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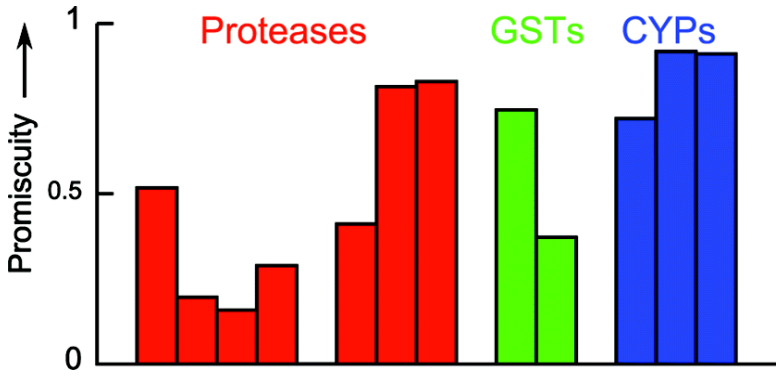

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A Quantitative Index of Substrate Promiscuity†

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ABSTRACT: Catalytic promiscuity is a widespread, but poorly understood, phenomenon among enzymes with particular relevance to the evolution of new functions, drug metabolism, and in vitro biocatalyst engineering. However, there is at present no way to quantitatively measure or compare this important parameter of enzyme function. Here we define a quantitative index of promiscuity (*I*) that can be calculated from the catalytic efficiencies of an enzyme toward a defined set of substrates. A weighted promiscuity index (*J*) that accounts for patterns of similarity and dissimilarity among the substrates in the set is also defined. Promiscuity indices were calculated for three different enzyme classes: eight serine and cysteine proteases, two glutathione S-transferase (GST) isoforms, and three cytochrome P450 (CYP) isoforms. The proteases ranged from completely specific (granzyme B, $J = 0.00$) to highly promiscuous (cruzain, $J = 0.83$). The four drug-metabolizing enzymes studied (GST A1-1 and the CYP isoforms) were highly promiscuous, with *J* values between 0.72 and 0.92; GST A4-4, involved in the clearance of lipid peroxidation products, is moderately promiscuous ($J = 0.37$). Promiscuity indices also allowed for studies of correlation between substrate promiscuity and an enzyme's activity toward its most-favored substrate, for each of the three enzyme classes.

Enzymes are traditionally (*1*−*3*) considered to be specific catalysts, capable of converting a single substrate to a single product with high efficiency. However, many enzymes are catalytically promiscuous, and they can metabolize structurally distinct substrates or convert a single substrate to multiple products. It is increasingly well-appreciated that functional promiscuity (*4*) is important for the evolution of new protein functions (*5*−*15*), the in vitro engineering of biocatalysts (*16*−*19*), and drug metabolism (*8*, *20*−*25*).

In particular, promiscuity may be extensively exploited during evolution of new protein functions from existing structural scaffolds (*6*−*8*, *10*−*15*). It has been suggested that point mutations cause substrate-specific enzymes to become more promiscuous; after gene duplication, the promiscuous templates can undergo further mutation to gain or optimize new function (*9*, *14*). The promiscuous evolutionary intermediates allow for sufficient "native" function that the organism forfeits little survival advantage. Additional survival advantage is gained upon gene duplication and mutation that optimizes the new "specific" enzyme.

Promiscuous templates may be desirable starting points for in vitro evolution strategies (*4*, *16*, *19*, *26*, *27*). The use of in vitro selection techniques with promiscuous "wild-type" proteins, in principle, could most efficiently yield new biocatalysts with novel function. However, this possibility has been underexploited, perhaps in part because appropriate templates for engineering have not been established.

In principle, detoxification enzymes are the result of evolutionary selection for promiscuity, rather than evolutionary intermediates en route to new function. The hallmark of native detoxification enzymes such as the cytochrome P450's (CYPs),[1] glutathione S-transferases (GSTs), uridyl diphosphate glucuronic acid transferases (UGTs), P-glycoprotein, and others is their remarkable ability to metabolize chemicals with widely disparate properties (*21*, *23*−*25*). Moreover, a single detoxification enzyme−substrate complex often yields multiple products, in marked contrast to the single substrate−single product paradigm of substrate-selective enzymes. That is, detoxification enzymes exhibit product promiscuity as well as substrate promiscuity, and it has been argued that product promiscuity could have a detoxification function (*28*, *29*). This promiscuous enzymology is not well accommodated by "traditional" enzymological theories developed on the basis of substrate selectivity. Furthermore, the regulatory systems responsible for induction of drug-metabolizing enzymes are highly promiscuous (*30*, *31*). In short, promiscuity is obvious at many levels of detoxification catalysis.

For the purpose of this paper, we define substrate promiscuity simply as the ability of an enzyme to metabolize different substrates: a highly promiscuous enzyme is one that metabolizes a range of substrates with similar catalytic efficiencies. There is currently no quantitative measure of an enzyme's substrate promiscuity, which makes it impossible to compare the promiscuous behavior of different proteins or to study correlations between promiscuity and catalytic efficiency or protein stability. For example, in our own work with GSTs it has been impossible to consider quantitatively how promiscuity of a series of mutants is related to their dynamic properties (*32*).

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[1] Abbreviations: CYP, cytochrome P450; GST, glutathione S-transferase; UGT, uridyl diphosphate glucuronic acid transferase. Substrate abbreviations are in Tables 1 and 2.

