.

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