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Here, we describe and implement an entropy-based metric that can be adapted to quantify substrate promiscuity. We apply this metric to three different classes of enzymes, proteases, glutathione S-tranferases, and cytochrome P450's, to demonstrate how promiscuity can be measured and compared within different enzyme classes. We also modify this metric to account for chemical diversity among the set of an enzyme's substrates.

THEORY

In information theory, entropy (*33*) is essentially a measure of uncertainty about the outcome of a process. If there are N possible outcomes, each with an associated probability p_i, the entropy for the set of outcomes (p_1, p_2, ..., p_N) is defined as

$$H = -\sum_{i=1}^{N} p_i \log p_i \tag{1}$$

If the probabilities of each of the N outcomes are equal, there is maximum uncertainty as to the outcome of the process, and entropy is at its maximum value (log N). On the other hand, if the nth outcome is certain (i.e., $p_{i=n} = 1$ and $p_{i\neq n} = 0$), entropy equals zero.

The concept of information entropy has been adopted in fields such as ecology as a measure of the diversity of an ecosystem (*34*, *35*). For example, if N different species occur in a region, each with an associated population density d, then p_i is defined by

$$p_i = \frac{d_i}{\sum_{i=1}^{N} d_i}$$

H then serves as a measure of the biodiversity of the region under consideration, tending toward log N when all species are equally abundant and tending toward 0 when one species predominates. The use of entropy to measure base specificity in the target sequences of DNA binding proteins is also well-established (*36*).

Analogously, we can define entropy to describe the diversity of an enzyme's catalytic efficiency toward various substrates. The catalytic efficiency e of an enzyme for a given substrate is conventionally defined (*37*) as

$$e = \frac{k_{cat}}{K_M} \tag{2}$$

k_{cat} is the rate at which the enzyme−substrate complex turns over to release product and free enzyme; it is the rate at which product is generated by an enzyme under saturating substrate concentrations (normalized for enzyme concentration). The Michaelis constant K_M is the concentration of substrate that yields a half-maximal rate. The rate of product formation becomes linearly dependent on e when the substrate concentration is much lower than K_M.

For a promiscuous enzyme with N substrates, each with an associated catalytic efficiency e_i, we define p_i:

$$p_i = \frac{e_i}{\sum_{i=1}^{N} e_i} \tag{3}$$

Here p_i can be conceptualized as the probability that the ith substrate will be the first to be metabolized when an enzyme is simultaneously exposed to equal, low concentrations of all N substrates. We can then define a promiscuity index I as

$$I = -\frac{1}{\log N}\sum_{i=1}^{N} \frac{e_i}{\sum_{j=1}^{N} e_j} \log \frac{e_i}{\sum_{j=1}^{N} e_j} \tag{4}$$

If all the substrates in the set are equally well-metabolized by an enzyme, the enzyme is perfectly promiscuous and $I = 1$; if the enzyme only turns over a single substrate, the enzyme is perfectly specific and $I = 0$. The promiscuity index is a functional parameter that is defined for a specified set of substrates, just as an enzyme's catalytic efficiency is a functional parameter defined for a single substrate. Promiscuity indices for two different enzymes are quantitatively comparable if they have been calculated using the same substrate set.

This description of promiscuity is incomplete because it is independent of similarities or dissimilarities between the various substrates in the set. Intuitively, an enzyme that metabolizes two chemically dissimilar substrates with equal rates is more promiscuous than an enzyme that metabolizes two similar substrates with equal rates. We now describe a modification of I that accounts for substrate similarity.

The type of metric used to measure similarity obviously depends strongly on the type of substrates under consideration. For example, in determining the promiscuity of proteases with respect to amino acids at the cleavage site, it is convenient to use one of several published residue similarity matrices. For this work, residue dissimilarities were calculated from the Miyata similarity matrix (*38*, *39*), which is based on various physicochemical properties of amino acids.

When comparing chemically more diverse substrates, we turn to one of the more general techniques (*40*) that have been developed to quantify small-molecule similarity. One common method is a keyset-based distance metric. A keyset is essentially a binary string derived from the structure of a small molecule that reflects the presence or absence of suitable "descriptors"—functional groups, chemical properties, or structural features. If, for instance, the 10th descriptor is the presence of an aldehyde, then the 10th bit in a molecule's keyset will be 1 if it contains at least one aldehyde and 0 if it does not. A well-known example of a keyset is the MDL key system (*41*) developed for substructure and chemical similarity searching. Keysets can be easily weighted to emphasize a given chemical feature that is thought to be biologically relevant by increasing the number of bits affected by the presence of that feature. For example, a keyset intended to distinguish steroid substrates might devote several bits to the presence of an aromatic A-ring or the presence of a side chain at C-17. For our comparisons of GST and

Table 1: Activities ($e = k_{cat}/K_M$) of GST Isoforms A1-1 and A4-4 toward 12 GST Substrates, along with the Mean Normalized Tanimoto Distances ($\langle\delta\rangle_i$) Calculated from the Distance Matrix in Table S3 (Supporting Information)

substrate	abbrev	*e*(A1-1) ($\mu M^{-1} s^{-1}$)	*e*(A4-4) ($\mu M^{-1} s^{-1}$)	$\langle\delta\rangle_i$	ref
androstenedione	AD	0.5	0.0004	0.72	*54*
monobromobimane	MBBR	0.011	0.0022	0.73	*32*
13-oxooctadecadienoic acid	OXO	0.0089	0.0021	0.62	*55*
1-chloro-2,4-dinitrobenzene	CDNB	0.13	0.0045	0.79	*56*, *57*
4-hydroxynonenal	HNE	0.111	3.1	0.50	*57*, *58*
4-hydroxydecenal	HDE	0.166	3.8	0.50	*57*, *58*
(11*S*,12*R*)-dihydroxy-(13*S*,14*R*)-epoxy-11,12,13,14-tetrahydrodibenzo[*a*,*l*]pyrene	DPBDE	0.464	0[a]	0.79	*59*
ethacrynic acid	EA	0.0021	0.03	0.63	*57*, *60*
crotonaldehyde	CROT	0.0001	0.031	0.60	*57*, *58*
hydroxypentenal	HPE	0.0004	0.046	0.54	*57*, *58*
hexenal	HEX	0.0012	0.037	0.54	*61*
nonenal	NON	0.005	0.485	0.51	*61*

[a] No detectable activity.

CYP substrates, we designed a relatively general 92-bit keyset that equally weighted several general structural characteristics and the presence of one or more of several functional groups. A detailed description of this keyset can be found in the Supporting Information, Table S1.

Once such a keyset is constructed for each substrate in a set, a bitwise dissimilarity metric such as the Tanimoto distance (*40*, *42*) can be applied to each pair of substrates. For a pair of chemicals A and B, where *a* is the number of features present only in A, *b* is the number of features present only in B, and *c* is the number of features present in both A and B, the Tanimoto distance is $\delta_{AB} = (a + b)/(a + b + c)$. For substrates in a set, we can also define $\overline{\delta_{ij}}$ as the mean Tanimoto distance from a member *i* to all the other members in the set. The overall set dissimilarity δ_{set} serves as an upper bound for $\overline{\delta_{ij}}$: if *k* is the number of features present in at least one but not all of the members of the set, and *l* is the number of features present in all members of the set, then $\delta_{set} = k/(k + l)$. Finally, $\langle\delta\rangle_i = \overline{\delta_{ij}}/\delta_{set}$ yields the normalized mean distance for each substrate *i*.

Small molecules that are chemically similar to each other are expected to be metabolized similarly by an enzyme. In information-theoretic terms, a correlation between items in a dataset reduces the uncertainty (i.e., entropy) of the set. Therefore, we weight each substrate's contribution to *I* by its normalized mean distance to all of the other members of the set to define a weighted promiscuity index *J*:

$$J = -\frac{N}{\left(\sum_{i=1}^{N}\langle\delta\rangle_i\right)\log N}\sum_{i=1}^{N}\langle\delta\rangle_i\frac{e_i}{\sum_{j=1}^{N}e_j}\ln\frac{e_i}{\sum_{j=1}^{N}e_j} \qquad (5)$$

Structurally dissimilar substrates contribute disproportionately to the value of *J*, consistent with the idea that an enzyme that metabolizes two chemically dissimilar substrates is more promiscuous than an enzyme that turns over two similar substrates. Like *I*, *J* can range from 0 (completely specific) to 1 (completely promiscuous).

MATERIALS AND METHODS

We calculated *I* and *J* values for members of three different protein classes (proteases, glutathione S-transferase, and cytochrome P450's) using kinetic parameters from the literature. In general, we have used data from studies that simultaneously measured the activity of multiple proteins for a given substrate, to minimize differences due to discrepancies in experimental conditions or methodology. All calculations of keyset bit strings, substrate dissimilarities, and promiscuity indices were performed using scripts in the Python programming language that are available upon request.

Proteases. All data regarding the activities of various proteases are taken from Harris et al. (*43*), in which protease activity was assayed using combinatorial libraries of fluorescently labeled oligopeptides (Figure 1a). The combinatorial nature of the assay ensured that catalytic efficiencies for each substrate were measured under precisely identical conditions. Data were obtained by digitizing Figure 2 of Harris et al.; the activity toward any substrate without detectable turnover was set to 0.01 to simplify the calculations. Substrate concentrations were well below K_M, so reported activities are directly proportional to the true catalytic efficiencies. For calculation of *J* values, similarity scores for amino acid pairs from the published Miyata matrix (*38*, *39*) were scaled between 0 (least similar, for this matrix, Gly and Trp) and 1 (identical), and mean distance values (denoted by $\langle\delta\rangle_i$) were calculated by taking the mean of each residue's similarity scores to all other residues and subtracting from 1.

GSTs. For the cytosolic GSTs A1-1 and A4-4, we collected published V_{max} and K_M values and calculated catalytic efficiencies (Table 1) for 12 small-molecule substrates (Figure 2a). A 92-bit keyset (Table S1) was calculated from the structure of each substrate in the set of 12 and used to calculate Tanimoto coefficients for each substrate pair. The set mean dissimilarities ($\langle\delta\rangle_i$ values) were calculated as described in the introduction.

CYPs. We selected 18 substrates from the Metabolism and Transport Drug Interaction Database (http://www.druginteractioninfo.org) (*44*) for which a single study has reported kinetic turnover parameters (K_M and V_{max}) by each of the three CYP isoforms in recombinant systems. This will minimize systematic discrepancies in kinetic parameters caused by differences in research groups' conditions or methodology. For each pair of substrate and isoform, published V_{max} values [(pmol/min)/pmol of P450] were divided by K_M (μM) to yield catalytic efficiencies (μM^{-1}

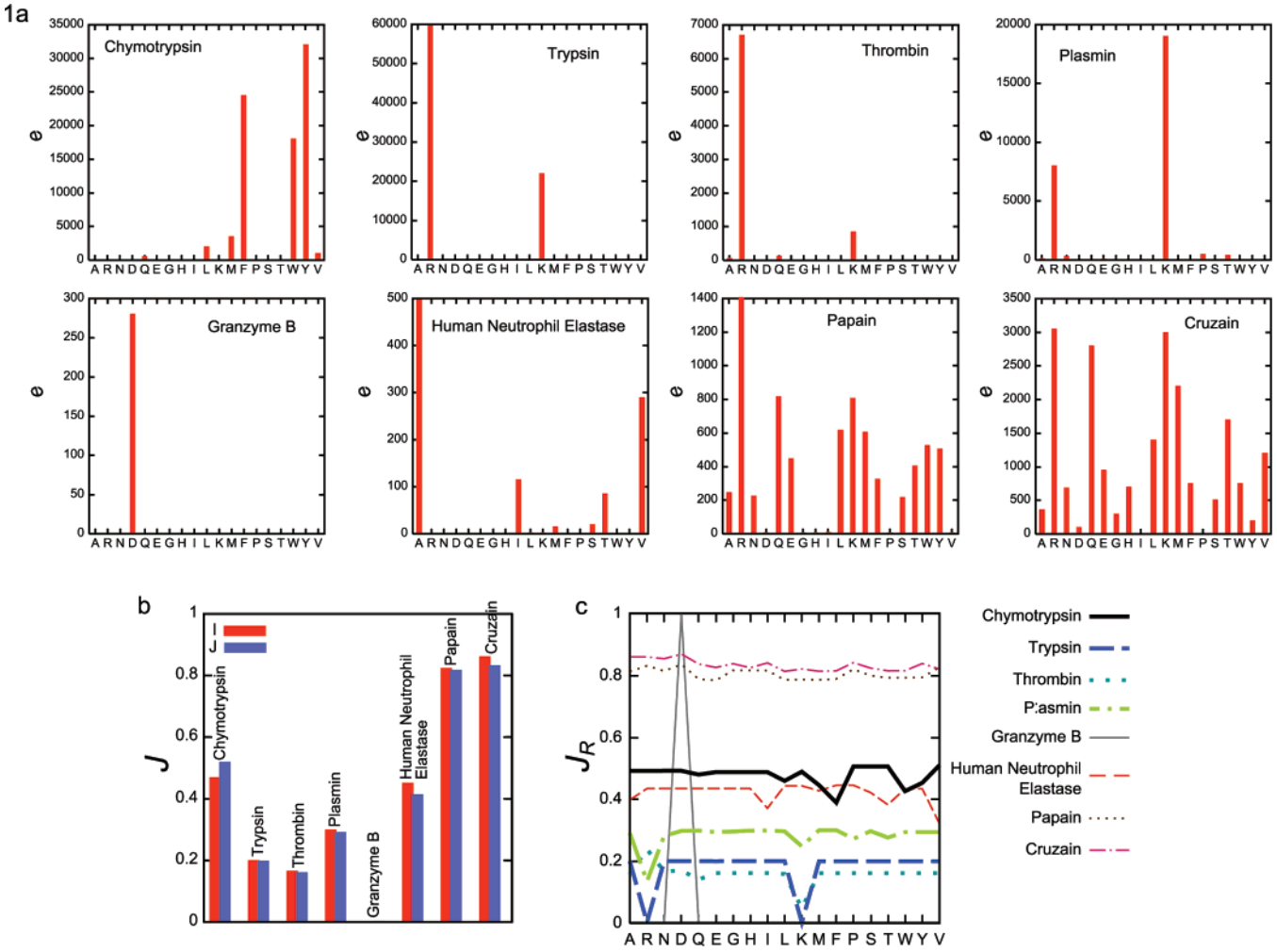

FIGURE 1: (a) Activities of eight different proteases as reported by Harris et al. (*43*) toward combinatorial libraries of tetrapeptides with one fixed residue (P1) immediately N-terminal to the cleavage site (on the *x*-axis) and three variable distal residues (P2, P3, P4). Activity (*e*) is in arbitrary fluorescence units and is directly proportional to k_{cat}/K_M. (b) Promiscuity indices of the eight proteases, calculated as described in the text. Higher values of *I* and *J* indicate higher promiscuity. *J* incorporates information from the Miyata similarity matrix (*38*, *39*) to emphasize promiscuous activity for chemically dissimilar amino acid residues. (c) Resampled promiscuity indices calculated by independently omitting one residue (on the *x*-axis) and recalculating *J* for each protease. As expected, omission of favored substrates has a greater effect on a protease's J_R value.

min^{-1}). Many reactions catalyzed by CYPs yield multiple products; in these cases the sum of the individual catalytic efficiencies for each product was taken to represent an overall catalytic efficiency.

RESULTS

Proteases. Figure 1a shows activities of eight different proteases toward 19 different mixtures of tetrapeptides with one fixed residue immediately N-terminal to the cleavage site and three variable distal residues. A simple visual inspection shows that these proteases vary considerably in specificity. Granzyme B strictly requires an Asp immediately N-terminal to the cleavage site, while the cysteine proteases papain and cruzain exhibit measurable activity with almost any residue at that position.

Figure 1b shows promiscuity index (*I*) and weighted promiscuity index (*J*) values calculated for each protease. Both parameters successfully describe the differing promiscuities of the various proteases, from the almost completely specific granzyme B ($J = 0.00$) to the highly promiscuous cruzain ($J = 0.83$) and papain ($J = 0.82$). Significantly, the promiscuity indices also allow the meaningful comparison of proteases whose promiscuities are more similar to each other. For instance, the fact that trypsin has a *J* value of 0.20 allows one to state quantitatively that it is more promiscuous than thrombin ($J = 0.16$) but less promiscuous than plasmin ($J = 0.29$); similarly human neutrophil elastase ($J = 0.41$) is modestly more specific than chymotrypsin ($J = 0.52$).

To estimate the influence of individual substrates on the calculated promiscuity indices for the entire set, we sequentially omitted each substrate from the set and recalculated *J* values for the subsets (J_R). J_R values for all eight proteases are plotted in Figure 1c. The fact that most of the resampled J_R values for each protease are close to *J* values for the complete substrate set indicates that the promiscuity index calculations are relatively robust with regard to the choice of substrate set. As expected, cognate substrates, or the accepted "most-favored" substrates, have the greatest effect on the calculated J_R values upon their removal from the set. This is most clearly seen for granzyme B: omission of Asp from the substrate set alters its J_R value from 0 (completely specific) to 1 (completely promiscuous—because it has no activity for any substrate in this subset). The most promiscu-

ous enzymes have index values that are relatively constant upon resampling. This behavior upon resampling supports the validity of the promiscuity index.

GSTs. The cytosolic GSTs are a family of enzymes involved in xenobiotic metabolism and in the response to oxidative stress. Here, we calculate *I* and *J* values for two members of this class: GST A1-1 is the primary drug-metabolizing GST and participates in phase II drug metabolism by conjugating glutathione to (and thereby solubilizing) numerous drugs and drug metabolites; GST A4-4 is involved in the clearance of lipid peroxidation products generated by oxidative stress. Figure 2a shows the structures of 12 different small molecules for which catalytic efficiencies have been reported in the literature for both A1-1 and A4-4.

Figure 2b shows how GST substrates are distributed in chemical space: the lipid peroxidation products 4-hydroxynonenal (HNE), 4-hydroxydecenal (HDE), and 13-oxooctadecadienoic acid (OXO) share many characteristics with similar aliphatic aldehydes and cluster together. The other substrates in the set are chemically more diverse. *I* and *J* values calculated from published activities (Table 1) indicate that A1-1 ($I = 0.61$, $J = 0.75$) is considerably more promiscuous than A4-4 ($I = 0.40$, $J = 0.37$). This agrees with what one would predict from the biological functions of these two proteins and on the basis of an intuitive inspection of the literature: A1-1 is responsible for xenobiotic detoxification and must metabolize a broad array of chemically diverse substrates; in contrast, A4-4 efficiently and rapidly clears structurally similar lipid peroxidation products.

CYPs. The CYPs are a superfamily of hemethiolate monoxygenases that, in mammals, dominate drug metabolism and participate in steroid biosynthesis. Hepatic CYPs are widely considered to be remarkably promiscuous with regard to their substrates and to the wide range of chemical reactions that they catalyze (*25*): the most common reactions catalyzed by CYPs include alkane hydroxylation, heteroatom oxidative dealkylation, and olefin epoxidation. Typically, a single CYP isoform can catalyze each of these reactions on structurally unrelated substrates. However, there has been no way to quantitatively compare the promiscuity of different CYP isoforms. To study how promiscuity varies across members of this class of enzymes, we focus on three CYP isoforms that play important roles in phase I drug metabolism in humans: CYP2C9, CYP2C19, and CYP3A4. Together they comprise the majority of CYP content in the liver (*45*) and small intestine (*46*), and CYP3A4 alone is estimated to be responsible for the metabolism of more than 50% of the drugs on the market (*47*).

Figure 3a contains the structures of each CYP substrate in the set, and Figure 3b shows the distribution of CYP substrates in chemical space (calculated using the same 92-bit keyset and algorithm described above). The steroids testosterone (TEST) and progesterone (PROG) are more similar to each other than any of the other 16 drugs in this set. Table 2 contains the substrates' $\langle\delta\rangle_i$ values, along with catalytic efficiencies from the literature. For this expansive set of substrates that represents a wide range of chemical space, all three CYPs are highly promiscuous, but CYP2C19 ($J = 0.92$) and CYP3A4 ($J = 0.91$) are significantly more promiscuous than CYP2C9 ($J = 0.72$). The high promiscuity index values of these three CYPs reflect their important

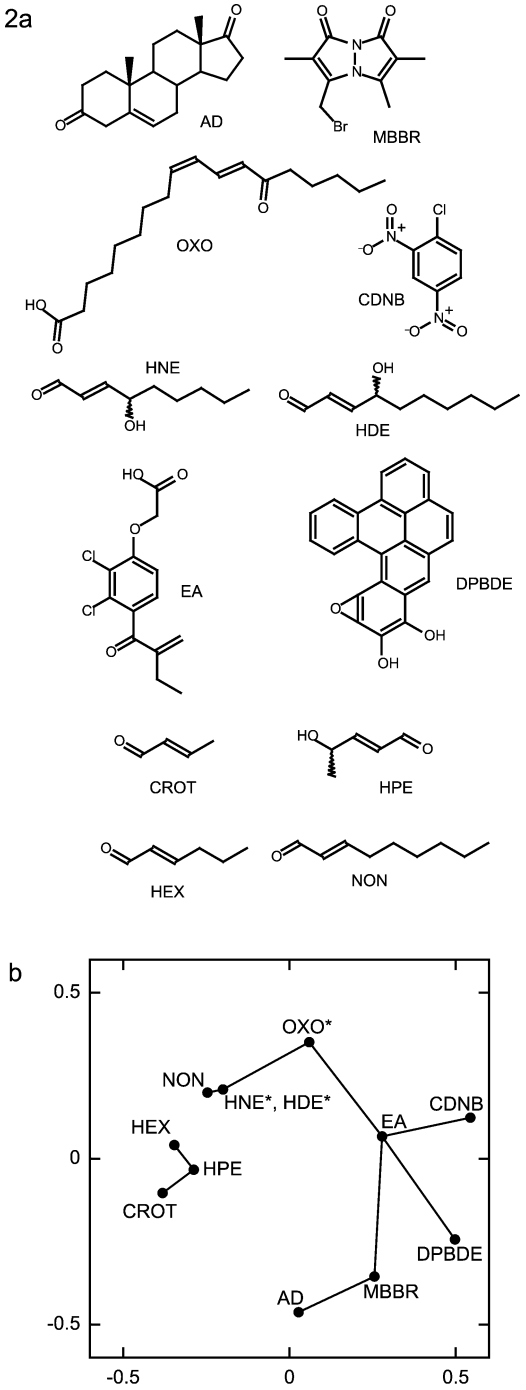

FIGURE 2: (a) Structures of GST substrates. (Refer to Table 1 for substrate abbreviations.) (b) Distribution of GST substrates in chemical space. Lipid peroxidation products HNE, HDE, and OXO cluster together with other aliphatic aldehydes and separately from the other more diverse GST substrates. Each substrate is connected to its nearest neighbor by a line. This 2-D representation was generated from the similarity matrix in Table S3 (Supporting Information) by multidimensional scaling using PERMAP (http://www.ucs.louisiana.edu/∼rbh8900/). Distances represent normalized chemical dissimilarity scores between each substrate.

physiological roles as phase I drug-metabolizing enzymes.

Notably, taking chemical similarity into account has a substantial effect on the calculated promiscuities of these enzymes: the corresponding unweighted promiscuity index (*I*) values are 0.80 for CYP2C19, 0.74 for CYP3A4, and

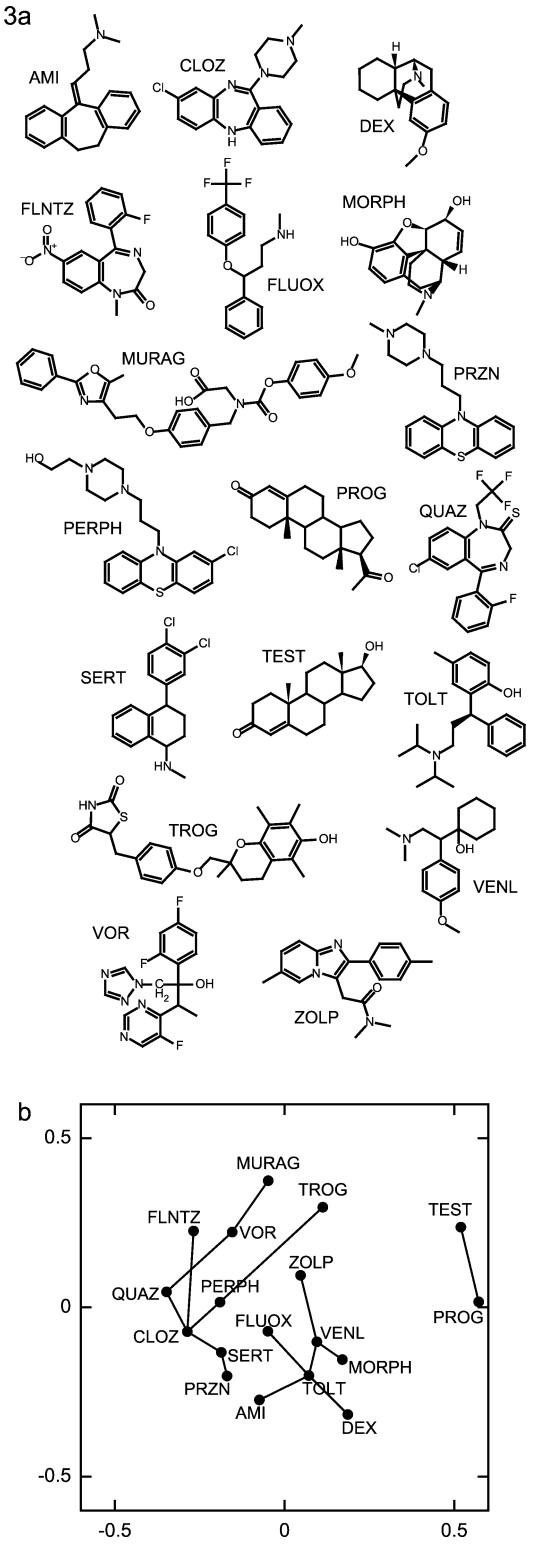

FIGURE 3: (a) Structures of CYP substrates. (Refer to Table 2 for substrate abbreviations.) (b) Distribution of CYP substrates in chemical space. The steroids TEST and PROG are more closely related to each other than to the other 16 substrates in this set. Each substrate is connected to its nearest neighbor by a line. This 2-D representation was generated from the distance matrix in Table S4 (Supporting Information) by multidimensional scaling using PERMAP (http://www.ucs.louisiana.edu/∼rbh8900/). Distances represent normalized chemical dissimilarity scores between each substrate.

0.63 for CYP2C9, reflecting the considerable chemical diversity of CYP substrates in the set.

DISCUSSION

We have defined an index to quantify promiscuity for a defined set of substrates and modified it to account for substrates representing different regions of chemical space. We have then applied these indices of promiscuity to three different sets of enzymes—proteases, GSTs, and CYPs. The proteases show a broad range of promiscuities that presumably correlate with these enzymes' diverse biological roles. Some proteases exhibit no promiscuity, whereas others are as promiscuous as the detoxification enzymes. Structural plasticity has been correlated with increased promiscuity in mutants of native proteases (*48*, *49*). Given that proteases share a highly conserved catalytic mechanism and yet display widely variable promiscuous behavior, they may serve as a useful system to study the structural and dynamic determinants of promiscuity. Several promiscuous intermediates in the evolution of serine proteases have already been characterized (*15*).

GST A1-1 is more promiscuous than GST A4-4, consistent with the former's role as a phase II drug-metabolizing enzyme and the latter's substrate-selective response to oxidative stress. Several biophysical techniques (*32*) indicate that A1-1 is also more structurally dynamic than A4-4, suggestive of a correlation between promiscuity and increased dynamic flexibility (and possibly decreased stability). The promiscuity index provides a quantitative basis for this comparison, which previously we could only describe "intuitively".

All three CYPs studied are highly promiscuous, as expected for detoxification enzymes, with CYP2C9 exhibiting less promiscuity than CYP2C19 and CYP3A4. It is particularly interesting that, on the basis of our index, CYP2C19 is as promiscuous as CYP3A4, which has not been previously appreciated. Further analysis of CYPs, using an even larger and more diverse substrate set, may provide valuable insights and predictions concerning their relative contributions to drug clearance in vivo. CYPs have also been used as templates for in vitro evolution by a number of groups, in part because of their innate promiscuity (*26*, *27*, *50*, *51*). The use of the proposed promiscuity indices in such experiments may prove useful in determining what the ideal level of promiscuity is for an initial template or what maximal promiscuity can be attained using a given protein fold.

We have focused exclusively on the rates at which substrates are metabolized, without regard to the chemistry of the reaction mechanisms employed by the various enzymes under consideration. Recent work on the quantitation of reaction similarity (*52*) may enable mechanistic studies of promiscuity, focusing on aspects of catalysis beyond merely the choice of substrate. Another possible application of similar indices is the description of redundancy in drug metabolism—quantifying the extent to which multiple enzymes are responsible for the metabolism of a given drug.

Promiscuous Enzymology. The ability of promiscuous enzymes to metabolize an extraordinary range of structurally dissimilar substrates is enigmatic when considered in the context of traditional enzymological theories based on transition-state stabilization. Intuitively, the selective transition-state stabilization of many structurally unrelated ground states would be difficult to achieve, and promiscuous

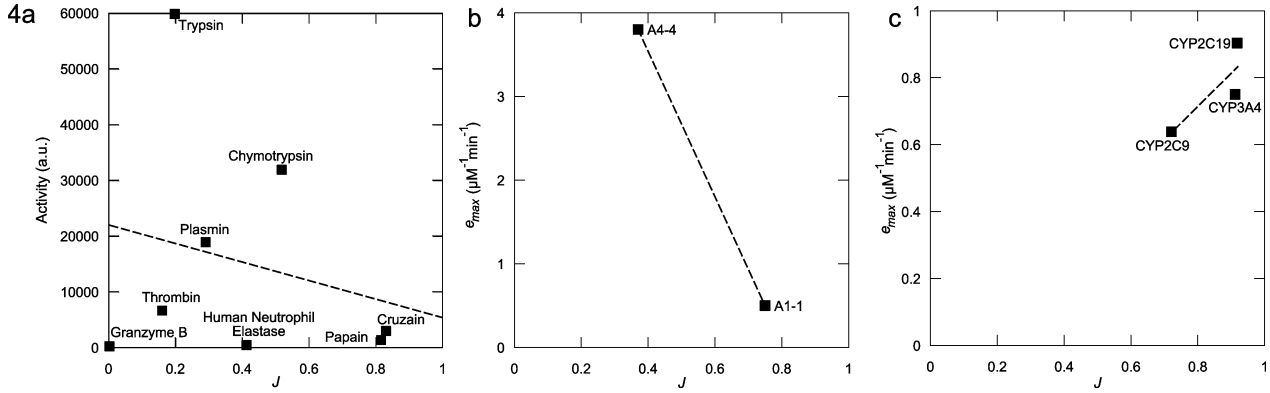

FIGURE 4: (a) Activity (in arbitrary fluorescence units directly proportional to k_{cat}/K_M) for each protease's cognate (most-favored) substrate plotted against its promiscuity index *J*. There is a slight negative correlation ($r = -0.26$) between peak activity and *J*. (b) Catalytic efficiency for each GST isoform's most-favored substrate (AD for A1-1, HDE for A4-4) plotted against the corresponding *J* value. A4-4 is the more specific isoform and is also more active. (c) Catalytic efficiency for each CYP isoform's most-favored substrate (FLUOX for CYP2C9, TROG for CYP2C19, QUAZ for CYP3A4) plotted against each enzyme's *J* value. There is a moderate positive correlation ($r = 0.83$), with CYP2C19 and CYP3A4 being both more promiscuous and more active than CYP2C9.

Table 2: Activities ($e = k_{cat}/K_M$) of CYP Isoforms CYP2C9, CYP2C19, and CYP3A4 toward 18 CYP Substrates, along with the Mean Normalized Distances ($\langle\delta\rangle_i$) Calculated from the Distance Matrix in Table S4 (Supporting Information)

substrate	abbrev	*e*(CYP2C9) ($\mu M^{-1} min^{-1}$)	*e*(CYP2C19) ($\mu M^{-1} min^{-1}$)	*e*(CYP3A4) ($\mu M^{-1} min^{-1}$)	$\langle\delta\rangle_i$	ref
amitryptiline	AMI	0.043	0.48	0.027	0.68	*62*, *63*
clozapine	CLOZ	0.038	0.027	0.63	0.68	*64*, *65*
dextromethorphan	DEX	0.0083	0.029	0.010	0.60	*66*
flunitrazepam	FLNTZ	0.0076	0.014	0.065	0.62	*67*
fluoxetine	FLUOX	0.64	0.23	0.43	0.72	*68*
morphine	MORPH	0.00021	0.0012	0.0020	0.67	*69*
muraglitazar	MUR	0.033	0.22	0.036	0.58	*70*
perazine	PRZN	0.029	0.32	0.15	0.70	*71*
perphenazine	PERPH	0.012	0.89	0.12	0.70	*72*
progesterone	PROG	0.12	0.57	0.52	0.39	*73*
quazepam	QUAZ	0.044	0.28	0.75	0.63	*74*
sertraline	SERT	0.057	0.12	0.013	0.71	*75*
testosterone	TEST	0.0074	0.021	0.38	0.39	*73*
tolterodine	TOLT	0.025	0.18	0.031	0.71	*76*
troglitazone	TROG	0.17	0.90	0.39	0.59	*77*
venlafaxine	VENL	0.0048	0.032	0.0027	0.72	*78*
voriconazole	VOR	0.0028	0.11	0.00060	0.65	*79*
zolpidem	ZOLP	0.017	0.026	0.0017	0.60	*80*

enzymes would therefore be less efficient than those evolved to recognize a single substrate. The extreme promiscuity of CYPs, for example, is often assumed to be achieved at the "cost" of slow turnover rates. On the other hand, highly specific enzymes may bear a cost associated with discriminating one desired substrate from several structurally similar chemical species (*53*).

Because *I* and *J* indices are independent of an enzyme's overall level of activity, they allow us to address the open question (*4*, *6*, *9*, *14*, *20*, *23*, *32*) of whether functional promiscuity can be achieved simultaneously with catalytic efficiency. If substrate specificity is correlated with catalytic efficiency, then a strong negative correlation between promiscuity and the catalytic efficiency for the most-favored substrate would be anticipated. For two of the three enzyme classes we studied, this correlation is weak: Figure 4 shows separate correlation plots of the *J* values for each enzyme and e_{max} (the highest catalytic efficiency for any substrate in the set) for proteases, GSTs, and CYPs. Proteases display a weak negative correlation (Pearson correlation coefficient $r = -0.24$). In fact, the most specific protease (granzyme B) also displays the lowest activity toward its cognate substrate. For the GSTs, the catalytic rate parallels specificity. GST A4-4 is both less promiscuous and more efficient than A1-1, suggesting that catalytic efficiency has been optimized in parallel with substrate specificity upon the evolution of GSTA4-4. However, it is obviously impossible to draw a definitive conclusion from a correlation between only two enzymes. Future studies comparing catalytic promiscuities of a broader range of GST isoforms (or of mutants of A1-1 and A4-4) may shed light on this correlation.

Interestingly, CYPs show a moderate positive correlation between promiscuity and activity ($r = 0.83$); the most promiscuous isoforms, CYP2C19 and CYP3A4, both have higher e_{max} values than CYP2C9. Again, investigations of more CYP substrates and isoforms may prove useful. Nonetheless, our analysis suggests that, within a defined protein fold, catalytic efficiency and promiscuity can coevolve.

CONCLUSIONS

Promiscuity is a critical feature of enzymatic detoxification, and it may be a critical feature of intermediates in the in vivo or in vitro evolution of new protein function. The relationship between functional promiscuity and protein

structure is not established, although protein "flexibility" is widely presumed to confer promiscuity. However, hypothesis-driven experiments aimed to further understand molecular mechanisms of promiscuity and its role in biology are impossible without a quantitative measure of functional promiscuity. The promiscuity indices presented here may prove generally valuable in future studies of promiscuous proteins, especially with regard to mechanistic aspects of the relationship between promiscuity and protein dynamics, stability, or enzymatic activity. For example, it is unclear how promiscuous a template needs to be to facilitate evolution of new function or whether the degree of structural plasticity required for high functional plasticity would be accompanied by thermal instability. These aspects of protein promiscuity have been intractable in the absence of any quantitative measure of promiscuity. The promiscuity indices described here, adapted to other sets of substrates, could facilitate our ability to systematically study this trait among many proteins.

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SUPPORTING INFORMATION AVAILABLE

Detailed description of the 92-bit keyset used for GST and CYP substrates, the resulting keysets for the 30 GST and CYP substrates, and similarity matrices calculated from the keysets. This material is available free of charge via the Internet at http://pubs.acs.org.

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